Published in final edited form as:
*Acta Histochem*. 2011 July ; 113(4): 428–435. doi:10.1016/j.acthis.2010.03.004.

# Ectopic expression of CD74 in *Ikkβ*-deleted mouse hepatocytes

**Katherine S. Koch**[a,#,*] and **Hyam L. Leffert**[a,#,*]

[a]Hepatocyte Growth Control and Stem Cell Laboratory, Department of Pharmacology, School of Medicine, University of California at San Diego, 9500 Gilman Drive MC 0636, La Jolla, CA 92093-0636, U.S.A.

## Abstract

CD74, a Type II membrane glycoprotein and MHC class II chaperone involved in antigen processing, is normally expressed by cells associated with the immune system. CD74 also forms heterodimers with CD44 to generate receptors to macrophage migration inhibitory factor (MIF), a proinflammatory cytokine. Following targeted Alb-Cre-mediated deletion of *Ikkβ* in $Ikk\beta^{\Delta hep}$ mice ($Ikk\beta^{F/F}$*:Alb-Cre*, a strain highly susceptible to chemically-induced hepatotoxicity and hepatocarcinogenesis), CD74 is expressed abundantly by adult hepatocytes throughout liver acini, albeit more intensely in midzonal-to-centrilobular regions. By comparison, CD74 expression is not observed in $Ikk\beta^{F/F}$ hepatocytes, nor is it augmented in the livers of $Ikk\beta^{+/+}$*:Alb-Cre* mice; CD74 is barely detectable in cultured embryonic fibroblasts from $Ikk\beta^{-/-}$ mice. Microarray profiling shows that constitutive CD74 expression in $Ikk\beta^{\Delta hep}$ hepatocytes is accompanied by significantly augmented expression of CD44 and key genes associated with antigen processing and host defense, including MHC class II I-Aα, I-Aβ, and I-Eβ chains, CIITA and CD86. Taken together, these observations suggest that $Ikk\beta^{\Delta hep}$ hepatocytes might express functional capacities for class II-restricted antigen presentation and heightened responsiveness to MIF-signaling, and also suggest further roles for intrahepatocellular IKKβ in the suppression or inactivation of molecules normally associated with the formation and differentiation of cells of the immune system.

### Keywords

Hepatocytes; *Ikkβ* deletion; CD74; MHC Class II

## Introduction

CD74 is recognized as the invariant chain chaperone (Ii) of MHC class II processing (Bertolino and Rabourdin-Combe, 1996). Its molecular sizes range between 31-45 kDa; the most abundant mouse isoforms, splice variants p31 and p41, are post-translationally modified with chondroitin sulfate side-chains to generate Type II membrane glycoproteins with 30 aa cytoplasmic tails (Bertolino and Rabourdin-Combe, 1996; Stumptner-Cuvelette and Benaroch, 2002). CD74 is expressed throughout the immune system by B cells, activated T cells, dendritic cells, monocytes and macrophages (Bertolino and Rabourdin-Combe, 1996; Calandra and Roger, 2003; Faure-André et al., 2008; Leng and Bucala, 2006; Lue et al., 2006; Stumptner-Cuvelette and Benaroch, 2002). In the liver, CD74 is expressed

[*]Corresponding authors: Tel: 858-534-2354; fax: 858-822-4184. kskoch@ucsd.edu (K.S. Koch), hleffert@ucsd.edu (H.L.Leffert).
[#]Both authors contributed equally to this study.

constitutively in dendritic and Kupffer cells (Momburg et al., 1986), and in resident hepatic stellate cells (HSCs) (Maubach et al., 2007); however, except for one isolated report of a scattered sub-population of CD74-positive hepatocyte clusters (containing 1 to 5 cells) in ♀ B10.BR H-$2^k$ mice (Momburg et al., 1986), CD74 is not expressed in hepatocytes. CD74 is also a component of heterodimeric CD74/CD44 cell surface receptors (Leng and Bucala, 2006; Lou et al., 2006) which mediate mitogenic and proinflammatory effects of macrophage migration inhibitory factor (MIF) (Calandra and Roger, 2003; Leng and Bucala, 2006; Lue et al., 2006), a 12.5 kDa cytokine secreted by a variety of T and B cells, epithelial cells, and liver Kupffer cells (Calandra and Roger, 2003; Kobayashi et al., 1999; Lan, 2008; Leng and Bucala, 2006; Lue et al., 2006). MIF exhibits intrinsic enzymatic activities; induces cyclin D1 expression, mitogenesis and NO production; and plays major roles in the pathogenesis of inflammatory disease (Javeed et al., 2008; Leng and Bucala, 2006; Swant et al., 2005). MIF expression is causally related to chronic hepatitis B virus (HBV) infection (Zhang et al., 2005), fulminant alcoholic hepatitis (Kumagi et al., 2001) and Bacille-Calmette-Guerin-primed lipopolysaccharide (LPS)-induced T cell-mediated liver failure (BLTLF) (Kobayashi et al., 1999). Anti-MIF antisera block BLTLF (Kobayashi et al., 1999) and attenuate experimentally-induced murine colitis (de Jong et al., 2001); anti-sense MIF cDNA attenuates LPS-induced liver injury (Iwaki et al., 2003); and MIF knockout mice are protected from concanavalin A (ConA), LPS- and acetaminophen-induced liver injuries (Bourdi et al., 2002; Bozza et al., 1999; Nakajima et al., 2006). CD74 also is cleaved by regulated intracellular proteolysis (RIP) to form a 10-kDa cytoplasmic polypeptide fragment which migrates into the nucleus and activates nuclear factor kappa B (NF-κB) expression (Becker-Herman et al., 2005). However, the relationship between CD74 and NF-κB in liver disease remains unclear.

