

Regulation of dipeptidyl peptidase 8 and 9 expression in activated lymphocytes and injured liver

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(B220⁺), human lymphoma cell lines and mouse splenocytes stimulated with pokeweed mitogen (PWM) or lipopolysaccharide (LPS), and in dithiothreitol (DTT) and mitomycin-C treated Raji cells. DPP8 and DPP9 expression were measured in epidermal growth factor (EGF) treated Huh7 hepatoma cells, in fibrotic liver samples from mice treated with carbon tetrachloride (CCl₄) and from multidrug resistance gene 2 (*Mdr2/Abcb4*) gene knockout (gko) mice with biliary fibrosis, and in human end stage primary biliary cirrhosis (PBC).

RESULTS: All three lymphocyte subsets expressed DPP8 and DPP9 mRNA. DPP8 and DPP9 expression were upregulated in both PWM and LPS stimulated mouse splenocytes and in both Jurkat T- and Raji B-cell lines. DPP8 and DPP9 were downregulated in DTT treated and upregulated in mitomycin-C treated Raji cells. DPP9-transfected Raji cells exhibited more annexin V⁺ cells and associated apoptosis. DPP8 and DPP9 mRNA were upregulated in CCl₄ induced fibrotic livers but not in the lymphocytes isolated from such livers, while DPP9 was upregulated in EGF stimulated Huh7 cells. In contrast, intrahepatic DPP8 and DPP9 mRNA expression levels were low in the *Mdr2* gko mouse and in human PBC compared to non-diseased livers.

CONCLUSION: These expression patterns point to biological roles for DPP8 and DPP9 in lymphocyte activation and apoptosis and in hepatocytes during liver disease pathogenesis.

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Abstract

AIM: To investigate the expression of dipeptidyl peptidase (DPP) 8 and DPP9 in lymphocytes and various models of liver fibrosis.

METHODS: DPP8 and DPP9 expression were measured in mouse splenic CD4⁺ T-cells, CD8⁺ T-cells and B-cells

Key words: Dipeptidyl peptidase; CD26; Lymphocytes; Liver fibrosis; Biliary fibrosis

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INTRODUCTION

The four enzyme members of the dipeptidyl peptidase (DPP) 4 gene family, *DPP4*, fibroblast activation protein (*FAP*), *DPP8* and *DPP9*, have attracted considerable research interest in recent years since *DPP4* inhibitors became a successful therapy for type 2 diabetes^[1,2]. *FAP* is a potential cancer therapeutic target^[2,3]. *DPP4*, the most well characterized family member, has ubiquitous cell surface and extracellular expression^[2,4-7]. *DPP8* and *DPP9* are the most recently discovered members of the *DPP4* gene family^[8-11]. *DPP4*, *DPP8* and *DPP9* are ubiquitously expressed cytosolic enzymes with *DPP4*-like activity^[8,11,12]. They are expressed by major epithelial organs including liver, colon, small intestine, stomach, lung, skin, tongue, kidney, testis and the lymphoid cells of lymph node, blood, thymus, and spleen^[13]. The biological functions of *DPP8* and *DPP9* are largely uncharacterized.

DPP4 is also known as *CD26* and has important roles in the immune system. It is a costimulatory molecule in T cell activation and proliferation and is critical in the development of T helper 1 responses to foreign antigens. It is expressed at detectable levels by some resting T cells but the cell surface expression increases 5-10 fold following stimulation with antigen or anti-CD3⁺ interleukin-2 or with mitogens such as phytohemagglutinin^[14-19]. However, the costimulatory role of *DPP4/CD26* is mediated by extra-enzymatic activities^[20-22]. Hence, some of the immunological effects observed in early *DPP4* inhibitor studies are now thought to be due to off-target non-selective inhibition of *DPP8* and *DPP9*^[2,23,24]. In support of this viewpoint, there is some evidence that *DPP8* and *DPP9* are functionally significant in the immune system. Their mRNA levels are elevated in activated human leukocytes^[25,26]. An inhibitor of *DPP8* and *DPP9* attenuates proliferation in *in vitro* models of human T-cell activation^[23]. An inhibitor selective for *DPP8* and *DPP9* vs related proteases can suppress DNA synthesis in mitogen-stimulated splenocytes from both wildtype *DPP4*^{+/+} and *DPP4*^{-/-} gene knockout (gko) mice^[27]. Moreover, *DPP8* and *DPP9* have been implicated in hematopoiesis and in inflammatory diseases including arthritis^[2,28,29]. Most importantly, *DPP8* and *DPP9* are involved in processing and degradation of peptides involved in antigen presentation by Major histocompatibility complex class I^[30].

Inflammatory and immune responses are important in liver injury. Improved understanding of immune response, inflammation and fibrogenic progression is needed to advance the understanding of liver disorders. *DPP8* and *DPP9* are expressed in hepatocytes and lymphocytes of human cirrhotic liver^[13]. Hepatocytes in the periportal area of regenerative nodules and lymphocytes in the portal tracts are strongly positive for *DPP8* and *DPP9* *in situ*

hybridization (ISH)^[13]. Bile ducts and ductular reactions are ISH positive for *DPP9* but not for *DPP8*^[13]. However, the role of *DPP8* and *DPP9* in liver is unknown. Other members of this protease family, *DPP4* and *FAP*, are altered in liver diseases and are potential disease markers and therapeutic targets^[31-36]. Despite the pleiotropic roles of *DPP4* and *FAP* in various biological processes, *DPP4* and *FAP* gko mice exhibit no spontaneous defects, suggesting that *DPP4* and *FAP* are not essential for normal functions, and hence, targeting them is likely to lack adverse side effects^[37,38].

DPP8 and *DPP9* have interesting properties in cell biological processes that may contribute to disease pathogenesis, such as apoptosis and cell migration^[39,40]. Their biological functions, especially in the immune system, are important considerations for the selectivity of *DPP4* inhibitors over *DPP8* and *DPP9* in clinical development of DPP antagonists. Here we studied the expression of *DPP8* and *DPP9* in lymphocyte activation, proliferation and apoptosis and in liver injury to elucidate their potential biological roles in the immune system and in liver diseases.

MATERIALS AND METHODS

Materials

Antibodies are detailed in Table 1. Other materials were from Sigma-Aldrich (St Louis, MO, United States) unless stated.

