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Exp Mol Pathol. Author manuscript; available in PMC 2012 March 29.

Published in final edited form as:
Exp Mol Pathol. 2010 June ; 88(3): 353–362. doi:10.1016/j.yexmp.2010.03.001.

SAME PREVENTS THE INDUCTION OF THE IMMUNOPROTEASOME AND PRESERVES THE 26S PROTEASOME IN THE DDC-INDUCED MDB MOUSE MODEL

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Abstract

Mallory-Denk Bodies (MDBs) form in the liver of alcoholic patients. This occurs because of the accumulation and aggregation of ubiquitinated cytokeratins, which hypothetically is due to the ubiquitin-proteasome pathway's (UPP) failure to degrade the cytokeratins. The experimental model of MDB formation was used in which MDBs were induced by refeeding DDC to drug-primed mice. The gene expression and protein levels of LMP2, LMP7 and MECL-1, the catalytic subunits in the immunoproteasome, as well as FAT10, were increased in the liver cells forming MDBs but not in the intervening normal hepatocytes. Chymotrypsin-like activity of the UPP was decreased by DDC refeeding, indicating that a switch from the UPP to the immunoproteasome had occurred at the expense of the 26S proteasome. The failure of the UPP to digest cytokeratins would explain MDB aggregate formation. SAMe prevented the decrease in UPP activity, the increase in LMP2, LMP7, and MECL-1 protein levels and MDB formation induced by DDC. DDC refeeding also induced the TNFα and IFNγ receptors. SAMe prevented the increase in the TNFα and IFNγ receptors, supporting the idea that TNFα and IFNγ were responsible for the up regulation of LMP2, LPM7, and FAT10. These results support the conclusion that MDBs form in FAT10 over-expressing hepatocytes where the up regulation of the immunoproteasome occurs at the expense of the 26S proteasome.

Keywords

26S proteasome; Immunoproteasome; TNF alpha; Interferon gamma; inflammatory response; Mallory-Denk Body

INTRODUCTION

The ubiquitin-proteasome pathway (UPP) is an ATP-dependent proteolytic pathway and is responsible for the degradation and removal of short-lived functional proteins and oxidatively damaged molecules. Following ubiquitination catalyzed by the ubiquitin activating enzyme (El), a ubiquitin-carrier protein (E2), and one of the cell's many ubiquitin-protein ligases (E3s), the protein substrates are targeted to the 26S proteasome for

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degradation into small peptides (Liu et al., 2007). The ubiquitination system and the 26S proteasome constitute the UPP. The UPP plays a critical role in cellular homeostasis because it is implicated in the regulation of the cell cycle (cyclins) (Coux et al., 1996), transcriptional regulators (i.e., p53, NFκB, HIF-1α) (Paul, 2008), and inflammation (Donohue et al., 2002; Fuchs, 2002; Starkova et al., 2000). The regulatory control of cellular protein levels by the UPP is essential as shown by the fact that the inhibition of 26S proteasome activities leads to the loss of cellular regulation, with the development of many pathological consequences (Schwartz et al., 2009). For instance, it has been shown that chronic ethanol feeding causes significant inhibition of the 26S proteasome in liver cells (Bardag-Gorce et al., 2000; Bardag-Gorce et al., 2004), which results in the accumulation of oxidatively damaged and ubiquitinated proteins. Aggregates of ubiquitinated proteins accumulate and form MDB-like bodies (Bardag-Gorce et al., 2006). MDBs are a characteristic of alcoholic and non alcoholic chronic liver disease and form in alcoholic hepatitis (Zatloukal et al., 2007).

The mechanism involved in MDB formation is still not fully defined. When microarray analysis was done on the liver of these mice fed DDC, it was found that FAT10, a ubiquitin-like modifier, was markedly up regulated in hepatocytes that had formed MDBs. FAT10 was induced 119 fold by refeeding DDC (Bardag-Gorce et al., 2007). These MDB-forming and FAT10 positive liver cells increased in number when the drug was refed for 7 days (Bardag-Gorce et al., 2007). These MDB forming liver cells showed a growth advantage over the neighboring normal hepatocytes when DDC was refed (Oliva et al., 2008) or when thioacetamide was administrated intraperitoneally (Roomi et al., 2006). The ability of drug-primed hepatocytes to form FAT10 and MDB by DDC refeeding persisted for at least 4 months after DDC withdrawal (Li et al., 2008). The response to DDC refeeding was prevented by feeding S-adenosylmethionine (SAMe) (Li et al., 2008). FAT10 positive hepatocytes persisted for up to 15 months after DDC withdrawal (Oliva et al., 2008). At this time, liver tumors formed, indicating that the epigenetic cellular memory of the change in FAT10 expression was heritable (Oliva et al., 2008). 90% of human hepatocellular carcinomas also over express FAT10 (Lee et al., 2003).

FAT10 expression is down regulated by p53 but the reverse is also true because FAT10 up regulation down regulates p53 expression (Zhang et al., 2006). This reciprocal relationship may be involved in liver tumors genesis.

FAT10 belongs to a family of ubiquitin-like (UBL) proteins and is an 18 kDa protein, originally named diubiquitin (UbD) (Fan et al., 1996). FAT10 is 29% identical to ubiquitin at its N-terminus and 36% identical at its C-terminus. Like ubiquitin, it has C-terminus Gly-Gly residues, which are used to form isopeptide covalent linkages to target proteins (Chiu et al., 2007; Raasi et al., 2001). It has a lysine 48 residue analogous to ubiquitin. However, unlike ubiquitin, this site cannot be used to form polyubiquitin-like chains of FAT10 and cannot be removed by deubiquitinase enzymes from proteins to which it had become conjugated. Proteins, which are covalently bound by FAT10, are digested along with FAT10 by the 26S proteasome via a pathway which is independent of the ubiquitin pathway (Hipp et al., 2005; Schmidtke et al., 2009).

The FAT10 gene is present at the HLA-F human genomic locus of the major histocompatibility complex (MHC) class 1 (Hipp et al., 2005). Heat shock protein 90 (HSP90) and FAT10 are involved in the generation of peptides by the immunoproteasome. Immunoproteasomes are used in antigen presentation by MHC-1 at the cell surface (Yamano et al., 2008).

FAT10 is induced by IFNγ or by TNFα and synergistically (Raasi et al., 1999) together with the induction of LMP2 and the immunoproteasome (Lukasiak et al., 2008). FAT10 plays an

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important role in regulating cell growth of malignant tumors. These tumors express a high level of LMP2 (Ho et al., 2007; Lukasiak et al., 2008). The other immunoproteasome subunits, LMP7 and MECL-1, are also induced by IFNγ and TNFα (Kloetzel et al., 2001). These catalytic subunits of the immunoproteasome replace the 3 catalytic subunits of the 26S proteasome, i.e., chymotrypsin-like (β5), trypsin-like (β1), and peptidylglutamyl peptide-hydrolase(PGPH), recently called caspase-like activity (β2) (Aki et al., 1994; Belich et al., 1994; Kloetzel et al., 2001; Oliva et al., 2009).

Thus, FAT10 and the catalytic subunits of the immunoproteasome may play a role in the pathogenesis of MDB-associated liver preneoplasia and tumor formation in the DDC mouse model (Oliva et al., 2008). To investigate this possibility, gene microarray data from the DDC refed mice were mined (Bardag-Gorce et al., 2007). DDC refeeding caused the up regulation of the immunoproteasome subunits, FAT10, TNFα, TNFα receptors and IFNγ receptors, as well as the other catalytic subunits of the immunoproteasome. These changes were prevented by feeding SAMe. Previous studies showed that SAMe prevented the formation of MDBs and FAT10 expression, which was induced by DDC refeeding in drug primed mice (Bardag-Gorce et al., 2007; Li et al., 2008; Roomi et al., 2006).