To understand how NF-κB, a transcription factor that plays anti-inflammatory and protective roles in hepatotoxic injury (Heyninck et al., 2003) and mediates liver disease, we have investigated an hepatocyte-targeted mouse strain (*Ikkβ*$^{\Delta hep}$) with Cre recombinase-mediated deletions of IκB kinase β *(Ikkβ)* exon 3 (Maeda et al., 2003). IKKβ, a subunit of the IκB Kinase (IKK) complex, is required for activation of NF-κB (Ghosh and Karin, 2002; Heyninck et al., 2003). *Ikkβ*$^{\Delta hep}$ mice exhibit defective activation of NF-κB and survival genes, and enhanced susceptibility to hepatotoxins (Maeda et al., 2003, 2005). Following ConA- or LPS+Galactosamine-induced inflammation, *Ikkβ*$^{\Delta hep}$ mice exhibit heightened susceptibility to liver failure, resulting partly from tumor necrosis factor (TNF)α-activation of TNF receptor 2 (TNFR2; Maeda et al., 2003), and from increased reactive oxygen species (ROS) formation. ROS inhibits mitogen activated protein kinase (MAPK) phosphatases and sustains c-JUN nuclear kinase 1 (JNK1) activation (Kamata et al., 2005; Maeda et al., 2003). Following treatment with diethylnitrosamine (DEN), *Ikkβ*$^{\Delta hep}$ mice exhibit increased susceptibility to hepatocellular carcinoma (HCC), resulting partly from cytokine-driven NF-κB activation-independent compensatory hepatocyte proliferation (Laurent et al., 2005; Maeda et al., 2005); from sustained JNK1 activation, accompanied by necrotic hepatocellular interleukin (IL)-1α release which activates IL-1 receptors in Kupffer cells and IL-6 secretion (Sakurai et al., 2008); and, as described most recently, from intrinsic *Ikkβ*$^{\Delta hep}$ hepatocellular growth advantages (Koch et al., 2009).

Neither CD74 nor MIF has been linked to the pathophysiological responses of *Ikkβ*$^{\Delta hep}$ hepatocytes, or to *Ikkβ*-deletion in other cells in other IKK*β*-deficient mice (Chen et al., 2006; Malato et al., 2008). However, CD74 is required for major histocompatibility (MHC) class II antigen processing (Bertolino and Rabourdin-Combe, 1996); and MIF has wide-ranging effects on hepatic inflammation, infection and tumor progression (Bourdi et al., 2002; Bozza et al., 1999; Calandra and Roger, 2003; de Jong et al., 2001; Iwaki et al., 2003; Nakajima et al., 2006; Zhang et al., 2005). In addition, because the proinflammatory effects elicited by the CD74/CD44 MIF receptor, like those by TNFR2 (Maeda et al., 2003), require

sustained kinase activation (Leng and Bucala, 2006), we investigated constitutive CD74 expression *in* $Ikk\beta^{\Delta hep}$ and $Ikk\beta^{F/F}$ mice.

Surprisingly, we find that $Ikk\beta^{\Delta hep}$ hepatocytes express CD74, whereas $Ikk\beta^{F/F}$ hepatocytes do not. In addition, as assessed by microarray profiling, the hepatocellular CD74-positive phenotype is accompanied by the expression of large numbers of genes normally associated with immune system processing and host defense mechanisms, including MHC class II I-Aα, I-Aβ, and I-Eβ chains, CD44, the transcriptional coactivator of MHC class II expression CIITA (Ting and Trowsdale, 2002), and coactivator CD86. These observations suggest that targeted *Ikkβ* deletion confers several unexpected and unusual immunologic phenotypes in hepatocytes which, if functionally active, might have significant implications for both the pathophysiology of liver disease and the regulation of animal cell differentiation.

## Materials and Methods

### Animals and cell culture

C57BL/6, $Ikk\beta^{+/+}$*:Alb-Cre*, $Ikk\beta^{F/F}$ and $Ikk\beta^{F/F}$*:Alb-Cre* (referred to as $Ikk\beta^{\Delta hep}$) mice were bred at UCSD (La Jolla, CA), maintained and sacrificed according to NIH guidelines (Koch et al., 2009; Maeda et al., 2003). Protocols used in all animal studies were approved by the UCSD IACUC animal ethics committee. Mouse embryonic fibroblasts (MEFs) from $Ikk\beta^{-/-}$ embryos were cultured under standard conditions (Chen et al., 2006).

### Isolation and analyses of primary hepatocytes, tissues and macromolecules

Freshly isolated hepatocytes and liver tissues were harvested from adult mice; DNA, RNA and proteins were isolated from hepatocytes, liver tissues and MEFs as described elsewhere (Chen et al, 2006; Koch et al., 2009; Maeda et al., 2003). *Ikkβ* deletion and genotyping tests were performed by polymerase chain reaction (PCR) using DNA from tails, livers and cultured hepatocytes and MEFs (Chen et al, 2006; Koch et al., 2009; Maeda et al., 2003). RNA and DNA were sized by gel-electrophoresis (Koch et al., 2009).

### Microarray profiling

Animals were sacrificed and tissues harvested at 5-6 weeks of age. RNA samples were labeled and hybridized by standard procedures (Feng et al., 2007) using two Illumina WG-6 V2 mouse bead-chips (San Diego, CA, cat. #BD 201-0202) in the UCSD BIOGEM Core (http://microarrays.ucsd.edu/). Each chip carried 6 microarrays housing 47,000 oligonucleotide targets representing ~5,000 genes/array (see: Fig. 1). Data were normalized and grouped (using the gene ontology databases from the Gene Ontology Consortium [www.genego.org] including pathways from KEGG, Biocarta, and Proteinlounge databases), and analyzed for statistical significance (Feng et al., 2007). Quantitative variation among data samples for individual genes was <1-2%. The original data (see Tables 1 and 2) have been entered into the NCBI GEO Repository (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE15476); a sample of selected data in Table 1 has been presented elsewhere (Koch and Leffert, 2010). For GenBank details and probes employed for these analyses, see the Illumina Reference Chip file, MouseWG-6_V2_0_R2_11278593_A (http://www.switchtoi.com/annotationfiles.ilmn).