Animal studies

Mice were maintained in the Centenary Institute animal facility under specific pathogen-free conditions. The Animal Ethics Committee of the University of Sydney approved experimental procedures and housing arrangements. *FAP* gko^[38] and *DPP4* gko mice^[37] (C57BL/6J background) were bred at the Animal Resource Centre (Perth, Australia). Female multidrug resistance gene 2 (*Mdr2/Abcb4*) gko mice (FVB/N background) with targeted disruption of *Mdr2*, were obtained from Jackson Laboratory (Jackson Laboratory, Bar Harbor, ME, United States)^[35]. Liver samples from the *Mdr2* gko and wild type (wt) mice were obtained at 4, 8 and 12 wk after birth, the time points that span the most active fibrosis progression^[35]. RNA were obtained as previously described^[41]. Lymphocytes from wt, *DPP4* gko and *FAP* gko mouse spleen, liver and lymph nodes were isolated as previously described^[42].

For the liver fibrosis mouse model, 8-wk-old female wt, *DPP4* gko and *FAP* gko mice were injected intraperitoneally with carbon tetrachloride (CCl₄) twice weekly for 3 wk. Each dose comprised 5.36 μ L of 12% CCl₄ (in paraffin oil) per gram of initial weight of each mouse. Significantly elevated alanine aminotransferase (ALT) (68 \pm 11.1 U/L vs untreated controls 32 \pm 1.2 U/L) indicated liver injury. ALT was performed by an auto-analyzer at the Clinical Biochemistry Department of the Royal Prince Alfred Hospital. Organs were collected 3 d after the final CCl₄ treatment.

Table 1 Antibodies used in immunoblot and flow cytometry

Name	Isotype	Conjugate	Dilution	Supplier	Catalogue no.
Primary antibodies					
CD4	Rat IgG _{2b}	FITC	1:50	BD Pharmingen, NJ, United States	557307
B220	Rat IgG	PE	1:50	Caltag Laboratories, CA, United States	RM2604-3
CD8	Rat IgG _{2a}	APC	1:50	BD Pharmingen, NJ, United States	553035
Annexin V		APC	1:50	BD Pharmingen, NJ, United States	550474
V5	Mouse monoclonal IgG _{2a}		1:5000	Invitrogen	R960-25
DPP8-catalytic domain	Rabbit polyclonal		1:2000	Abcam Inc	Ab42077
DPP8-catalytic domain	Rabbit polyclonal		1:2000	Abcam Inc.	Ab42076
DPP9-catalytic domain	Rabbit polyclonal		1:2000	Abcam Inc.	Ab42080
GAPDH	Mouse monoclonal		1:1000	EnCor biotechnology	MCA-1D4
β-actin	Rabbit polyclonal		1:1000	Sigma	A2103
Secondary antibodies					
Anti-rabbit IgG	Goat	HRP	1:3000	DAKO, Carpinteria, CA, United States	PO448
Anti-mouse	Goat IgG	R-PE	1:400	Molecular Probes	P852

DPP: Dipeptidyl peptidase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IgG: Immunoglobulin G; FITC: Fluorescein; PE: Phycoerythrin; APC: Allophycocyanin; HRP: Horse radish peroxidase; R-PE: R-phycoerythrin.

Human liver samples

Human liver tissues were obtained from liver transplant recipients in accordance with National Health and Medical Research Council guidelines under Royal Prince Alfred Hospital Human Ethics Committee approvals. Non-diseased liver donors had an age range of 56-58 years and mixed genders. Cirrhotic livers were from primary biliary cirrhosis (PBC) patients of average age 51.7 ± 13.3 years (range 27-67 years; 10 females, 2 males) and end stage alcoholic liver disease patients of average age 49.3 ± 8 years (range 34-60 years, 9 males) as described previously.^[40]

In vitro stimulation assays

Human B lymphocyte Burkitt's lymphoma cell line (Raji) (ATCC, CCL-86) and human T cell leukemia cell line (Jurkat) (ATCC, TIB-153) were cultured in Roswell Park Memorial Institute (RPMI) Medium 1640 (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal calf serum (FCS) and Penicillin-Streptomycin (100 units of penicillin and 100 µg/mL of streptomycin) ($1 \times$ P/S) and human liver hepatocellular carcinoma cell line Huh7 were grown in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% FCS and $1 \times$ P/S.

Lymphocytes at 1×10^6 cells/mL RPMI were treated with either 5 µg/mL pokeweed mitogen (PWM), 20 µg/mL lipopolysaccharide (LPS), 50 µg/mL Mitomycin C or 10 mmol/L dithiothreitol (DTT). Human liver hepatocellular carcinoma cell line, Huh7 cells were serum starved for 20 h before stimulation with 0, 1, 10, 100 ng/mL epidermal growth factor (EGF; R-D Systems, MN, United States) for 4 h.

Apoptosis assay

To determine if *DPP9* overexpression induces apoptosis, Raji cells were transiently transfected with wt*DPP9*-V5-His, mut*DPP9*-V5-His or vector control (pcDNA3.1/V5-HisA; Invitrogen, Carlsbad, CA, United States) as described previously,^[39] then cultured. The lymphocytes

were transfected by electroporation using Amaxa® Cell Line Nucleofector® Kit V (Lonza, Basel, Switzerland) on a Lonza-amaxa Nucleofector device (Lonza). Forty hours post transfection, cells were washed with annexin binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl₂, pH 7.4). Staining involved incubating cells with annexin V antibody (Table 1) for 30 min at room temperature in the dark followed by 4',6-diamidino-2-phenylindole (DAPI), Sigma Aldrich) at 100 ng/mL. Cells were enumerated using flow cytometry. Analysis was performed using FlowJo software (Tree Star Inc., Ashland, OH, United States).

Fluorescence activated cell sorting

To isolate mouse lymphocyte subsets, 3×10^7 splenocytes were resuspended in primary antibody diluted in phosphate buffered saline (PBS) containing 1% FCS and incubated in the dark, on ice for 30 min. The primary antibodies used are listed in Table 1. Following antibody staining, cells were washed with PBS containing 1% FCS. Cells underwent a final resuspension of 2×10^7 cells/mL of PBS with 5% FCS and 2 mmol/L ethylene diamine tetraacetic acid (EDTA) to minimize clumping of cells. Twenty-five µL/mL of DAPI was added prior to cell sorting. Cell sorting was performed using the Fluorescence Activated Cell Sorting Vantage™ SE (BD Bioscience, NJ, United States). Cells were gated to exclude doublets and DAPI⁺ (dead) cells. Three-way sort was performed to collect CD4⁺ cells, CD8⁺ cells and B220⁺ cells into separate collection tubes.