In the present study, drug primed mice were refed DDC after DDC withdrawal. The activity of the 26S proteasome, the formation of the immunoproteasome, and the induction of MDBs were investigated. The results supported the concept that the shift of the 26S proteasome to form the immunoproteasome leads to MDB formation because the shift is at the expense of the 26S proteasome. The results of this study have been reported in part in an abstract (Oliva et al., 2009a).

MATERIAL AND METHOD

Animals

One-month-old C3H male mice (Harlem Sprague Dawley, San Diego, CA) were divided into 4 groups. Group 1: 3 control mice were fed a protein rich semi-synthetic, complete standard control diet (Teklad, Madison, WI). Group 2: 5 mice were fed the control diet containing 0.1% diethyl 1, 4-dihydro-2,4,6,-trimethyl-3,5-pyridinedicarboxylate (DDC, Aldrich, St. Louis, MO), for 10 weeks to induce MDB formation *in vivo*. Group 3: 5 mice were fed DDC for 10 weeks, then withdrawn from DDC for 4 weeks, at which time the MDBs had mostly disappeared (drug primed mice). Group 4: 6 mice were fed DDC for 10 weeks, and withdrawn 4 weeks. Then 3 mice were refed DDC (Yuan et al., 1996), and 3 mice were refed DDC+SAMe (4 g/kg/d) for 7 days. Additional group 1 and 4 mice were used for primary tissue culture studies.

The DDC refeeding over 6–7 days is a pulse from the base line model. It is used to start MDB formation in the liver of mice that were primed to form MDBs by feeding DDC previously. The same approach is used for the primary tissue culture in vitro study. All mice were treated in a humane manner, as approved by the Animal Care Committee at Harbor-UCLA LABioMed Research and Education Institute, according to the Guidelines of the National Academy of Science.

Liver Homogenates

Mouse liver homogenates were prepared by homogenizing 100 mg of liquid nitrogen frozen liver in 2 ml of 20 mM Tris-HCl pH 7.5; glycerol 10%; EGTA 1 mM; DTT 1 mM; sodium-fluoride 50 mM; protease and phosphatases inhibitor cocktail (Sigma, St Louis. MO). The livers were homogenized using the Ultra-Turrax T25 homogenizer. Protein concentrations were quantitated using the Bradford method (Bradford, 1976).

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Microarray analysis

Data mining was done using data derived from microarray performed and analyzed in a previous publication (Bardag-Gorce et al., 2007).

Proteasome Chymotrypsin-like Activity

1 μg of total protein from the cytosol fraction was used. The reaction mixture contained 50 mM Tris–HCl pH 8, 1 mM DTT, and 40 μM Suc-LLVY-AMC substrate for chymotrypsin-like activity or 40 μM of Boc-Leu-Ser-Thr-Arg-AMC for trypsin-like activity. To distinguish the 26S proteasome activity from the 20S proteasome activity, 5 mM ATP was added to the reaction mixture. The mixture was then 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 (λ excitation: 355 nm, λ emission: 430 nm), using a Perkin Elmer LS 30 spectrofluorometer.

Western Blot Analysis

Proteins (50 μg) from liquid nitrogen frozen stored livers were separated by SDS-PAGE gels and transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1 hr. in 25 mM Tris-HC1 (pH 8.3), 102 mM glycine and 20% methanol. The membranes were stained using primary antibodies against LMP2, LMP7 and MECL-1 (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA). Primary antibody against poly-ubiquitinated proteins was obtained from BIOMOL (Plymouth Meeting, PA). Appropriate species antipolyclonal and monoclonal HRP-conjugated antibodies were used as the second antibodies. The membranes were examined for chemiluminescence using luminal, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). The results were normalized by stripping the membranes and staining for GAPDH (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA).

Quantitative Real-time RT-PCR

Total liver RNAs were extracted with Trizol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA). Synthesis of cDNAs was performed with 5 μg total RNA, and 50 ng random hexamer primers, using SuperSriptIII RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA). RT-PCR primers were designated using Primer Express software (Applied Biosystems, Foster City, CA).

Sense and anti-sense: Quantitative PCR was done using the SYBR Green JumpStart™ Tag ReadyMix (Sigma, St. Louis, MO) on an ABI PRISM 7700 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 signal associated with an exponential growth for PCR product starts at $\Delta Ct = Ct_{target\ gene} - Ct_{18S}$. For each target gene, the highest ΔCt was assigned as ΔCt_{max}. The relative mRNA levels were calculated as a 2ΔΔCt, $\Delta\Delta Ct = \Delta Ct_{max} - \Delta Ct$.

List of Primer Sequences Used for RT-PCR

1-LMP2 (PSMB9): NM_013585,	Forward: TGGAGCTACACGGGTTGGA, Reverse: AGATGTTCTTCACCACGTTTGC

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2-LMP7 (PSMB8): NM_010724,	Forward: GGCTCTCGGGACAGATGTTTT, Reverse: ACCACTGTCCATCACCCCATA
3-MECL1: NM_013640,	Forward: TTGTGTTCCGAGATGGAGTCAT, Reverse: GCCACAACCGAATCGTTAGTG
4-IFNGR1: NM_010511,	Forward: GTAGTAACCAGTCAGGCCCTTGTAG, Reverse: CCACGAGGCCACTGTCAGA
5-IFNGR2: NM_008338,	Forward: TCCTCGCCAGACTCGTTTTC, Reverse: ATCTGCTCATCATTGTACAGGTGAA
6-TNFR21: NM_178589,	Forward: CCCTGACTCCCACCCAGAA, Reverse: CTGCTACAAGCTTCAAGATGTCAATAC
7-TNFR12a: NM_013749,	Forward: GAGAGAAAAGTTTACTACCCCCATAGAG Reverse: GGGTGCTCCTCACTGGATCA
8-TNFR1a: NM_011609,	Forward: GTCCATTCTAAGAACAATTCCATCTG, Reverse: GCTCGGACAGTCACTCACCAA

Liver Cell Isolation and Tissue Cultures

Livers from DDC primed mice and controls were used to isolate liver cells for primary cultures of hepatocytes. The mice were anesthetized with 33% ketamine (Phoenix, St. Joseph, MD). Surgery was performed to insert a catheter into the hepatic vein. The livers were then perfused in a retrograde manner with PBS containing 100 U/ml collagenase type 1 (Sigma, St Louis, MO) and 0.1 U/ml elastase (Worthington, Lakewood, NJ). After perfusion, the livers were removed, and the cells dispersed in William's E serum-free medium (Sigma, St. Louis, MO) containing fatty acid free bovine serum albumin (5 mg/ml), insulin (24 U/ml), dexamethasone (3.9 μg/ml), ornithine (67 μg/ml), and streptomycin/penicillin 10 ml/L. The hepatocytes were plated at a density of 10^5 cells per well on six-well plates containing fibronectin-coated glass coverslips. The cells were cultured for 6 days with or without IFNγ (100 U/ml) added. The cells on the coverslips were fixed with 100% ethanol for morphology analysis.

Immunohistochemistry and Confocal Microscopy

Liver sections, cell suspensions and tissue culture on coverslips were immunostained with primary antibodies. Liver tissue was fixed in 10% buffered zinc formalin. Cell suspensions were fixed in cold acetone for 10 min., and were then kept at 4°C until they were stained. Tissue cultures on coverslips were fixed in 100% ethanol, and kept at 4°C. The liver sections were incubated with primary antibodies to CK-8 (Fitzgerald Industries International, Concord, MA) LMP2, LMP7, and MECL1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and double stained with a mouse monoclonal antibody to ubiquitin to stain MDBs (CHEMICON, Millipore, Billerica, MA). They were also stained with an antibody to FAT10 (BIOMOL International, L.P., Plymouth Meeting, PA). Texas-red and FITC-conjugated secondary antibodies were used. DAPI was used as the nuclear stain. Fluorescent antibody stains were viewed using a Nikon 400 fluorescent microscope with a FITC filter cube and a triple color band filter cube to detect FITC and Texas-red labeled antibodies. Confocal microscopy was used to show colocalization of antibodies using a Leica confocal microscope.