### Western blots

Animals were sacrificed and tissues harvested at 58 and 48 days (see Figures 2A [lanes 3-6] and 2B [lanes 1-4], both panels, respectively), and at 125 days (see Figure 2B [lanes 5 and 6], both panels) of age. Liver extracts (30 μg proteins/lane) and cultured MEF extracts (40 μg) were analyzed on non-reducing prefabricated 10% SDS-PAGE gels (Invitrogen, Carlsbad, CA; Koch et al., 2009). Proteins were transferred to nitrocellulose membranes

using an Xcell II blot module (Invitrogen, Carlsbad, CA). Membranes were incubated overnight with either primary ('1°') rat anti-mouse CD74 antibody (clone In-1, BD Biosciences Pharmingen, San Diego, CA, catalog (cat.) #555317) or with matched rat IgG2b,κ isotype control (BD Biosciences Pharmingen, San Diego, CA, cat. #553986), washed, incubated 2 hr with secondary ('2°') horse radish peroxidase (HRP)-conjugated goat anti-rat IgG (H+L) (Pierce, ThermoScientific, Rockford, IL, cat. #31470), washed, and incubated 1 hr with ECL-Western-Blotting substrate according to the supplier's instructions (Pierce, ThermoScientific, Rockford, IL, cat. #32106). Loading control blots using identical extracts were performed with anti-GRP94 (gp96) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, cat. #sc-71182). Chemiluminescence was visualized by 5-30 sec exposure to Amersham ECL-Hyperfilm (GE Healthcare Biosciences, Piscataway, NJ, cat. #28-9068-36). Results of Western blots were obtained from different extracts from two independent experiments.

### Immunohistochemistry

Animals were sacrificed and liver tissues (portions of the right lobe) harvested at 58 days of age. Thin sections (5 μm) of paraffin blocks of fixed tissues (4% paraformaldehyde in PBS, 24 hr at 4 °C), and routine hematoxylin and eosin (H&E) stained sections were prepared by standard automated procedures in the UCSD Cancer Center Histology Core (http://cancer.ucsd.edu/Research/Shared/histology.asp). For immunohistochemical staining, endogenous peroxidase activity was blocked in deparaffinized sections with 3% $H_2O_2$. Sections were pretreated with Retrievagen A* according to the supplier's protocol (BD Pharmingen, San Diego, CA, cat. #550524), followed by 10% fetal bovine serum (Gibco Invitrogen, Carlsbad, CA) to block non-specific binding. Slides were washed, and incubated with 1° anti-CD74 antibody or with matched isotype control rat IgG2b,κ antibody at final concentrations of 5 μg/mL for 1 hr at 21°C. Slides were washed, treated with biotinylated goat 2° anti-rat Ig (BD Pharmingen, San Diego CA, cat. #51-7605KC) for 30 min at 21 °C, washed and treated with Streptravidin-HRP (BD Pharmingen, San Diego CA, cat. #551013) for 30 min at 21 °C. After further washing, the slides were incubated with diaminobenzidine solution (BD Pharmingen, San Diego CA, cat. #551013) for 0.5-5 min (or, as needed), washed, counterstained in Meyer's hematoxylin, dehydrated and coverslipped. Digital images and information (available on request) were acquired with NIS-Elements F 2.30 software, by Nikon DS-Ri1 camera, from Nikon Diaphot with 20× (Fig. 3) or 4× (Fig. 4) objectives. Independent immunohistochemical staining experiments utilizing sections from at least two animals of each genotype were performed multiple times.

## Results

### Microarray profiling shows upregulation of immune phenotypic expression in Ikkβ$^{\Delta hep}$ hepatocytes

Liver tissue and isolated hepatocytes from both genotypes (four separate samples) were compared to see whether profiling differences would be more or less enriched in hepatocytes compared to total liver. RNA sizing and DNA genotyping were performed prior to profiling studies. Intact RNA of high quality (Fig. 1A), and PCR products consistent with wild type and *Ikkβ*-deleted genomes were observed (Fig. 1B) (Maeda et al., 2003). Microarray hybridizations were performed in triplicate with formatting as diagrammed in Figure 1C.

Key findings in the largest expression group ('Biological Process') are shown for freshly isolated livers and freshly isolated hepatocytes in Tables 1 and 2, respectively. Two functional sub-families topped both lists at very high levels of statistical significance (albeit in reverse order): **liver** (immune system process, $p \sim 6.5 \times 10^{-28}$; defense response, $p \sim 2 \times 10^{-27}$); **hepatocytes** (defense response, $p = 1.4 \times 10^{-21}$; immune system process, $p \sim 1.2 \times$

$10^{-18}$). Examination of the 6-19 genes topping these lists showed that all were expressed at relatively higher levels in *Ikkβ*$^{\Delta hep}$ samples, except for two which were higher in *Ikkβ*$^{F/F}$ liver tissue (histocompatibility 2, blastocyst H2-Bl [both sub-families]) and one which was apparently higher in *Ikkβ*$^{F/F}$ hepatocytes (CD163 antigen [defense response]).

Enhanced CD74 expression in *Ikkβ*$^{\Delta hep}$ samples ranked highest throughout; CD74 was enriched ~38-fold in isolated *Ikkβ*$^{\Delta hep}$ hepatocytes, and ~8-fold in *Ikkβ*$^{\Delta hep}$ liver tissue (a 5-fold enrichment following hepatocyte isolation; see both Tables 1 and 2). Profiling also indicated that additional immune process and defense genes were significantly upregulated >4-10-fold in *Ikkβ*$^{\Delta hep}$ hepatocytes, including MHC class II I-Aα, I-Aβ, and I-Eβ chains, CD44, Cxcl9, guanylate nucleotide binding proteins 1-3, TAP, Tlr2, interferon gamma (IFN-γ) inducible protein 47 and IFN-γ regulatory factor 1 (see both Tables 1 and 2). Direct inspection of the microarray data also revealed augmented expression levels of *Ikkβ*$^{\Delta hep}$ hepatocyte CIITA, STAT1, cathepsin S, co-activator CD86 and programmed death ligand-1 (PD-L1) (> 6-, 5.4-, 3.6-, 2- and 5.5-fold, respectively), all significantly greater than isolated liver tissue. As shown in Table 3, the available data from microarray comparisons between *Ikkβ*$^{\Delta hep}$ hepatocytes and *Ikkβ*$^{-/-}$ MEFs revealed few similarities (Chen et al., 2006).