Real time quantitative polymerase chain reaction

RNA from cells was extracted using the RNAqueous-Micro™ kit (Ambion, TX, United States) following manufacturer's instructions. Total RNA (1 µg) was then reverse-transcribed to cDNA using 10 pmol of oligo(dT)₁₂₋₁₈ primer (Invitrogen, Carlsbad, CA, United States), 10 mmol/L deoxyribonucleotide triphosphates and SuperScript III reverse transcriptase (Invitrogen). Real time quantitative poly-

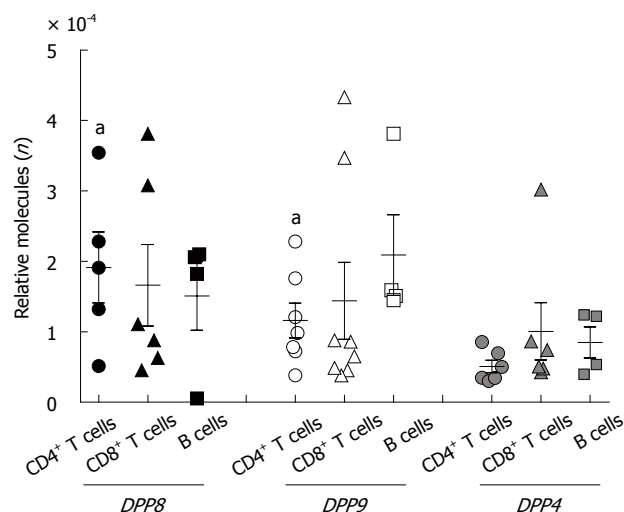


Figure 1 Dipeptidyl peptidase mRNA expression in C57BL/6 mouse splenic lymphocyte subpopulations. Number of molecules relative to 18S RNA ($n = 4-6$ mice). ^a $P < 0.05$ vs dipeptidyl peptidase (*DPP*) 4.

merase chain reaction (PCR) by Taqman[®] gene expression assays was performed using the Stratagene[®] Mx3000PTM System (La Jolla, CA, United States) according to manufacturer's recommendations. Taqman primers used for the assays were mouse *DPP4* (Mm00494548_mL), *DPP8* (Mm00547049_mL) and *DPP9* (Mm00841122_mL). The samples were run in duplicates. The gene expression level was analyzed using a standard curve of serially diluted known numbers of molecules of the same gene and then normalized relative to 18S (Hs99999901_s1). Quantitative PCR on human samples were performed using sequence detector (Prism, model 7700; Life Technologies, NY, United States) and were analyzed using sequence detector software (Prism, Version 1.6.3; Applied Biosystems Inc.). Primers used for human *DPP8* were forward: 5' CCAGATGGACCTCATTCAGACAG-3' and reverse: 5'GGTTGTTGCGTAAATCCTTGTGG-3' and for human *DPP9* were forward: 5'AGAAGCACCCACCGTCCTCTTTG-3' and reverse: 5'AGGACCAGCCATGGATGGCAACTC-3'. The number of molecules was normalized with human aldolase B (forward: 5'-CCTC-GCTATCCAGGAAAAC-3' and reverse: 5'TTGTAGACAGCAGCCAGGAC-3').

Immunoblotting assay

Cells were washed with ice-cold PBS three times and then lysed with ice-cold lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 1mmol/L MgCl₂, 300 μ L of 150 mmol/L NaCl, 1% Triton-114, 10% glycerol and 1 \times Roche complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and stored at -80 $^{\circ}$ C. Protein concentration was determined using the micro BCA protein assay kit (Thermo Scientific, CA, United States) following the manufacturer's protocol. 50 μ g total of each cell lysate in LDS sample buffer (catalogue No. NP0007, Invitrogen) with reducing agent (catalogue No. NP0004, Invitrogen) in conditions that retain *DPP8* and *DPP9* dimerization^[8,9,40] was resolved on 3%-8%

Tris-acetate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen) followed by immunoblotting. Antibodies for immunoblotting are listed in Table 1. Relative band intensities were quantified using Image J and normalized against control proteins as indicated^[40].

Statistical analysis

Results are expressed as individual replicates. Horizontal lines represent mean and error bars represent standard error. Differences among groups were analyzed using Mann-Whitney *t*-test by GraphPad Prism 5 software. *P* values < 0.05 were considered significant.

RESULTS

To investigate which lymphocyte subsets express *DPP8* and *DPP9*, their transcript levels were quantified in the major lymphocyte subpopulations, CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells and B220⁺ (B cells) from normal C57BL/6 mouse splenocytes. All three lymphocyte subsets expressed *DPP8* and *DPP9* mRNA (Figure 1). *DPP8* and *DPP9* transcripts were expressed to significantly greater levels than *DPP4* transcripts in CD4⁺ T cell subpopulation ($P = 0.02$) and *DPP9* mRNA was significantly more abundant than *DPP4* mRNA in B cells ($P = 0.03$).

To examine whether, like *DPP4*^[7], *DPP8* and *DPP9* are upregulated upon lymphocyte activation, mouse splenocytes were stimulated *in vitro* with PWM^[43-45] and LPS^[46,47]. *DPP8* and *DPP9* mRNA was markedly upregulated in PWM stimulated mouse splenocytes in a time dependent manner (Figure 2A). To examine whether the increased mRNA levels corresponded to protein expression, *DPP8* and *DPP9* proteins were measured in Jurkat (T cells) stimulated in-vitro with PWM. Both *DPP8* and *DPP9* were upregulated in a time dependent manner (Figure 2B and C).

Similarly, mRNA levels of *DPP8* and *DPP9* were upregulated in LPS stimulated mouse splenocytes (Figure 3A). Also, LPS stimulated Raji (B cells) had upregulated *DPP8* and *DPP9* protein expression in a time dependent manner (Figure 3B and C).

Immunoblots of *DPP8* exhibited a slow mobility band at 150-180 kDa, which probably represents dimer or processed dimer, in addition to the faster mobility bands at 95-100 kDa that are likely to be monomer and truncated or trimmed monomer (Figure 2B and 3B). *DPP9* showed a slow mobility band of monomer at 110 kDa and faster mobility bands, which are possibly truncated or trimmed monomers at 75-95 kDa (Figure 2C and 3C)^[8,9,40,48]. The intensity of all three *DPP8* bands increased in a time dependent manner with PWM stimulation in Jurkat cells (Figure 2B). However, in LPS stimulated Raji cells the intensity of only the 150 kDa band increased in a time dependent manner (Figure 3B). The intensity of all the *DPP9* bands increased with time in both PWM stimulated Jurkat cells and LPS stimulated Raji cells (Figure 2C and 3C). In PWM stimulated Jurkat cells, *DPP8* and *DPP9* expression both peaked at 48 h (Figure 2B and C). In Raji cells, increased expression of *DPP8* was observed at 72 h post LPS stimulation (the longest time point of the