Statistical Analysis

Data were obtained from 3 animals for each group. Bars represent mean values ± SEM. P values were determined by one-way ANOVA and Student-Newman Keuls for multiple group comparisons (Sigma-Stat software, San Francisco, CA). p= < 0.05 was used for

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establishing a significant difference. Correlation of data was done by linear regression analysis using Pearson's period momentum method.

RESULTS

It well known that the induction of proinflammatory cytokines, such as TNFα and IFNγ, causes the catalytic beta subunits β1, β2 and β5 of the constitutive 20S proteasome to be replaced by the LMP2, MECL-1 and LMP7 subunits (28–31), thus forming the immunoproteasome (Figure 1(a)). Microarray analysis data mining of control vs. DDC refed comparisons on 3 mice per group showed that DDC refeeding caused up regulation of various immunoproteasome catalytic subunits (Figure 1(b)). In addition, the PA28 alpha subunit, which is part of the regulatory complex 11S in the immunoproteasome (Figure 1(a)), was up regulated. It is postulated that there was a proinflammatory response associated with DDC refeeding that stimulated the formation of the immunoproteasome at the expense of the 26S proteasome. MDBs formed because of the loss of the turnover of the cytokeratins by the 26S proteasome.

It has been shown that FAT10 is significantly induced in the liver of mice fed DDC for 10 weeks, as well as in the liver of mice refed DDC for 1 week (Oliva et al., 2008). To analyze the effect of DDC on the immunoproteasome subunits, liver sections were double stained for ubiquitin and FAT10, as well as for ubiquitin and LMP2. FAT10 and LMP2 were found to be highly over expressed in hepatocytes, forming MDBs when mice were refed DDC (Figure 2). There was some colocalization of LMP2 and FAT10 in the MDB aggregates (Figures 2). The confocal microscopy analysis showed that LMP2 and FAT10 co-localized in the cytoplasm of hepatocytes (Figure 3B, merged yellow photograph) in a DDC primed mouse liver. Individual hepatocytes over expressed both LMP2 and FAT10 (Figure 3B: FITC for FAT10 and Texas Red for LMP2). Note that intervening normal hepatocytes that did not form MDBs, and did not over express either LMP2 nor FAT10. Confocal analysis also showed that DDC feeding caused the increase of the other immunoproteasome subunits LMP7 and MECL-1 (Figure 3C and D), along with the induction of FAT10. Therefore, DDC feeding for 10 weeks, as well as DDC refeeding for 1 week, caused an up regulation of FAT10, as well as an up regulation of each immunoproteasome subunit, LMP2, LMP7, and MECL-1, in hepatocytes forming MDBs. To test the hypothesis that DDC feeding causes a switch from the 26S proteasome to the immunoproteasome, the function of the 26S proteasome and the catalytic subunits of the immunoproteasome were measured in MDB forming livers of drug primed mice. Figure 4 illustrates the effects of both DDC feeding and DDC refeeding. It shows that there was no difference between DDC feeding and DDC refeeding. The immunoproteasome subunits were up regulated and the beta 5 subunits that carry the chymotrypsin-like activity for the 26S proteasome was down regulated, whereas there was no significant change in the alpha type subunits. The rationale of using DDC refeeding was to demonstrate that the hepatocytes have a memory, and that the same phenotype, obtained in 10 weeks of DDC feeding, was again obtained in 1 week of re-exposure to the drug. These results indicated that an epigenetic memory is formed in the hepatocytes following the first exposure, and that SAMe is a powerful modulator of this memory (Bardag-Gorce et al., 2007; Li et al., 2008).

Western blot analysis and RT-PCR showed that LMP2 (Figure 5(a)) and LMP7 (Figure 5(b)) were up regulated in the livers of mice refed DDC. SAMe supplementation in the diet prevented the up regulation of the immunoproteasome subunits (Figures 5(a) and (b)). MECL-1, the 3rd immunoproteasome catalytic subunit, was also up regulated by DDC refeeding, and SAMe also prevented this up regulation (Figure 5(c)).

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The gene expression of TNFα was also analyzed by RT-PCR. It was up regulated by DDC refeeding. This up regulation was prevented by adding SAMe to the DDC diet (Figure 6). Likewise, TNFα and IFNγ receptors were up regulated, which could enhance the response to TNFα and IFNγ.

The above results suggested that DDC refeeding caused an up regulation of the proinflammatory cytokine response, which induces the formation of the immunoproteasome. Therefore, to further investigate the effects of DDC feeding on the proteasome activity, proteasome chymotrypsin-like activity was measured, and the results showed that the 26S proteasome activity was significantly decreased by DDC refeeding (Figure 7(a)), while the 20S proteasome activity showed no change. These results indicated that DDC refeeding specifically affected the polyubiquitin 26S proteasome pathway responsible for the degradation of ubiquitinated proteins (Figure 7(b)). In addition, when mice were withdrawn from the DDC treatment, 26S proteasome activity recovered. However, when the mice were re-exposed to DDC for 7 days, the 26S proteasome activity was, again, significantly decreased (Figure 7(a and b)). These results indicated that there was a cellular memory of the first DDC exposure in the hepatocytes. An epigenetic mechanism was involved in the DDC-induced proinflammatory response and accumulation of polyubiquitinated proteins. Figure 7(b) shows that the addition of SAMe to the DDC diet, a major methyl donor in the remethylation pathway, prevented the inhibition of the 26S proteasome. It also prevented the accumulation of polyubiquitinated proteins caused by the inhibition of the 26S proteasome both by feeding DDC (Figure 7 c) and by refeeding DDC (Figure 7(d)), as determined by Western blot analysis. The proteasome subunit composition strongly influences the proteolytic functions of the proteasome complex. The types of proteins digested by the two types of proteasome are different.

To determine the mechanism of MDB formation and test the role of IFNγ induction in MDB formation, primary cell cultures, isolated from 1 month withdrawn DDC primed mice, were treated with IFNγ (200 U/ml) (Shenandoah, Warwick, PA) (Figure 8). The cultures were incubated for 6 days, and the cells were double stained for ubiquitin (Texas red) and FAT10 (FITC green). MDBs formed in gigantic hepatocytes (120 μm in diameter) after IFNγ treatment, and stained positive for ubiquitin (red) (Figure 8). The number of the MDBs in the hepatocytes treated with IFNg was 4 fold higher than the number of MDBs in the hepatocytes with no treatment (Figure 8 D).

DISCUSSION

SAMe has been shown to prevent MDB formation when added to the DDC refeeding diet (Li et al., 2008). The mechanism by which SAMe prevents MDBs is an epigenetic mechanism, because DDC drug refeeding caused a decrease in trimethylation of histone 3 lysine 4 and histone 3 lysine 9 (H3K4 and K9) (Bardag-Gorce et al., 2008), and SAMe prevented this decrease (Bardag-Gorce et al., 2008). In addition, the DDC diet caused a significant down regulation of SAMe metabolizing enzymes, i.e. Mat2α, AMD, AHCY and Mthfr, and particularly GNMT; The later is responsible for SAMe methyl transfer. SAMe treatment prevented the decreased expression of all these enzymes caused by DDC feeding (Bardag-Gorce et al., 2008). Betaine treatment, which generates methionine synthesis from homocysteine by increasing the activity of betaine homocysteine methyltransferase (BHMT), also prevented the decreased expression of these enzymes as well as BHMT, which is also decreased by DDC refeeding (Oliva et al., 2009b).

The disruption of the remethylation pathway is a major cause of changes in gene expression. A previous report, using microarray analysis, showed dramatic changes in the expression of a large number of genes when DDC was fed or refed. When SAMe was added to the DDC

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diet, the changes in gene expression were prevented (Bardag-Gorce et al., 2008). Of importance here is that FAT10 up regulation by DDC was also prevented by SAMe treatment (Oliva et al., 2008).