## Ectopic expression of CD74 proteins is upregulated in Ikkβ$^{\Delta hep}$ hepatocyte extracts

Four specific bands ($M_r$ ~ 31, 35, 41 kDa, and a 10-12 kDa doublet) were detected in *Ikkβ*$^{\Delta hep}$ liver extracts by antibody specific to cytosolic CD74 epitopes (Figs. 2A and 2B [left panels]). CD74-specific bands were not seen in similar extracts probed with isotype control antibody (Figs. 2A and 2B [right panels]). Band intensities were similar in ♂ and ♀ samples; the relative intensities of p31 were ~2.5-fold higher than p41 as expected (Bertolino and Rabourdin-Combe, 1996). In contrast, some (p31 and p41) but not all (p35, p45 and the 10-12 kDa doublet) CD74-specific bands were detectable at significantly lower levels in *Ikkβ*$^{F/F}$ liver extracts (Figs. 2A and 2B [left panels]); these bands were likely due to CD74 expressed by non-parenchymal cells (NPCs) in the intact livers (Fig. 3). The finding of CD74-specific expression in *Ikkβ*$^{\Delta hep}$ liver extracts was not an artifact of albumin promoter-driven Cre expression, as CD74 expression levels in liver extracts from *Ikkβ*$^{+/+}$*:Alb-Cre* mice were similar to those from *Ikkβ*$^{F/F}$ mice, with no detectable p35, p45, or 10-12 kDa doublet (Fig. 2B).

As expected from profiling (Chen et al., 2006), CD74 was undetectable in wildtype MEF extracts whereas extracts from *Ikkβ*$^{-/-}$ MEFs variably expressed very low levels of p31 and p41 bands but undetectable levels of p35 or the 10-12 kDa doublet (Figs. 2A and 2B [left panels]). The significance of these observations is currently unclear.

## Ectopic and specific expression of CD74 is observed *in situ* in Ikkβ$^{\Delta hep}$ hepatocytes

To localize hepatic CD74 expression, livers from ♂ C57BL/6, and ♂ and ♀ *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta hep}$ mice were analyzed by routine histology and immunohistochemistry. In all cases, the same 1° antibody used for Western blots was employed; with respect to *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta hep}$ mice, portions of the same tissue samples used for Western blots (Fig. 2A) were fixed for histochemistry (Figs. 3 and 4).

Normal histology was observed by H&E staining of C57BL/6 liver (Fig. 3, row 1). As expected (Maubach et al., 2007;Momburg et al, 1986), specific CD74 staining was observed in NPCs bordering a portal triad, and also scattered sparsely throughout the liver. In contrast, specific staining was not observed either in hepatocytes, or in cells bordering the central vein, or in sections incubated with isotype control antibody (Fig. 3, row 1). The faint CD74-positive p31 and p41 bands on Western blots of liver extracts from *Ikkβ*$^{+/+}$*:Alb-Cre* and *Ikkβ*$^{F/F}$ mice (Figs. 2A and 2B) may reflect NPC staining. Further work is needed for

precise identification of NPCs, whether dendritic, HSC, Kupffer, sinusoidal endothelial and/or biliary ductular epithelial cells, which are responsible for this CD74-positive staining.

As shown in Fig. 3 (rows 2-5), H&E staining showed no morphological differences between $Ikk\beta^{\Delta hep}$, and control $Ikk\beta^{F/F}$, ♀ or ♂ livers. However, intense CD74 staining was observed in virtually all $Ikk\beta^{\Delta hep}$ hepatocytes throughout liver acini and regardless of gender. Specific CD74 staining in $Ikk\beta^{\Delta hep}$ hepatocytes was associated with cytoplasmic and with membrane sites. In contrast, although scattered periportal and acinar staining was observed in NPCs, specific CD74 staining was undetectable in $Ikk\beta^{F/F}$ hepatocytes. No CD74 staining was observed in any liver sections incubated with isotype control antibody (Fig. 3) or in the absence of 1° antibody (data not shown).

By observation of liver sections at lower magnification (Fig. 4), specific CD74 staining in $Ikk\beta^{\Delta hep}$ livers appeared to be more intense in hepatocytes bordering central veins, and to decrease slightly and gradually in intensity from centrilobular to periportal zones.

## Discussion

Immunohistochemical, microarray profiling, and Western blot findings show ectopic and gender-independent expression of major, minor and putatively processed CD74 isoforms in hepatocytes deleted in *Ikkβ* ($Ikk\beta^{\Delta hep}$). Immunohistochemistry suggests that antibody-specific CD74 expression occurs in all hepatocytes, as might be expected from *Ikkβ*-deletion analyses indicating no undeleted product in a population of isolated $Ikk\beta^{\Delta hep}$ hepatocytes (Maeda et al., 2003; Koch et al, 2009); however, the isoform(s) responsible for the observed immunochemical reactivities have not been specifically identified. The prominent 35 kDa band in $Ikk\beta^{\Delta hep}$ liver extracts might represent another CD74 murine isoform, so far unreported; the nature of the 45 kDa band observed in younger but not older mice (compare Fig. 2A with Fig. 2B) is unclear. The 10-12 kDa doublet, also observed in spleens from $Ikk\beta^{+/+}$*:Alb-Cre* mice (Fig. 2B [left panel]), might be a form of RIP-processed CD74 (Becker-Herman et al., 2005); if functional, this might account for residual NF-κB activity in $Ikk\beta^{\Delta hep}$ hepatocytes (Maeda et al., 2003).

The occurrence of multiple isoforms complicates interpretation of immunohistochemical observation of enriched midzonal-to-centrilobular localization of hepatocellular CD74, since site-enrichment could be due either to increased expression of all or selected isoforms, or to increased availability of antigenic sites of one or more equally abundant isoforms. Alternatively, owing to the relatively short paraformaldehyde fixation time, and to subsequent variably effective antigen retrieval steps, zonal staining intensity differences might reflect differential fixation of antigenic sites accessible to the anti-CD74 antibody employed. Whereas longer fixation times (> 24 hr) clearly reduce antigen detection (data not shown), zonal differences were still visible. Therefore, because the zonal differentiation of hepatocytes is responsible for many functional attributes of liver under both normal, and pathological conditions, further experiments are needed to clarify these observations as well as to eliminate other possible fixation artifacts.