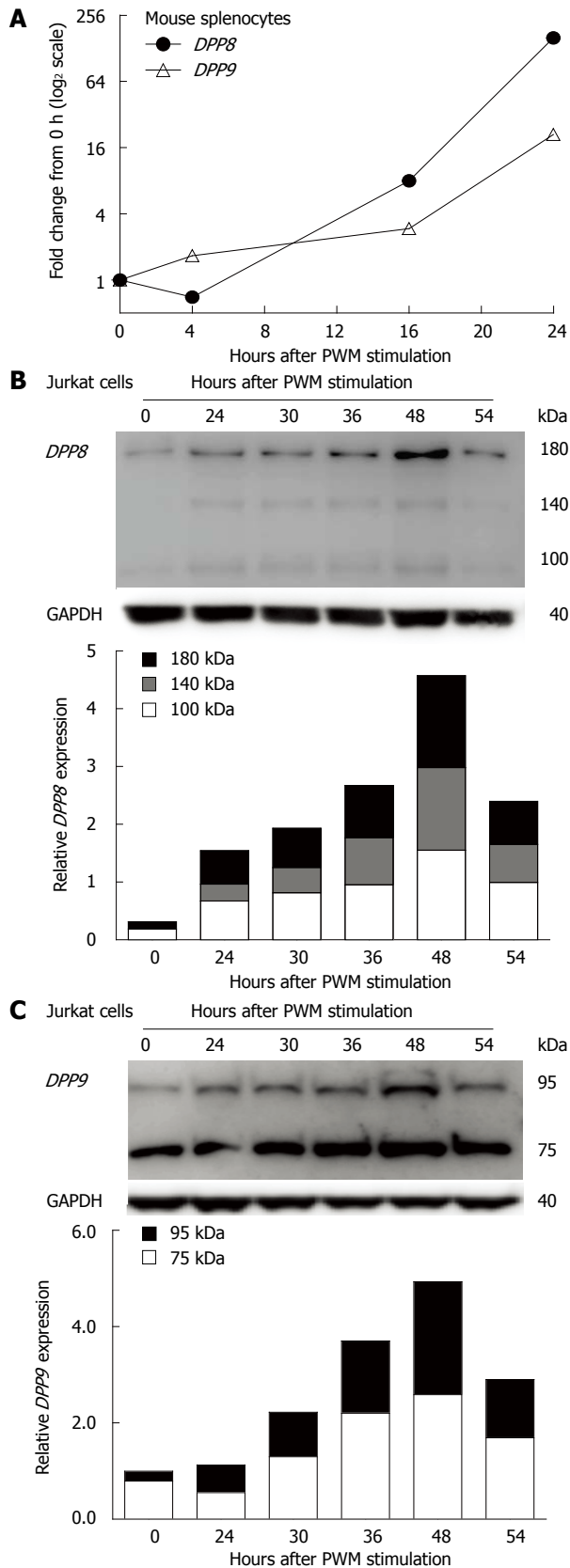


Figure 2 Dipeptidyl peptidase 8 and dipeptidyl peptidase 9 upregulation in pokeweed mitogen stimulated lymphocytes. A: Dipeptidyl peptidase (DPP) 8 and DPP9 mRNA in mouse splenocytes (representative data from one of three mice); B: Immunoblot of DPP8 and densitometry analysis of DPP8 bands; C: Immunoblot of DPP9 and densitometry analysis of bands. Densitometry data shown are relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PWM: Pokeweed mitogen.

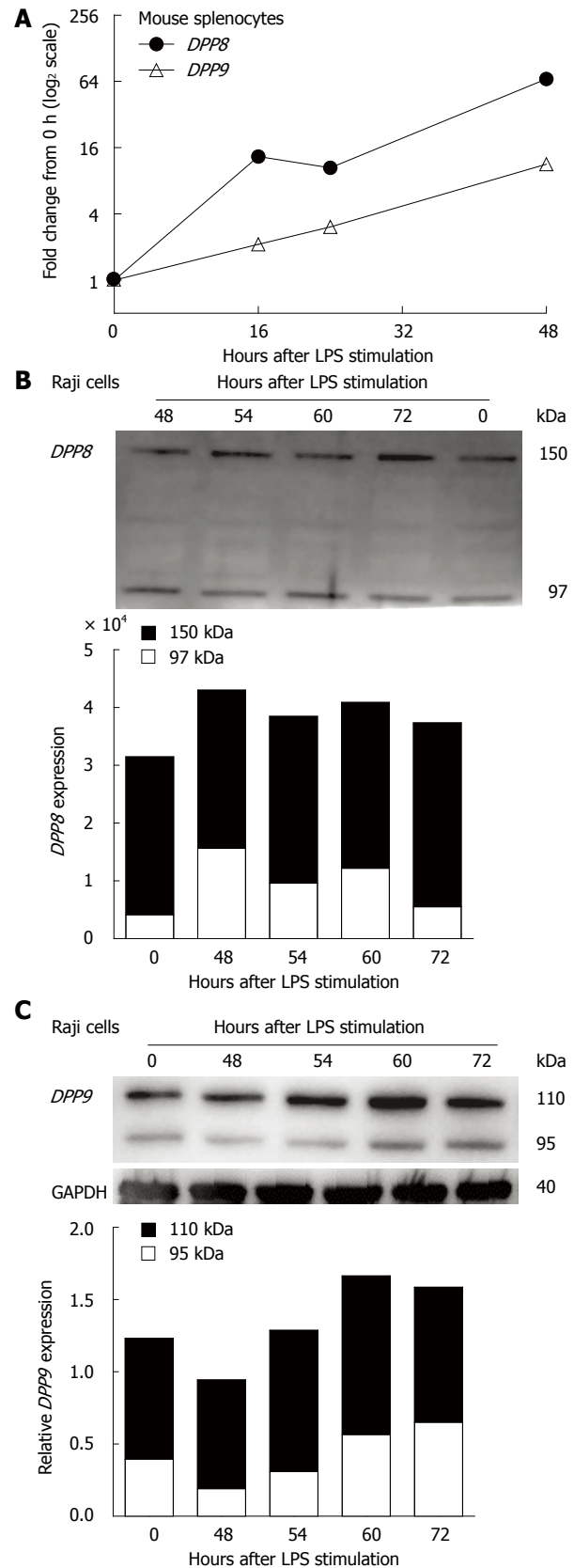


Figure 3 Dipeptidyl peptidase 8 and dipeptidyl peptidase 9 upregulation in lipopolysaccharide stimulated lymphocytes. A: Dipeptidyl peptidase (DPP) 8 and DPP9 mRNA in mouse splenocytes (representative data from one of three mice); B: Immunoblot of DPP8 and densitometry analysis of DPP8 bands; C: DPP9 immunoblot and densitometry analysis of DPP9 bands relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). LPS: Lipopolysaccharide.