Since FAT10 was reported to be jointly over expressed with the IFNγ and TNFα induced immunoproteasome subunit LMP2 (Lukasiak et al., 2008), the focus became the effect of DDC on proteasome activity where it was shown that DDC decreased the activity of the 26S proteasome.

The induction of the immunoproteasome catalytic subunits caused the immunoproteasome formation and decreased the formation of the 26S proteasome. These changes would lead to a reduction in the rate of degradation of ubiquitinated proteins by the 26S proteasome and an increase in the formation of the immunoproteasome. A reduction in the turnover of CK-8 and CK-18 could result in aggregation of accumulating cytokeratins leading to MDB formation in these cells. The cleavage site specificity of the immunoproteasomes is required for efficient antigen processing and presentation by major histocompatibility complex (MHC) class I molecules (Groettrup et al., 1996; Groettrup et al., 1997; Busse et al., 2008).

The results showed an increase in gene expression of the TNFα and IFNγ receptors in the liver of DDC refed mice. Since the immunoproteasome subunits LMP2, LMP7, and MECL-1, have all been shown to be up regulated by TNFα and IFNγ (Kloetzel, 2001), it was postulated that DDC refeeding caused an enhancement of proinflammatory cytokines, which trigger the switch of the constitutive 26S proteasome to the immunoproteasome. The immunoproteasome does not degrade damaged and ubiquitinated proteins, such as the cytokeratins. The 26S proteasome activity responsible for ATP-ubiquitin degradation of proteins was significantly inhibited when the mice were fed or refed DDC. This would cause an accumulation of altered and ubiquitinated proteins, which would aggregate to form MDBs. Indeed, a striking increase in ubiquitinated proteins was induced by DDC and this change was blocked by SAMe feeding. TNFα was increased in the liver by DDC refeeding and this was prevented by SAMe feeding. NFκB is activated by TNFα to activate gene expression including proinflammatory genes (38). NFκB activation in the drug primed mice model has been previously described (Nan et al., 2005; Yuan et al., 2000; Nagao et al., 1998).

When SAMe was added to the DDC diet, the proinflammatory proteins were not up regulated, and the 26S proteasome activity was preserved. Moreover, the number of MDBs was significantly lowered (Li et al., 2008), and the accumulation of polyubiquitinated proteins with high molecular weight was also decreased. SAMe prevented the DDC up regulation of TNFα and IFNγ receptors, which correlates positively with the reported results by Veal *et al.* (Veal et al., 2004). The beneficial effects of SAMe in reducing the inflammatory response has also been reported by Ara *et al.* (Ara et al., 2008). It is reported here, for the first time, that SAMe supplementation preserved the ubiquitin 26S proteasome activity. Since the activation of the IFNγ gene expression has been demonstrated to be controlled by the methylation levels of IFNγ promoter (Dong et al., 2007), the effects of SAMe treatment may be the result of stimulating the global remethylation of histone and DNA to include the IFNγ promoter. This would lead to silencing the gene expression of IFNγ (Dong et al., 2007). Nevertheless, in vitro experiments need to be done using siRNA to determine the specific role of the 26S proteasome switch to the immunoproteasome where MDB are formed.

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List of Abbreviations

LMP2 and LPM7	Large Multifunctional Protease 2 and 7
MECL-1	Multicatalytic Endopeptidase Complex-Like 1
MDBs	Mallory-Denk Bodies
DDC	Dihydro-2,4,6,-trimethyl-3,5-pyridinedicarboxylate (DDC)
UPP	ubiquitin-proteasome pathway
SAMe	S-adenosylmethionine
UBL	ubiquitin-like

Acknowledgments

The authors would like to thank Mr. Emmanuel J. Gorce who worked on the word processing and the illustrations of the manuscript. This study was supported by the grant from NIH/NIAAA 8116 and the Alcohol Center grant to study liver and pancreas, PA50-1991.

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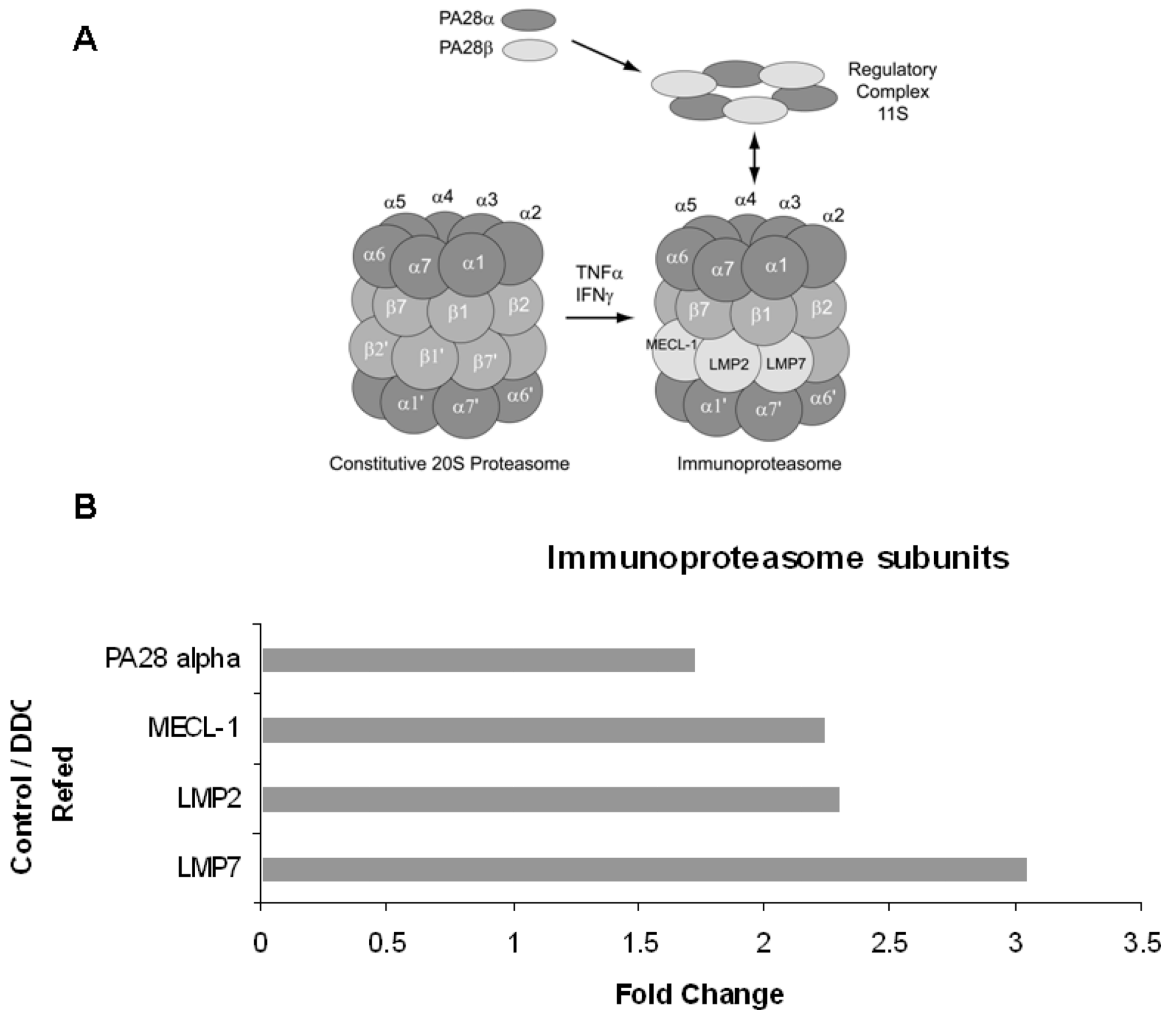

Figure 1.

A: Diagram showing the location of the immunoproteasome subunits, which substitute for the catalytic β1, β 2, and β 5 subunits of the 20S proteasome (26S proteasome); B: Gene microarray data mining showed that the immunoproteasome subunits LMP2, LMP7, and MECL 1, were up regulated by DDC drug refeeding. Note that the subunit PA28a of the 11S regulatory complex of the immunoproteasome was also induced.