Although the upregulation of CD74 expression in hepatocytes appears to be a consequence of targeted hepatocellular deletion of *Ikkβ* in $Ikk\beta^{\Delta hep}$ mice, *Ikkβ* deletion alone is not a uniformly sufficient condition of CD74 upregulation, because $Ikk\beta^{-/-}$ MEFs express only barely detectable levels of CD74, as revealed by Western blots (Fig. 2), and none by RNA profiling (Chen et al., 2006). While the mechanisms responsible for ectopic hepatocellular CD74 expression remain to be determined, they do not appear to be random or isolated. This conclusion is supported by liver micorarray profiling results which reveal, with very high statistical significance, that augmented CD74 expression in $Ikk\beta^{\Delta hep}$ hepatocytes is

concomitant with enriched expression of two large families of immune system-related genes, among them CD44, MHC class II I-Aα, I-Aβ, and I-Eβ chains, CIITA and PD-L1 mRNAs, the functions of which are involved in antigen processing, host defense and liver tolerance (Tiegs and Lohse, 2009). This enrichment is selectively greater in isolated hepatocytes compared to that in unfractionated liver extracts. Although further work is needed to show the translation of these mRNAs directly and to characterize the cellular locations of these putatively translated proteins, these additional findings are surprising and intriguing because the expression of all five of these molecules, like CD74, is usually restricted to lymphoid (Abbas et al., 2010;Rafi-Janajreh et al., 1998) and NPCs (Tiegs and Lohse, 2009).

The collective, and significantly augmented, expression of CD74 and these two gene families in *Ikkβ*$^{\Delta hep}$ hepatocytes suggests several interrelated hypotheses about ectopic immunologic functions and susceptibility to HCC in these mice, as discussed elsewhere (Koch and Leffert, 2010).

## Acknowledgments

This work was supported by NIH (CA113602, AI067354) and the Superfund Basic Research Program (P42ES010337). Breeding colonies were started with mice generously supplied by M. Karin (UCSD), whom we also thank for the suggestion to examine *Ikkβ*$^{+/+}$*:Alb-Cre* mice. We thank K. Juson, M. Zhang and A. Elbehti for technical assistance; Anne Chang (UCSD, Karin Laboratory) for wildtype and *Ikkβ*$^{-/-}$ MEFs and *Ikkβ*$^{+/+}$*:Alb-Cre* mice; and R. Sasik (UCSD BIOGEM Core) for statistical analyses and organization of microarray profiling data.

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## Abbreviations

| | |
|---|---|
| **Alb** | refers to albumin-specific gene promotor |
| **BLTLF** | Bacille-Calmette-Guerin-primed lipopolysaccharide-induced T cell-mediated acute liver failure |
| **ConA** | lectin concanavalin A |
| **CIITA** | transcriptional coactivator of MHC class II expression |
| **Cre** | cre recombinase |
| **DEN** | diethylnitrosamine |
| **HBV** | hepatitis B virus |
| **HCC** | hepatocellular carcinoma |
| **H&E** | hematoxylin and eosin |
| **HRP** | horse radish peroxidase |
| **HSC** | hepatic stellate cell |
| **IFN-γ** | interferon γ |
| **Ii** | invariant chain CD74 chaperone |
| **IKK** | IκB complex |
| **IKKβ** | protein kinase subunit of IKK |
| ***Ikkβ$^{F/F}$*** | mouse strain carrying Cre-recombinase sites (F/F) spanning *Ikkβ* exon 3 |
| ***Ikkβ$^{\Delta hep}$*** | mouse strain *Ikkβ$^{F/F}$:Alb-Cre*, specifically deleted in hepatocyte *Ikkβ* |
| **IL** | interleukin |
| **JNK** | c-JUN nuclear kinase |
| **LPS** | lipopolysaccharide |
| **MAPK** | mitogen-activated protein kinase |
| **MEFs** | mouse embryonic fibroblasts |
| **MHC** | major histocompatibility |
| **MIF** | macrophage migration inhibitory factor |

| | |
|---|---|
| **NPC** | non-parenchymal cell |
| **NF-κB** | nuclear factor kappa B |
| **PD-L1** | programmed death ligand |
| **RIP** | regulated intramembrane proteolysis |
| **ROS** | reactive oxygen species |
| **STAT** | signal transducers and activator of transcription |
| **TNF** | tumor necrosis factor |
| **TNFR2** | tumor necrosis factor receptor 2 |

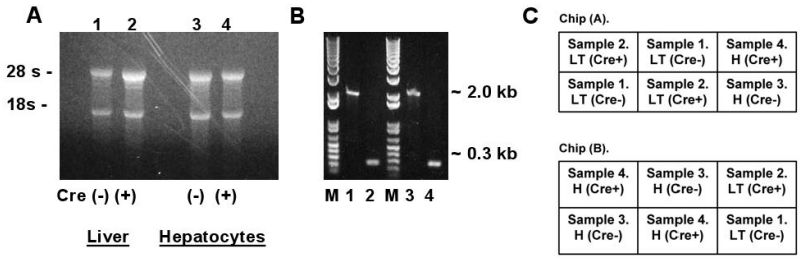


**Figure 1.**

Analysis of RNA and DNA from male Cre(-) *Ikkβ*$^{F/F}$ and Cre(+) *Ikkβ*$^{\Delta hep}$ mouse livers and freshly isolated hepatocytes used for microarray profiling. (A) EtBr-stained RNA-sizing gel. (B) Deletion analysis. EtBr-stained DNA-sizing gel. Genotyping for deletion confirmation was performed by PCR using standard primers (Koch et al., 2009). M, size markers (bps); lanes #1-#4 as in panel (A); Cre(-) ~2-kb, Cre(+) ~0.3-kb. (C) Hybridization format: chips 1 (A) and 2 (B). Each RNA sample (liver tissue [LT], or isolated hepatocytes [H]) was obtained from a separate mouse: Samples #1 and #2, Cre(-)- and Cre(+)-tissues; #3 and #4, Cre(-)- and Cre(+)-isolated hepatocytes. Each sample was hybridized 3×, on 3 separate sectors across the 2 chips, as diagrammed.