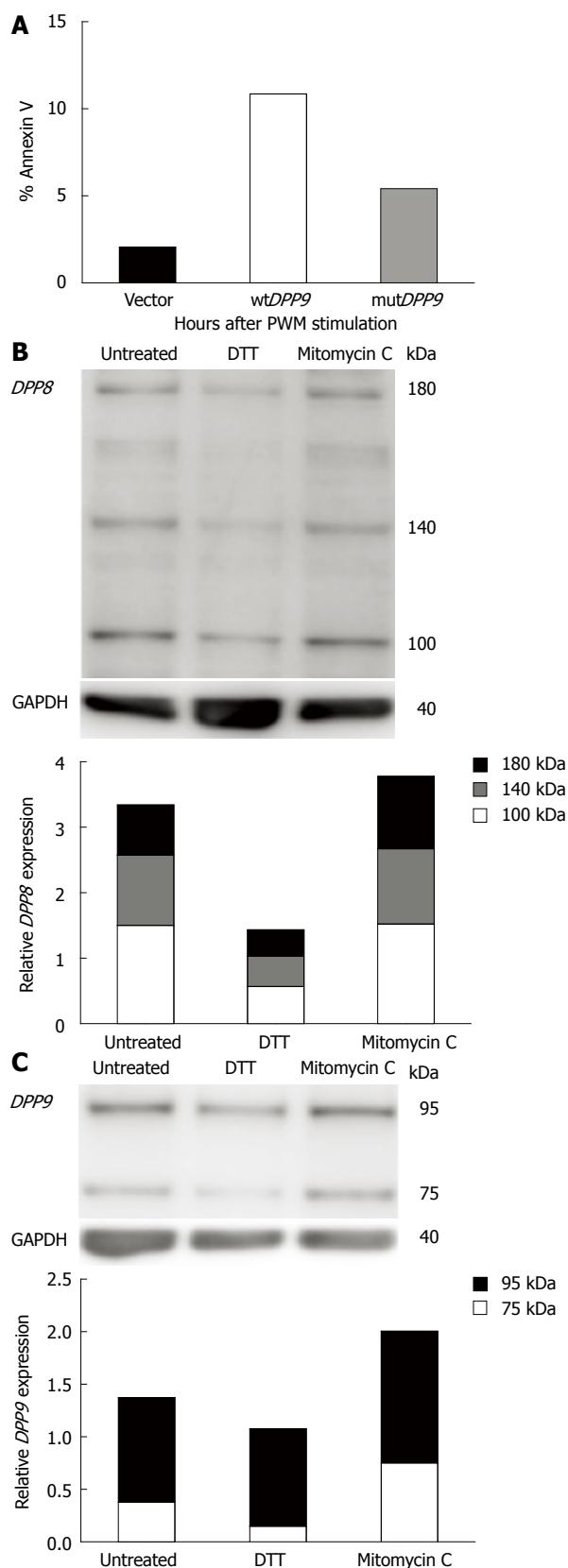


Figure 4 Diptidyl peptidase 8 and diptidyl peptidase 9 were associated with lymphocyte apoptosis. **A:** Percentage of annexin V + Raji cells 40 h after transfection with vector, wild type (wt) diptidyl peptidase (*DPP*) 9-V5-His or enzyme-negative mutant (mut) *DPP*9-V5-His. Annexin V staining was enumerated by flow cytometry; **B:** Immunoblot of *DPP*8 and its densitometry (**C**) immunoblot of *DPP*9 and its densitometry in Raji cells untreated and treated with dithiothreitol (DTT) or mitomycin C for 24 h. Densitometry are shown as relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

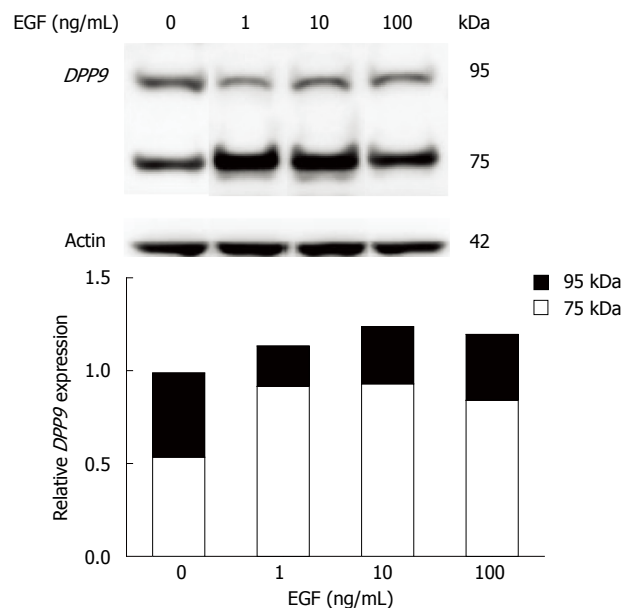


Figure 5 Diptidyl peptidase 9 upregulation in epidermal growth factor treated Huh7 cells. Diptidyl peptidase (*DPP*) 9 immunoblot of untreated and epidermal growth factor (EGF)-treated Huh7 cells at 4 h. Cells were serum starved overnight before EGF treatment. Densitometry of *DPP*9 is shown relative to actin.

study) (Figure 3B), and *DPP*9 expression peaked at 60 h (Figure 3C).

*DPP*8 and *DPP*9 in lymphocyte apoptosis

We have previously shown that *DPP*9 overexpression induces intrinsic cell apoptosis in human hepatoma and embryonic kidney cell lines^[39,40]. Similar to epithelial cells, *DPP*9 overexpression induced increased cell death in Raji cells (Figure 4A). This effect was less pronounced when Raji cells were transfected with mutant *DPP*9 lacking *DPP* activity (Figure 4A), suggesting that the enzyme activity of *DPP*9 influences lymphocyte apoptosis.

Interestingly, Raji cells treated with DTT, an antioxidant that impairs cell apoptosis, had less *DPP*8 and *DPP*9 expression compared to untreated cells (Figure 4B and C). Conversely, treatment of Raji cells with mitomycin C, a lectin that impairs cell proliferation, resulted in increased *DPP*8 and *DPP*9 expression in Raji cells (Figure 4B and C). Intensities of all *DPP*8 and *DPP*9 band sizes were less with DTT treatment and greater with Mitomycin C treatment compared to untreated cells (Figure 4B and C).

*DPP*9 in EGF stimulated hepatocytes

EGF is a regulatory factor in cell survival, growth, proliferation and differentiation^[49]. Previously, we have shown that *DPP*9 overexpression impairs EGF-stimulated cell proliferation in HepG2 and Huh7 human hepatoma cell lines^[40]. *DPP*9 expression at 75 kDa was greater in Huh7 cells after EGF stimulation (Figure 5). This study expands the association of *DPP*9 with EGF in this hepatoma cell line.