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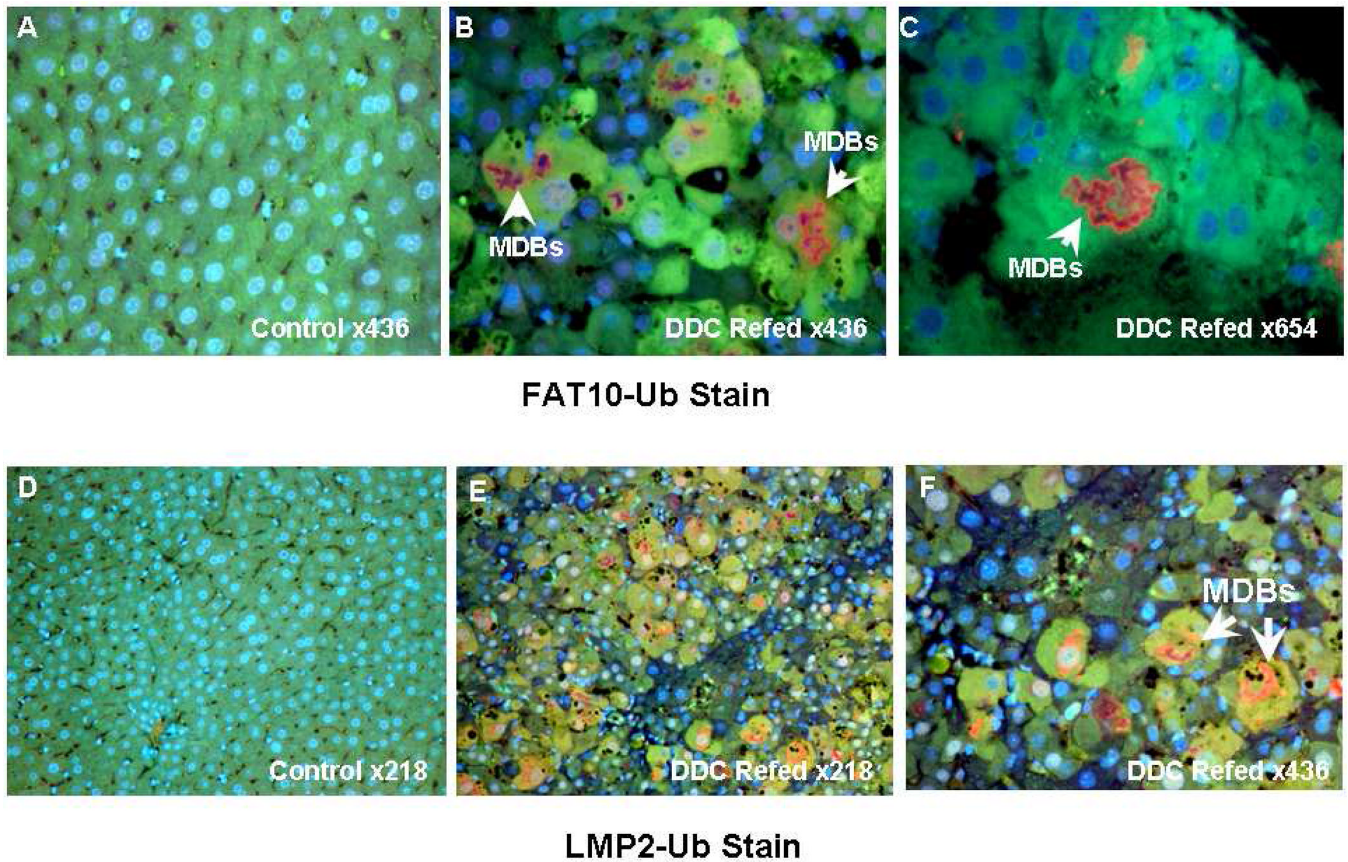

Figure 2.

Antibodies to FAT10 (green) and Ubiquitin (red) (A, B and C) were used to double stain hepatocytes from DDC treated mice. Note that the MDB forming cells cytoplasm stained positive for FAT10 (green). MDBs were stained with ubiquitin (red). Normal hepatocytes are scattered between FAT10 over expressing cells. D, E and F : LMP2 (green) and Ubiquitin (red) double staining of hepatocytes from DDC treated mice. Note that the MDB forming cells cytoplasm stained positive for LMP2 (green). MDBs were stained with ubiquitin (red). Some LMP2 over expressing cells that formed MDBs showed colocalisation with ubiquitin (yellow). MDBs showed colocalisation of ubiquitin and LMP2 at the border of the MDBs (yellow). Magnifications are written on each photograph.

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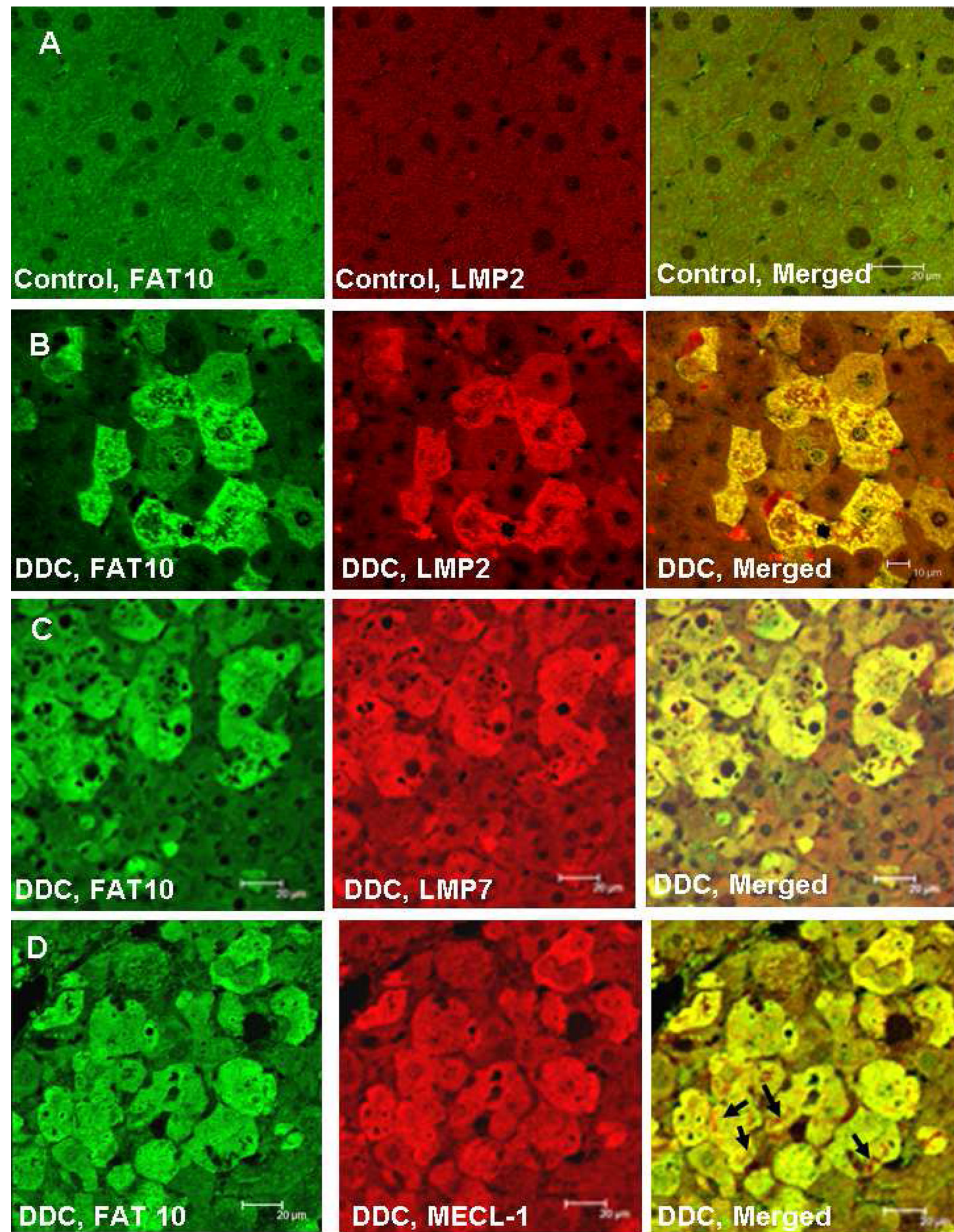

Figure 3.