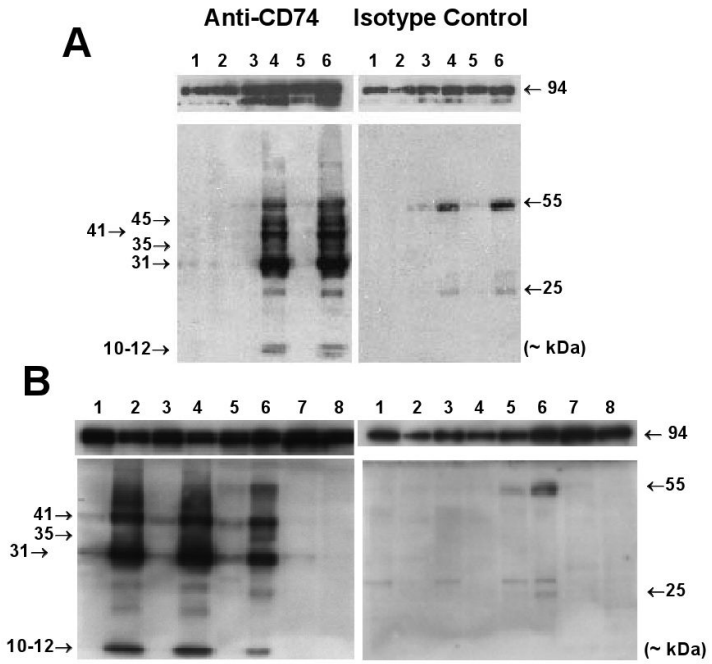


**Figure 2.**

Detection of CD74 in cell extracts on Western Blots. (A) *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta hep}$ mouse livers, and cultured MEFs (each lane from a separate mouse). Lanes: 1 (wildtype MEFs), 2 (*Ikkβ*$^{-/-}$ MEFs), 3 (♂*Ikkβ*$^{F/F}$ liver), 4 (♂*Ikkβ*$^{\Delta hep}$ liver), 5 (♀*Ikkβ*$^{F/F}$ liver), 6 (♀*Ikkβ*$^{\Delta hep}$ liver). Non-specific ~25 kDa and ~55 kDa bands in isotype control panels (right upper and lower) likely reflect non-specific cross-reactions with serum or reagent light and heavy IgG chains. (B) *Ikkβ*$^{+/+}$*:Alb-Cre* mouse livers and spleens, and cultured MEFs (each lane from a separate mouse). Lanes: 1 (♂ #1, *Alb-Cre* liver), 2 (♂ #1, *Alb-Cre* spleen), 3 (♂ #2, *Alb-Cre* liver), 4 (♂ #2, *Alb-Cre* spleen), 5 (♂*Ikkβ*$^{F/F}$ liver), 6 (♂*Ikkβ*$^{\Delta hep}$ liver), 7 (*Ikkβ*$^{-/-}$ MEFs), 8 (wildtype MEFs). Loading controls (both panels) showed comparative intensities of 94-96 kDa bands in all lanes.

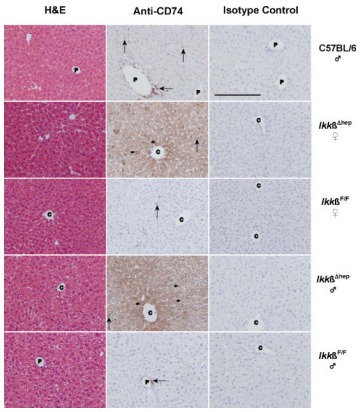


**Figure 3.**

Histochemical studies of CD74 in livers from C57BL/6 ♂, and *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta hep}$ ♀ and ♂ mice. Photomicrographs of liver tissue sections stained with H&E, anti-CD74 antibody, or isotype control antibody are shown in the left-, middle-, and right-hand columns, respectively. The sections are from individual C57BL/6 (row 1), *Ikkβ*$^{\Delta hep}$ ♀ (row 2), *Ikkβ*$^{F/F}$ ♀ (row 3), *Ikkβ*$^{\Delta hep}$ ♂ (row 4), and *Ikkβ*$^{F/F}$ ♂ (row 5) mice. Annotations indicate hepatocyte membrane (small →) and NPC (large →) staining; C, central vein; P, portal triad. (The unannotated H&E stained *Ikkβ*$^{\Delta hep}$ ♀ liver section appears to approach a central region.) Specific CD74 staining is present in hepatocytes from *Ikkβ*$^{\Delta hep}$ mice but not in hepatocytes from C57BL/6 and *Ikkβ*$^{F/F}$ mice. Tissue-specific CD74 staining is not seen in sections treated with isotype control antibody. Objective magnification = 20× (the solid bar, lower left, in the C57BL/6 isotype control panel = 200 μm; the scale bar is identical for experimental panels).

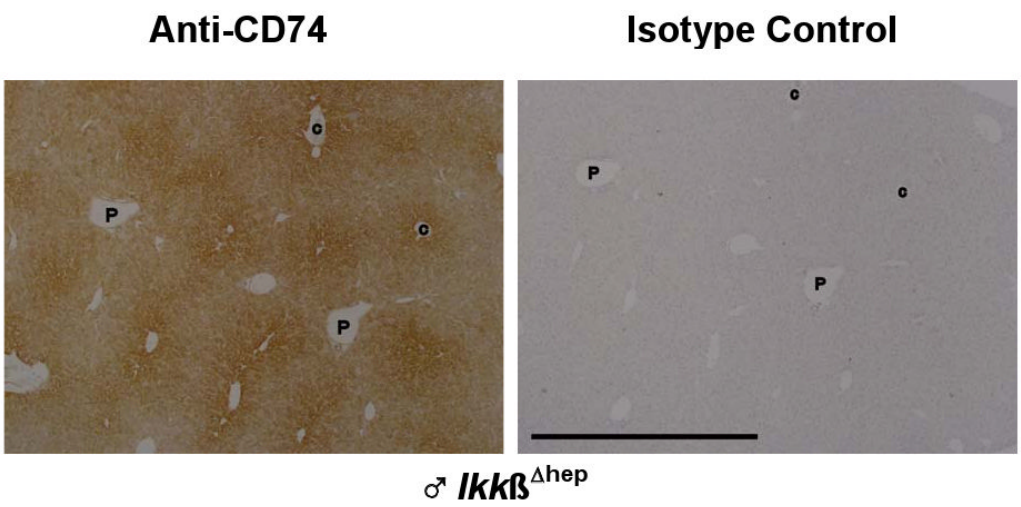


**Figure 4.**

Enriched CD74 staining in *Ikkβ*$^{\Delta hep}$ hepatocytes in midzonal-to-centrilobular regions. Serial sections from a ♂ *Ikkβ*$^{\Delta hep}$ liver, stained with either anti-CD74 or isotype control antibodies as described in the text and in Fig. 3, are here placed in register. Low objective magnification (4×) was used to illustrate the zonal gradient of staining intensity. Hepatocytes proximal to central veins (C) are more intensely stained than hepatocytes proximal to portal triads (P). At lower magnification, scattered NPC staining is more difficult to visualize. The few notations are placed within the same vessels of both serial sections to facilitate registration of the two panels. The solid bar, lower left, in the isotype control panel = 1000 μm (the scale bar is identical for the experimental panel).