Intrahepatic *DPP*8 and *DPP*9 upregulation in CCl₄ induced liver injury

To examine *DPP*8 and *DPP*9 expression in liver injury,

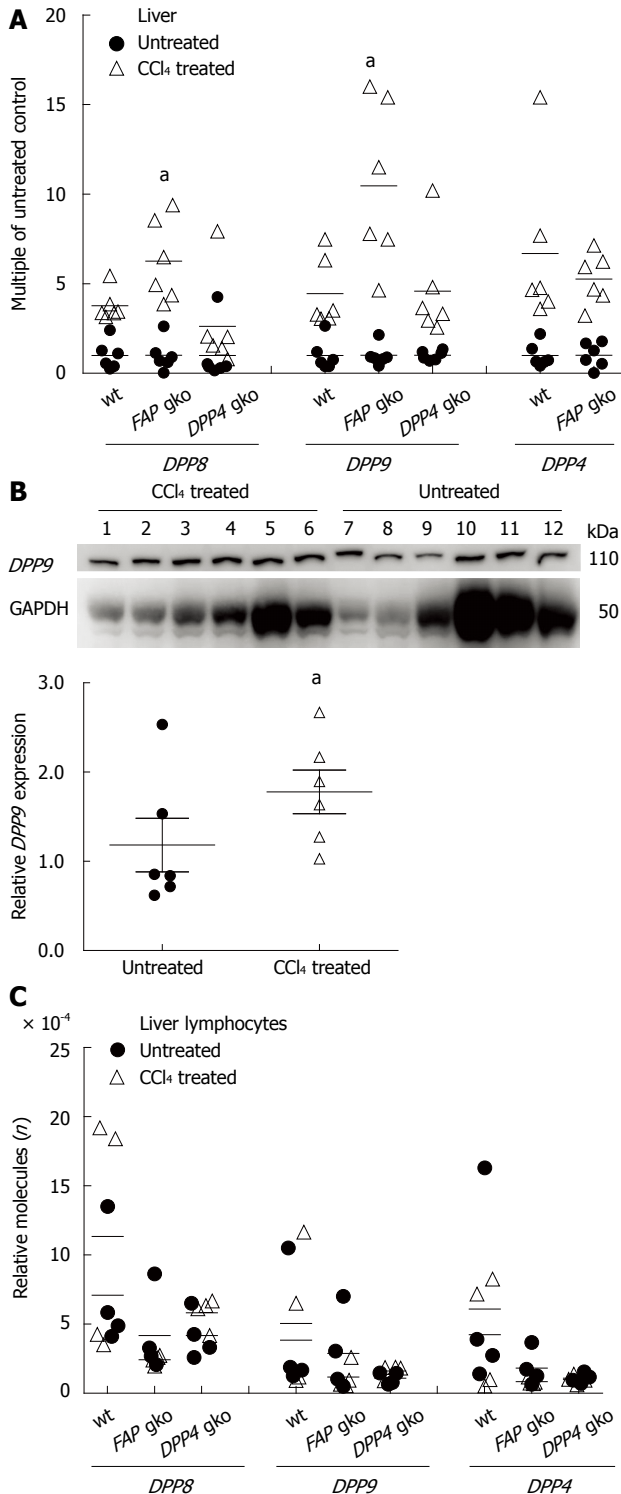


Figure 6 Dipeptidyl peptidase mRNA upregulation in carbon tetrachloride induced liver injury. **A**: Multiple of intrahepatic mRNA in carbon tetrachloride (CCl₄) treated mice to mean of untreated control mice; **P* < 0.05 in CCl₄ treated fibroblast activation protein (FAP) gene knockout (gko) vs wild type (wt); **B**: Dipeptidyl peptidase (DPP) 9 immunoblot of livers from CCl₄ treated (lanes 1-6) and untreated mice (lanes 7-12) (*n* = 6 per group); Densitometry of intrahepatic DPP9 relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **P* < 0.05 vs untreated controls; **C**: mRNA quantitation from isolated hepatic lymphocytes relative to 18S.

CCl₄ was used to induce liver fibrosis in wt, *DPP4* gko and *FAP* gko mice. *DPP4*, *DPP8* and *DPP9* mRNA were

significantly more abundant in the livers from CCl₄ treated mice of all three genotypes compared to the untreated controls (Figure 6A). *DPP8* and *DPP9* mRNA expression in the CCl₄ treated livers were greater in the *FAP* gko mice compared to wt (*DPP8* *P* = 0.02; *DPP9* *P* = 0.02), suggesting that *DPP8* and *DPP9* might have compensatory roles in the absence of *FAP* (Figure 6A). The increase in *DPP9* mRNA in the fibrotic livers was consistent with protein expression in wt mice (*P* = 0.05) (Figure 6B). An appropriate antibody to mouse *DPP8* is not available.

Since *DPP8* and *DPP9* are expressed by human hepatic lymphocytes^[13] and because there is an increase of infiltrating lymphocytes in liver fibrosis, we examined whether the mouse hepatic lymphocytes were likely to contribute to the observed upregulation of *DPP* expression. However, *DPP* mRNA in the mouse hepatic lymphocytes was similar in the fibrotic and normal livers (Figure 6C).

Intrahepatic *DPP8* and *DPP9* downregulation in biliary liver disease

The *Mdr2* gko mouse strain is deficient in the canalicular phospholipid flippase and is a model of periportal biliary fibrosis resembling primary sclerosing cholangitis^[41]. These mice develop spontaneous hepatomegaly as early as 2 wk after birth and significant biliary fibrosis with a fivefold increased liver collagen content by 12 wk of age, when no further fibrosis progression occurs^[41]. Measuring *DPPs* in these mice at 4, 8 and 12 wk of age showed that *DPP* mRNA expression was surprisingly very low at wk 4, significantly lower than in wt (*DPP8* *P* = 0.03; *DPP9* *P* = 0.03; *DPP4* *P* = 0.03) (Figure 7A). At 8 and 12 wk of age, *DPP* expression levels were similar to wt.

In human end-stage PBC livers, *DPP9* mRNA expression was significantly less than in the non-diseased livers (*P* = 0.03) (Figure 7B). This finding is consistent with the results in the *Mdr2* gko mice and with the human *DPP9* Western blot data^[40]. *DPP8* mRNA expression levels in the non-diseased and PBC livers were not statistically different (*P* = 0.057).