Confocal microscopic analysis of LMP2-FAT10 (A and B), LMP7-FAT10 (C), and MECL-1-FAT10 (D) double antibody staining of hepatocytes from a DDC primed mouse liver. The merged photo (yellow) showed colocalisation of LMP2, LMP7 and MECL-1 with FAT10 in the cytoplasm and nuclei indicating that the hepatocytes forming MDBs had shown an up regulation of the immunoproteasome subunits. Control liver confocal images are shown for comparison (A), magnification bar is 10–20 um).

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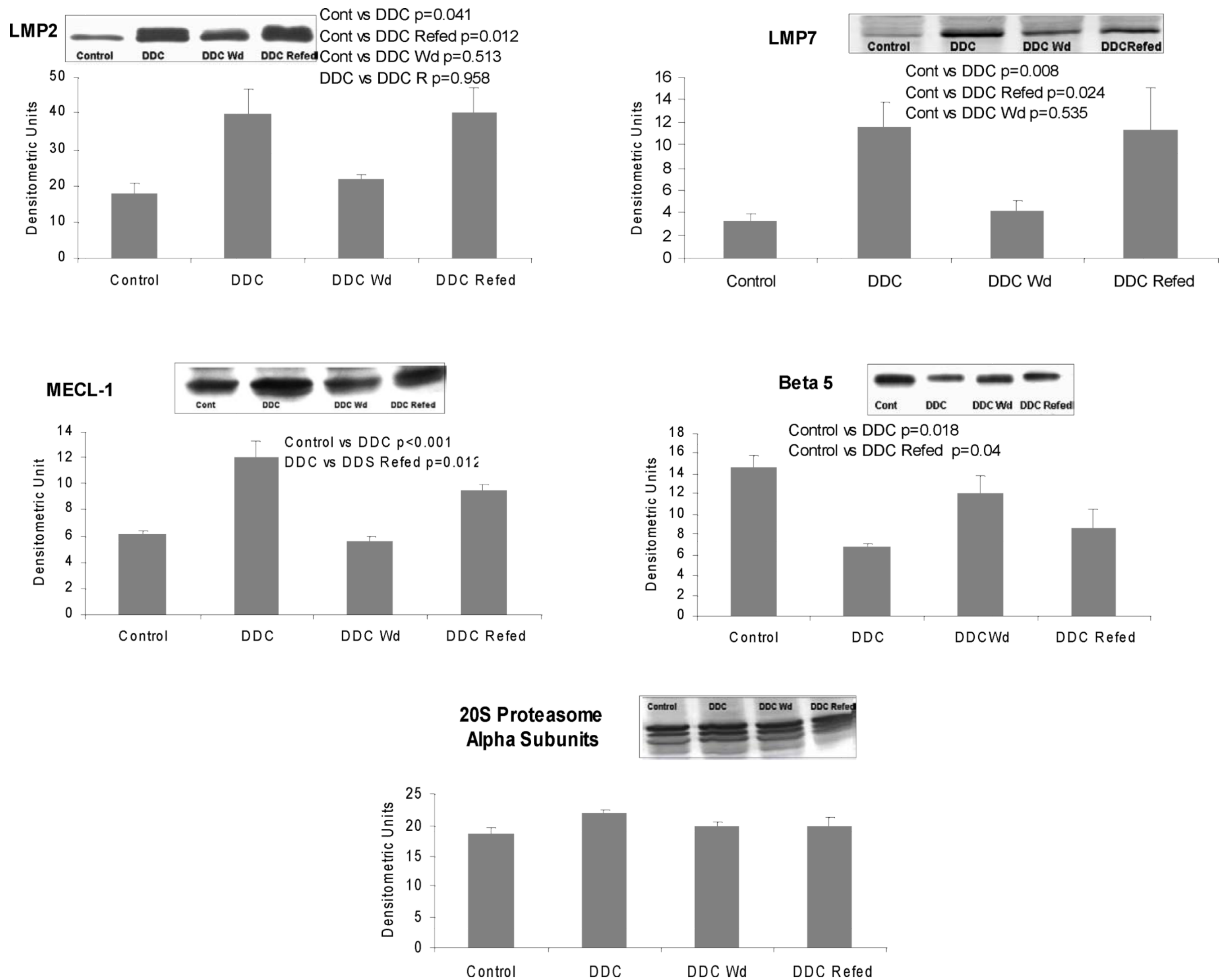

Figure 4.

DDC feeding for 10 weeks and DDC refeeding for 1 week caused a significant up regulation of the immunoproteasomes subunits LMP2, LPM7 and MECL-1. However the level of 26S proteasome beta5 subunit was decreased indicating there was a switch of the 26S proteasome to form the immunoproteasome. The proteasome alpha subunits did not change. (Mean +\− SEM, n=3).

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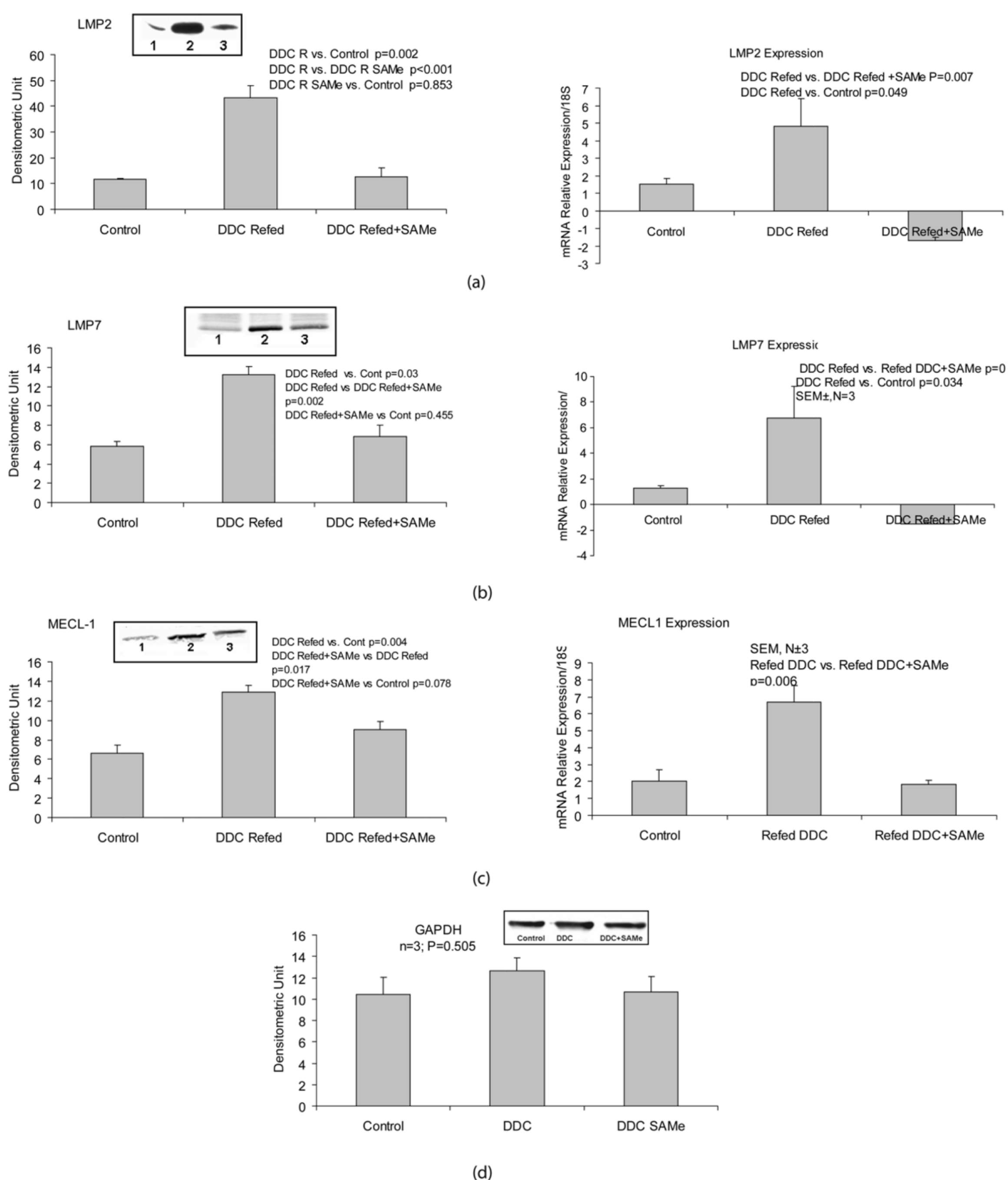