## Table 1

Partial results of microarray profiling: Freshly Isolated Liver Tissues.*

**IMMUNE SYSTEM PROCESS**
**p = 6.46685868E-28**

| Rank | Locus | GenBank Acc.# | Description | E** |
|---|---|---|---|---|
| 2 | 14961 | NM_207105.1 | Histocompatibility 2, class II antigen A, beta 1 (H2-Ab1) | -8.86 |
| 3 | 14969 | NM_010382.1 | Histocompatibility 2, class II antigen E beta (H2-Eb1) | -8.65 |
| 4 | 16149 | NM_010545.2 | CD74 antigen (invariant polypeptide of major histocompatibility complex) | -8.37 |
| 5 | 14960 | NM_010378.2 | Histocompatibility 2, class II antigen A, alpha (H2-Aa) | -6.21 |
| 6 | 14963 | NM_008199.1 | Histocompatibility 2, blastocyst (H2-Bl) | +5.72 |
| 8 | 14998 | NM_010386.2 | Histocompatibility 2, class II, locus DMa (H2-DMa) | -5.11 |

**DEFENSE RESPONSE**
**p = 2.07679921E-27**

| Rank | Locus | GenBank Acc.# | Description | E** |
|---|---|---|---|---|
| 4 | 16149 | NM_010545.2 | CD74 antigen (invariant polypeptide of major histocompatibility complex) | -8.37 |
| 6 | 14963 | NM_008199.1 | Histocompatibility 2, blastocyst (H2-Bl) | +5.72 |
| 10 | 17329 | NM_008599 | Chemokine (C-X-C motif) ligand 9 (Cxcl9) | -4.97 |
| 18 | 15019 | NM_023124 | Histocompatibility 2, Q region locus 8 (H2-Q8) | -3.82 |
| 20 | 20210 | NM_011315 | Serum amyloid A 3 (Saa3) | -3.74 |
| 29 | 55985 | NM_018866.1 | Chemokine (C-X-C motif) ligand 13 (Cxcl13) | -3.31 |
| 30 | 20304 | NM_013653.1 | Chemokine (C-C motif) ligand 5 (Ccl5) | -3.29 |
| 34 | 12262 | NM_007574.1 | Complement component 1, q subcomponent, C chain (C1qc) | -3.08 |
| 37 | 17105 | NM_017372 | Lysozyme (Lyzs) | -2.9 |
| 42 | 12260 | NM_009777.1 | Complement component 1, q subcomponent, beta polypeptide (C1qb) | -2.85 |
| 43 | 17069 | NM_008529.2 | Lymphocyte antigen 6 complex, locus E (Ly6e) | -2.82 |
| 53 | 14127 | NM_010185.2 | Fc receptor, IgE, high affinity I, gamma polypeptide (Fcer1g) | -2.55 |
| 67 | 24088 | NM_011905.2 | Toll-like receptor 2 (Tlr2) | -2.31 |
| 72 | 21356 | NM_009318.1 | TAP binding protein (Tapbp) | -2.29 |
| 74 | 15945 | NM_021274.1 | Chemokine (C-X-C motif) ligand 10 (Cxcl10) | -2.29 |
| 78 | 17110 | NM_013590.2 | Lysozyme (Lyzs) | -2.25 |
| 82 | 12505 | AK045226 | CD44 antigen (Cd44) | -2.22 |

*Selected expression comparisons between markers from $Ikk\beta^{F/F}$ and $Ikk\beta^{\Delta hep}$ freshly isolated liver tissues are listed; the markers with these comparatively most disparate expression levels are clustered within two functional groups.

**Enrichment, E**, as measured by relative microarray chip hybridization signals, is defined as follows: a negative (−) E value signifies that $Ikk\beta^{\Delta hep} > Ikk\beta^{F/F}$, and a positive (+) E value signifies that $Ikk\beta^{F/F} > Ikk\beta^{\Delta hep}$ (because the initial comparative chip data were expressed as X-fold enrichment of hybridization markers from $Ikk\beta^{F/F}$ compared to those from $Ikk\beta^{\Delta hep}$ RNA samples).

## Table 2

Partial results of microarray profiling: Freshly Isolated Hepatocytes.*

**DEFENSE RESPONSE**
**p = 1.38089039E-21**

| Rank | Locus | GenBank Acc.# | Description | E** |
|---|---|---|---|---|
| 1 | 16149 | NM_010545.2 | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) (Cd74) | -38.31 |
| 19 | 15953 | NM_008330.1 | Interferon gamma inducible protein 47 (Ifi47) | -7.15 |
| 23 | 15944 | NM_008326.1 | Immunity-related GTPase family, M (Irgm) | -6.51 |
| 24 | 17329 | NM_008599 | Chemokine (C-X-C motif) ligand 9 (Cxcl9) | -6.51 |
| 27 | 93671 | NM_053094.1 | CD163 antigen (Cd163) | +5.7 |
| 34 | 21356 | NM_001025313.1 | TAP binding protein (Tapbp) | -5.23 |
| 45 | 17105 | NM_017372 | Lysozyme (Lyzs) | -4.5 |
| 53 | 12505 | AK045226 | CD44 antigen (Cd44) | -4.25 |
| 55 | 15024 | NM_010395.5 | Histocompatibility 2, T region locus 10 (H2-T10) | -4.17 |
| 64 | 21354 | NM_013683.1 | Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP) (Tap1) | -3.96 |
| 65 | 24088 | NM_011905.2 | Toll-like receptor 2 (Tlr2) | -3.96 |
| 66 | 12260 | NM_009777.1 | Complement component 1, q subcomponent, beta polypeptide (C1qb) | -3.96 |
| 67 | 12774 | NM_009917.2 | Chemokine (C-C motif) receptor 5 (Ccr5) | -3.92 |
| 69 | 17110 | NM_013590.2 | Lysozyme (Lyzs) | -3.87 |