DISCUSSION

This study significantly promotes our understanding of the novel proteases *DPP8* and *DPP9* in lymphocytes, hepatocytes and liver injury. We showed that *DPP8* and *DPP9* are widely expressed in lymphocyte subpopulations and upregulated in mitogen activated lymphocytes in a time dependent manner. Besides lymphocyte activation, we demonstrated their potential involvement in lymphocyte apoptosis. In liver, we showed that *DPP8* and *DPP9* expression levels were altered in liver injury and confirmed their role in the regulation of EGF in hepatocytes, a mitogen that is considered crucial for hepatocyte proliferation and liver regeneration. The interestingly variable expression patterns of *DPP8* and *DPP9* in different conditions in lymphocytes and in liver injury suggest that these proteases may have important regulatory roles in the immune system and in liver disease pathogenesis.

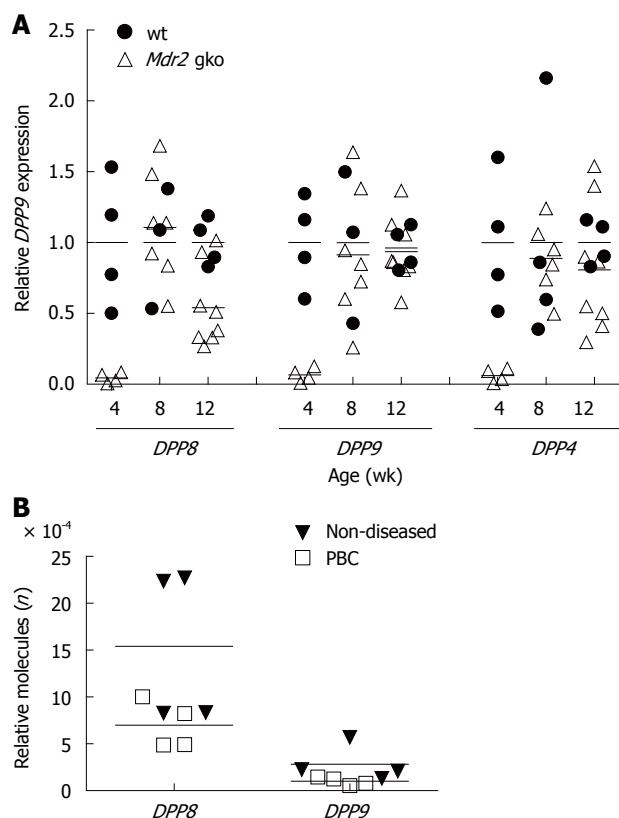


Figure 7 Dipeptidyl peptidase mRNA in mouse and human biliary liver diseases. A: Multiple of Intrahepatic dipeptidyl peptidase (*DPP*) mRNA in multidrug resistance gene 2 (*Mdr2*) gene knockout (*gko*) female mice to mean of wild type (*wt*) controls; B: Human end-stage primary biliary cirrhosis (*PBC*) and non-diseased control livers. Data from each individual is shown as the number of molecules relative to aldolase B ($n = 4$ per group).

DPP8/9 activity and expression in lymphocytes have been reported previously^[8,30,50], but which lymphocyte subpopulations express *DPP8* and *DPP9* remained unknown. Here we show that all the lymphocyte subpopulations tested, $CD4^+$ T cells, $CD8^+$ T cells and $B220^+$ B cells express *DPP8* and *DPP9*. The wide expression of *DPP8* and *DPP9* in lymphocyte subpopulations suggests that these proteases have essential roles in the immune system. As it is now known that immune roles of *DPP4* are mainly extraenzymatic (such as protein-protein interaction), greater abundance of *DPP8* and *DPP9* compared to *DPP4/CD26* in the lymphocytes further supports the hypothesis that the immune effects with non-selective *DPP4* inhibitors in earlier studies were more likely due to *DPP8* and *DPP9* inhibition^[2].

We demonstrated a quantitative time-dependent up-regulation of *DPP8* and *DPP9* in mitogen-stimulated mouse splenocytes and human Jurkat $CD4^+$ T cells, as well as in polyclonally activated Raji B cells. Therefore, *DPP8* and *DPP9* might have roles in both T and B cell activation. *DPP8* and *DPP9* were upregulated in lymphocytes following acute mitogen stimulation, but with prolonged stimulation, they were downregulated. Hence, the role of *DPP8* and *DPP9* perhaps differ in recently activated lymphocytes compared to persistently activated lymphocytes.

DPP9 enzyme activity induces intrinsic cell apoptosis in epithelial cells through the phosphatidylinositol-3-kinase/protein kinase B (Akt) signaling pathway^[39,40]. Our data on Raji cells suggest that *DPP9* could similarly have a role in intrinsic lymphocyte apoptosis. Moreover the increase in *DPP8* and *DPP9* expression in mitomycin C treated cells is perhaps a hallmark of increased apoptosis in the absence of cell proliferation^[51,52]. *DPP9* substrates and ligands involved in these processes have not been identified.

The modulation of *DPP8* and *DPP9* expression with varying lymphocyte activation, proliferation and apoptosis, implies that *DPP8* and *DPP9* have important regulatory roles in lymphocytes that deserve further investigation. Their role in lymphocyte activation is likely to differ from that of *DPP4*. While the role of cell surface *DPP4* in lymphocyte proliferation appears to be mainly extra-enzymatic^[22], enzyme inhibition of intracellular *DPP8* and *DPP9* affects lymphocyte proliferation^[23]. The observation of less annexin V staining in Raji cells overexpressing *DPP9* enzyme mutant compared to wild type *DPP9* suggests that enzyme activity of *DPP9* is important for its role in apoptosis. *DPP9* modulates Akt phosphorylation in hepatoma cell lines^[40], so *DPP8* and *DPP9* might similarly modulate the activity of signaling molecules that are crucial in lymphocyte activation pathways. *DPP8* can cleave several chemokines, stromal cell-derived factor (SDF)-1 α , SDF-1 β , inflammatory protein 10 and interferon-inducible T-cell alpha chemoattractant, *in vitro*^[12], however since *DPP8* is an intracellular protease, the biological relevance of this cleavage is unknown.

The association of *DPP4* and *FAP* with liver fibrosis is well documented^[24,53]. Here we have demonstrated possible involvement of *DPP8* and *DPP9* in liver fibrosis, too. Treatment of mice with CCl_4 for 3 wk, which represents early fibrosis with mild hepatic injury, increased intrahepatic *DPP8* and *DPP9* expression. This association with early stage disease may suggest pro-fibrogenic roles of *DPP8* and *DPP9*. Though *DPPs* have been implicated in inflammation and inflammatory diseases^[28,29,54], no change in *DPP* expression was observed in hepatic lymphocytes in this early stage fibrosis, suggesting that hepatocytes, which constitute more than 80% of the liver cell population, are probably the major source of upregulated *DPP8* and *DPP9* in this liver fibrosis model.