Figure 5.

Immunoproteasome LMP2, LMP7, and MECL-1 subunits induction in the liver homogenates of mice refed DDC and mice refed DDC + SAMe, as shown by Western blot ((a) and (c), left), and by RT-PCR ((b) and (c), right). (d) is the loading control. The striped membranes were stained for GAPDH antibody. When SAMe was fed to the mice along with DDC, there was a significant decrease in the levels of these subunits when compared to DDC refed mice livers. Mean ± SEM, n=3.

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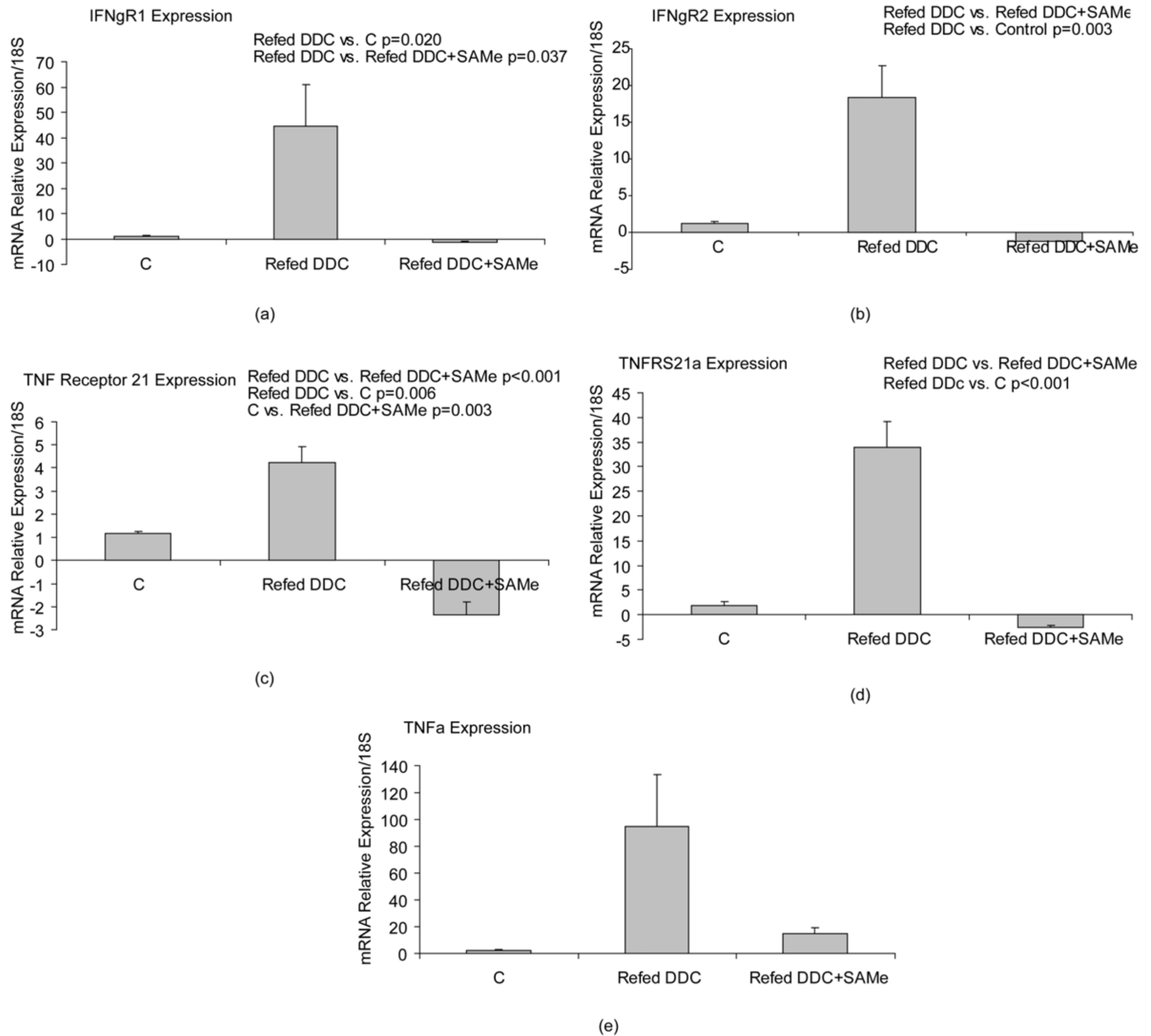

Figure 6.

The gene expression of the receptors for IFNγ, analyzed by PCR, showed a significant increase of both IFN receptor isoforms R1 and R2 (a,b). They form the IFN heterodimer in the liver of mice refed DDC. SAMe prevented this increase. Tumor necrosis factor (TNF) gene expression analyzed by PCR, showed a significant increase in TNFα, as well as the TNF receptors mRNA (c,d, and e), in the liver of mice refed DDC. SAMe prevented this increase. Mean ± SEM, n=3.