**IMMUNE SYSTEM PROCESS**
**p = 1.17768030E-18**

| Rank | Locus | GenBank Acc.# | Description | E** |
|---|---|---|---|---|
| 1 | 16149 | NM_010545.2 | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) (Cd74) | -38.31 |
| 3 | 14961 | NM_207105.1 | Histocompatibility 2, class II antigen A, beta 1 (H2-Ab1) | -22.48 |
| 4 | 14969 | NM_010382.1 | Histocompatibility 2, class II antigen E beta (H2-Eb1) | -18.24 |
| 6 | 14998 | NM_010386 | Histocompatibility 2, class II, locus DMa (H2-DMa) | -15.82 |
| 7 | 14960 | NM_010378.2 | Histocompatibility 2, class II antigen A, alpha (H2-Aa) | -14.39 |
| 8 | 21822 | NM_011579.2 | T-cell specific GTPase (Tgtp) | -13.26 |
| 9 | 14468 | NM_010259.1 | Guanylate nucleotide binding protein 1 (Gbp1) | -11.94 |
| 11 | 14999 | NM_010387.2 | Histocompatibility 2, class II, locus Mb1 (H2-DMb1) | -10.6 |
| 13 | 15000 | NM_010388 | Histocompatibility 2, class II, locus Mb2 (H2-DMb2) | -9.36 |
| 14 | 55932 | NM_018734.2 | Guanylate nucleotide binding protein 3 (Gbp3) | -8.96 |
| 20 | 14469 | NM_010260.1 | Guanylate nucleotide binding protein 2 (Gbp2) | -7.02 |
| 24 | 17329 | NM_008599 | Chemokine (C-X-C motif) ligand 9 (Cxcl9) | -6.51 |
| 30 | 60533 | NM_021893.2 | CD274 antigen (Cd274) | -5.54 |
| 33 | 16912 | NM_013585.1 | Proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2) (Psmb9) | -5.31 |
| 34 | 21356 | NM_001025313.1 | TAP binding protein (Tapbp) | -5.23 |
| 49 | 16913 | NM_010724 | Proteasome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7) (Psmb8) | -4.38 |
| 56 | 16362 | NM_008390.1 | Interferon regulatory factor 1 (Irf1) | -4.16 |

**IMMUNE SYSTEM PROCESS**
**p = 1.17768030E-18**

| Rank | Locus | GenBank Acc.# | Description | E** |
|---|---|---|---|---|
| 64 | 21354 | NM_013683.1 | Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP) (Tap1) | -3.96 |
| 65 | 24088 | NM_011905.2 | Toll-like receptor 2 (Tlr2) | -3.96 |

* Selected expression comparisons between markers from $Ikk\beta^{F/F}$ and $Ikk\beta^{\Delta hep}$ freshly isolated hepatocytes are listed; the markers with these comparatively most disparate expression levels are clustered within two functional groups.

** Enrichment, E**, as measured by relative microarray chip hybridization signals, is defined as follows: a negative (−) E value signifies that $Ikk\beta^{\Delta hep} > Ikk\beta^{F/F}$, and a positive (+) E value signifies that $Ikk\beta^{F/F} > Ikk\beta^{\Delta hep}$ (because the initial comparative chip data were expressed as X-fold enrichment of hybridization markers from $Ikk\beta^{F/F}$ compared to those from $Ikk\beta^{\Delta hep}$ RNA samples).

## Table 3

Microarray profiling: Partial comparisons between mouse embryo fibroblasts (MEFs, *Ikkβ*$^{-/-}$ vs. wild type MEFs) and freshly isolated hepatocytes (*Ikkβ*$^{\Delta hep}$ vs. *Ikkβ*$^{F/F}$).

| Gene | MEFs (*Ikkβ*$^{-/-}$ vs. wt) | Hepatocytes (*Ikkβ*$^{\Delta hep}$ vs. *Ikkβ*$^{F/F}$) | GenBank Acc. # | Function |
|---|---|---|---|---|
| | X-fold enrichment* | | | |
| CARP | +156.3 | -1.21 | AF041847 | Cardiac ankyrin repeat protein, binding to myopalladin |
| Versican | +9.7 | NR | D45889 | Anti-cell adhesive molecules |
| α-Actin | +2.6 | +1.28 | X13297 | Cytoskeleton, stress fibers |
| Prothymosin β4 | +2.34 | -1.98 | U38967 | Prevents actin polymerization |
| S100A4 | +2.2 | NR | M36579 | Tumor metastasis, calcium-binding protein |
| Tropomyosin 2 | +2.0 | -1.47 | M22479 | Actin-binding protein |
| IGFBP2 | -134 | -1.79 | X81580 | Inhibitor of cell mitogenesis |
| TIMP-2 | -5.8 | -2.32 | X62622 | Inhibitor of matrix metalloproteinases |
| TIMP-3 | -4.0 | -1.99 | U26437 | Inhibitor of matrix metalloproteinases |
| VCAM | -2.9 | +5.62 | M84487 | Vascular cell adhesion molecule |
| Integrin β5 | -2.0 | -1.2 | AF022110 | Cell adhesion |

X-fold enrichment values for MEFs were taken from Chen et al., 2006. The X-fold enrichment value is here in Table 3 defined as the relative comparison of RNA hybridization expression of MEF *Ikkβ*$^{-/-}$ to MEF *Ikkβ*$^{+/+}$, or of *Ikkβ*$^{\Delta hep}$ to *Ikkβ*$^{F/F}$ such that a positive (+) value signifies that MEF *Ikkβ*$^{-/-}$ > MEF *Ikkβ*$^{+/+}$, or that *Ikkβ*$^{\Delta hep}$ > *Ikkβ*$^{F/F}$; and, a negative (−) value signifies that MEF *Ikkβ*$^{+/+}$ > MEF *Ikkβ*$^{-/-}$, or that *Ikkβ*$^{F/F}$ > *Ikkβ*$^{\Delta hep}$. Note that this definition is here the reverse of that in Tables 1 and 2, in order to establish congruence with the E values previously published for the MEF *Ikkβ*$^{-/-}$ and *Ikkβ*$^{+/+}$ pair.