Unlike the CCl_4 induced liver fibrosis model, *DPP8* and *DPP9* were downregulated in end stage human *PBC* and in the *Mdr2 gko* mice. This suggests that *DPP8* and *DPP9* expression varies with the pathophysiology of liver diseases. The mouse CCl_4 model represents zone 3 fibrosis whereas *Mdr2 gko* represents a zone 1 fibrosis model^[41,55]. *DPP8* and *DPP9* show a zonal distribution pattern, with stronger staining in zone 3, the periportal hepatocytes and periportal lymphocytes^[13]. Hence, the zonal injury pattern may be important for *DPP8* and *DPP9* expression. Another possibility could be that activated cholangiocytes downregulate *DPP8* and *DPP9* expression. In the *Mdr2 gko* mice, *DPP8* and *DPP9* expression was least at week 4, when the cholangiocytes are

most active^[41]. Hence, this could be the reason why *DPP8* and *DPP9* expression was downregulated in human PBC and *Mdr2* gko mice.

Alternatively, the differential expression of *DPP* in the different liver diseases could be due to acute *vs* chronic stimuli. CCl₄ induces acute liver injury with hepatocyte damage followed by a repair phase that involves increased collagen deposition^[55]. Administration of CCl₄ twice per week for 3 wk leads to repeated cycles of injury and repair that results in fibrosis. We collected liver samples from the CCl₄ treated mice at day 3 after the last CCl₄ injection. At day 3, hepatocyte apoptosis is waning whereas fibrosis is developing^[55]. In contrast, the *Mdr2* gko mice and human end stage PBC represent chronic liver injury, whereby there is persistent (mild) hepatocyte damage, a fibrogenic cholangiocyte/progenitor cell response and downregulation of collagenolytic activity resulting in continuing progression of biliary fibrosis until week 12 of age^[41]. Thus, our data are consistent with the paradigm that *DPP8* and *DPP9* are upregulated in acute disease states then downregulated with progression to chronic disease states.

This distinctive *DPP* expression pattern in different liver diseases suggests that *DPP8* and *DPP9* have important regulatory roles in the pathogenesis of liver diseases, perhaps in modulating liver regeneration and apoptosis, which are important processes in liver disease progression. *DPP9* impairs EGF-stimulated hepatoma proliferation^[40]. Our observation that *DPP9* is upregulated in the presence of EGF is perhaps part of a regulatory mechanism of *DPP9* in hepatocyte proliferation. *DPP9* can induce intrinsic apoptosis in hepatoma cell lines *via* the Akt signaling pathway^[40]. Furthermore, *DPP8* and *DPP9* influence cell-extracellular matrix (ECM) interactions *in vitro*^[39] and in liver fibrogenesis, cell-ECM interaction is responsible for disrupting wound healing and progressive scarring in liver disease^[56].

The upregulated expression of *DPP8* and *DPP9* in acute conditions and less expression in chronic or persistent conditions in the immune system and in liver injury suggests that *DPP8* and *DPP9* are crucial for early cellular responses to stimuli. The mechanisms of *DPP8* and *DPP9* are yet to be elucidated. One obstacle in *DPP8* and *DPP9* studies is the poor availability of appropriate tools, such as monoclonal antibodies and selective inhibitors^[57].

In conclusion, our study suggests that *DPP8* and *DPP9* have fundamental roles in the immune system, in lymphocyte activation and in apoptosis and they could be involved in liver fibrogenesis. A better understanding of the biological functions of *DPP8* and *DPP9* could help reveal their therapeutic potential for liver diseases, cancer, inflammatory and autoimmune diseases.

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COMMENTS

Background

The four enzyme members of the dipeptidyl peptidase (*DPP*) 4 gene family, *DPP4*, fibroblast activation protein (*FAP*), *DPP8* and *DPP9* have attracted considerable research interest in recent years since *DPP4* inhibitors became a successful therapy for type 2 diabetes and *FAP* a potential cancer therapeutic target. *DPP8* and *DPP9* are the more recently discovered members of the *DPP4* gene family. They are ubiquitously expressed cytoplasmic enzymes with *DPP4* like enzyme activity. Many compounds intended to inhibit *DPP4* or *FAP* also inhibit *DPP8* and *DPP9*, but the compounds that became successful diabetes drugs are *DPP4*-selective.

Research frontiers

DPP4 is also known as CD26 T cell differentiation marker and has roles in T cell activation and proliferation. *DPP8* and *DPP9* are in lymphoid tissues and may have functional significance in the immune system. *DPP8* and *DPP9* are expressed in hepatocytes and expression is elevated in damaged hepatocytes near the septum of human cirrhotic liver. However, potential roles of *DPP8* and *DPP9* in liver disease are unknown. Here the authors studied the expression of *DPP8* and *DPP9* in lymphocyte activation, proliferation and apoptosis and in liver injury models to elucidate their potential biological roles in the immune system and in liver diseases. Models included hepatotoxicity from CCl₄, and the multidrug resistance gene 2 knockout mouse that spontaneously develops biliary fibrosis.

Innovations and breakthroughs

This study significantly promotes our understanding of the novel proteases *DPP8* and *DPP9* in lymphocytes, hepatocytes and liver injury. The authors showed that *DPP8* and *DPP9* were widely expressed in lymphocyte subpopulations and were upregulated in activated lymphocytes in a time dependent manner. The authors also demonstrated potential involvement of *DPP8* and *DPP9* in lymphocyte apoptosis. In liver, the authors showed that *DPP8* and *DPP9* expression levels were altered in liver injury and confirmed their role in the regulation of epidermal growth factor in hepatocytes, a mitogen that is considered crucial for hepatocyte proliferation and liver regeneration.

Applications

This study suggests that *DPP8* and *DPP9* have fundamental roles in the immune system, in lymphocyte activation and in apoptosis and they could be involved in chronic liver injury pathogenesis.

Terminology

DPP4 enzyme activity is a specialized proteolytic enzyme activity that cuts two amino acids from the N-terminus of each target peptide, usually cutting after a proline residue; Lymphocyte activation is a cellular process that leads to a radical shift in cell behavior to a more active and proliferative one. The activation of lymphocytes serves two purposes, augmenting the number of cells to respond to a particular antigen (clonal expression), and specializing to produce cytokines, and produce antibodies against a pathogen; cell apoptosis is the process of cell death mediated by an intracellular program. Apoptosis is important for normal cell turnover and organ remodeling.

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The manuscript deals with regulation of *DPP8* and *DPP9* expression in activated lymphocytes and injured liver. Here the authors focus on the expression levels of *DPP8* and *DPP9* in lymphocyte subpopulations in a time dependent manner. The authors have confirmed the altered expression level of *DPP8* and *DPP9* in liver injury and also confirmed their role in the regulation of epidermal growth factor in hepatocytes. The work has been carefully conducted and the experiments are clearly described in the vast majority of the cases.

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