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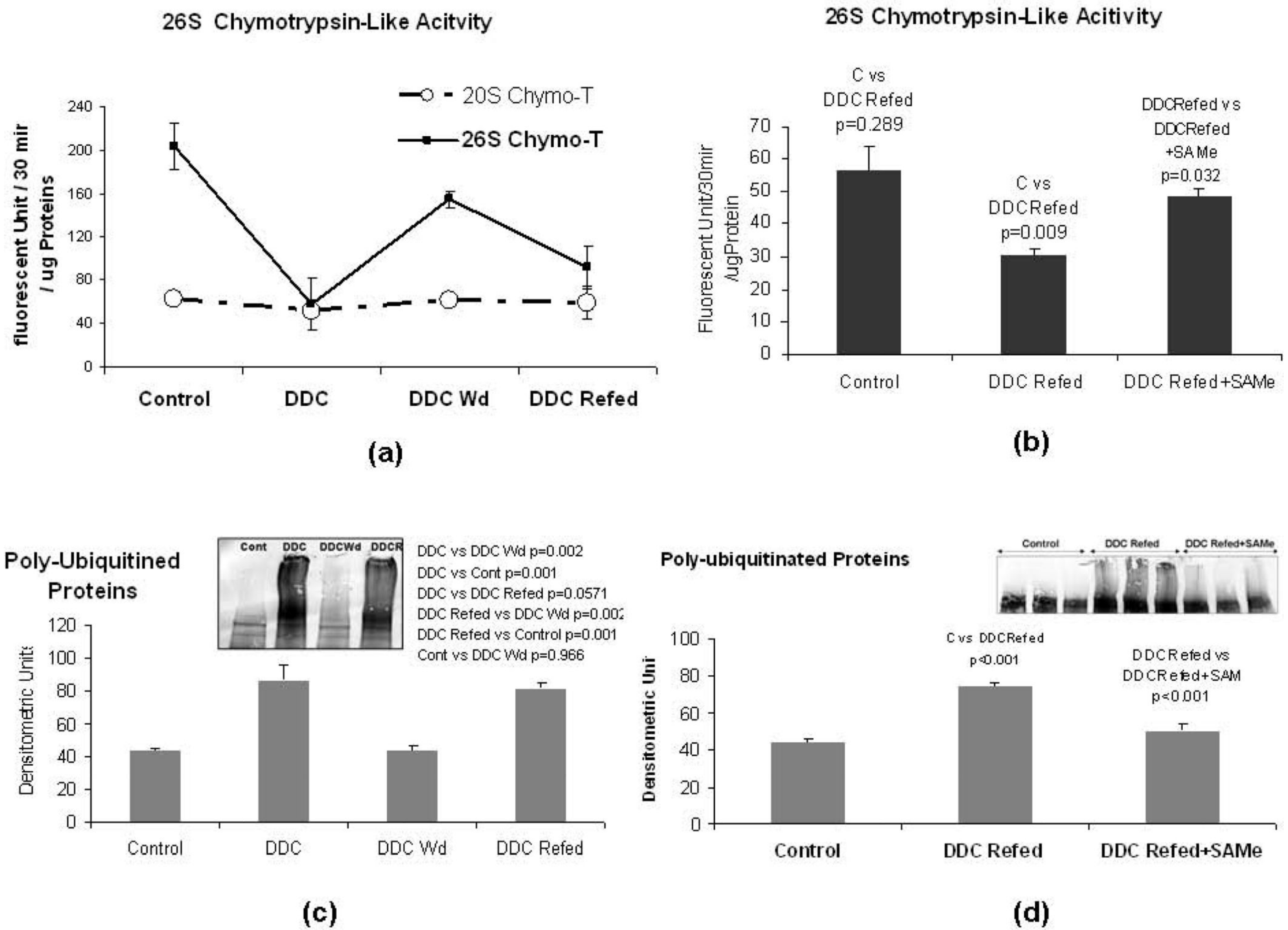

Figure 7.

(a) 26S proteasome activity in the cytosolic fraction from liver homogenates of mice fed DDC for 1 month (DDC), mice fed DDC 1 month, then withdrawn for 1 month (DDC Wd), and mice fed DDC, withdrawn, and then refed DDC for 7 days (Refed DDC). Control mice were fed the control diet. DDC feeding caused a significant 26S proteasome inhibition [control vs. DDC p=0.003, control vs. DDC Refed p=0.008, control vs. DDC Wd p=0.108, DDCWd vs. DDC p=0.018, DDCWd vs. DDC Refed p=0.049]. (b) 26S proteasome inhibition, induced by DDC, was prevented by the addition of SAMe in the DDC diet. (c) Accumulation of polyubiquitinated high molecular weight proteins due to DDC and to DDC refeeding (d), 26S proteasome inhibition, which causes the accumulation of polyubiquitinated proteins was prevented by SAMe supplementation. Mean ± SEM, n=3.

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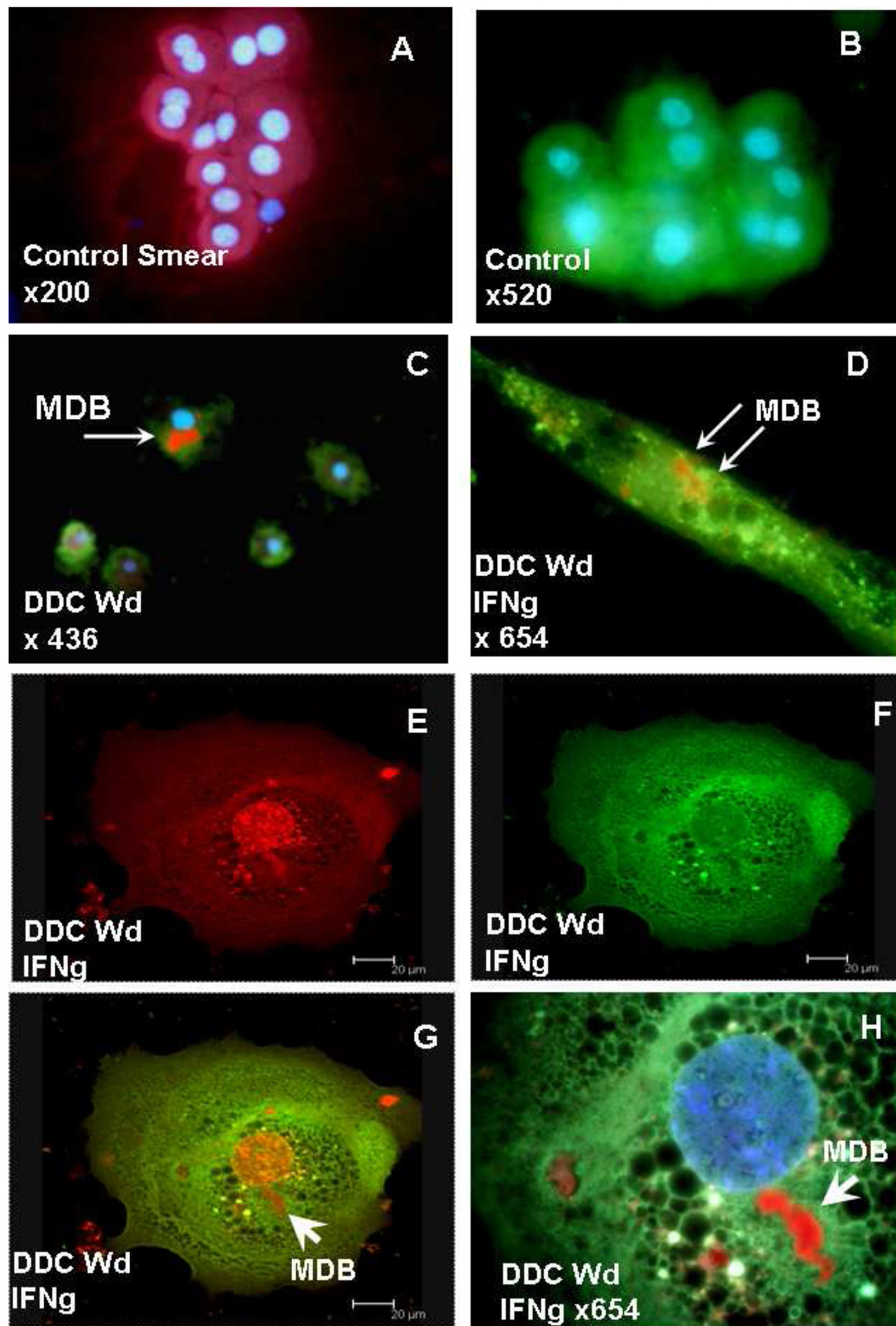

Figure 8.

Double stain for ubiquitin (Red) and CK-8 (Green) was performed on primary liver cells cultures grown for 6 days with or without IFNγ (100 U/ml) added. The cells were isolated from the liver from a DDC-primed mouse and a normal control mouse. A : Cell suspension of a control mouse showing a smear of isolated hepatocytes suspension. B: Control liver cells after 6 days in culture. C is the hepatocytes from the DDC drug-primed mouse. D is the DDC drug primed mouse hepatocytes treated with IFNg. E, F, G and H: Double stain for ubiquitin (Red) and FAT10 (Green) was performed on cover slips of primary liver culture cells grown for 6 days in the presence of 100 U/ml of IFNγ. The confocal microscopy analysis of a giant hepatocyte (120 µm in diameter), which had formed a Mallory-Denk

body (MDB, arrow), is illustrated in the merged picture (magnification bar is 20 um) (G). Note that the nucleus stained positive for ubiquitin (Red) and FAT10 (Green) with colocalisation (orange). MDBs that formed in the giant liver cell (arrows) were best seen using a Nikon triple color filter (H). The nucleus stained blue with DAPI.

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