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# Plasmacytoid Dendritic Cell-Derived IFN-α Promotes Murine Liver Ischemia/Reperfusion Injury by Induction of Hepatocyte IRF-1

Antonino Castellaneta,[1]* Osamu Yoshida,[1]* Shoko Kimura,[1] Shinichiro Yokota,[1] David A. Geller,[1,2] Noriko Murase,[1] and Angus W. Thomson[1,3]

**Plasmacytoid dendritic cells (pDC) constitute the body's principal source of type I interferon (IFN) and are comparatively abundant in the liver. Among various cytokines implicated in liver ischemia and reperfusion (I/R) injury, type I IFNs have been described recently as playing an essential role in its pathogenesis. Moreover, type I IFNs have been shown to up-regulate hepatocyte expression of IFN regulatory factor 1 (IRF-1), a key transcription factor that regulates apoptosis and induces liver damage after I/R. Our aim was to ascertain the capacity of IFN-α released by liver pDC to induce liver damage through hepatic IRF-1 up-regulation after I/R injury. Our findings show that liver pDC mature and produce IFN-α in response to liver I/R. Liver pDC isolated after I/R induced elevated levels of IRF-1 production by hepatocytes compared with liver pDC isolated from sham-operated mice. Notably, hepatic IRF-1 expression was reduced significantly by neutralizing IFN-α. *In vivo,* IFN-α neutralization protected the liver from I/R injury by reducing hepatocyte apoptosis. This was associated with impaired expression of IRF-1 and proapoptotic molecules such as Fas ligand, its receptor (Fas) and death receptor 5, which are regulated by IRF-1. Furthermore, pDC-depleted mice failed to up-regulate hepatic IFN-α and displayed less liver injury associated with reduced levels of hepatic interleukin (IL)-6, tumor necrosis factor-α, and hepatocyte apoptosis after I/R compared with controls. *Conclusion:* these data support the hypothesis that IFN-α derived from liver pDC plays a key role in the pathogenesis of liver I/R injury by enhancing apoptosis as a consequence of induction of hepatocyte IRF-1 expression. (HEPATOLOGY 2014;60:267-277)**

Hepatocellular injury caused by ischemia/reperfusion (I/R) is the result of a highly complex cascade of events that is triggered when the liver is exposed transiently to hypoxia then reperfused with oxygenated blood. I/R injury is unavoidable during transplant surgery, and adversely affects patient and graft outcomes.[1,2] In a recent large study of living- and deceased-donor liver transplant patients, extended cold ischemic time was associated with elevated rates of early organ failure and acute cellular rejection.[3] Moreover, because of the greater susceptibility of extended criteria liver grafts to ischemic insult, I/R injury exacerbates the donor organ shortage problem. No specific treatment is available to prevent or reduce hepatic I/R injury and current management is based on supportive care. Thus, extensive efforts are warranted to better understand the mechanisms that underlie liver I/R injury.

*Abbreviations: ALT, alanine aminotransferase; DC, dendritic cell(s); DR5, death receptor 5; IFN, interferon; IFNAR, type 1 interferon receptor; I/R, ischemia/reperfusion; NPC, nonparenchymal cell(s); pDC, plasmacytoid dendritic cell(s).*

*From the [1]Thomas E. Starzl Transplantation Institute, Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA; [2]Liver Cancer Center, University of Pittsburgh Medical Center, Pittsburgh, PA; [3]Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA.*

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*Present address for A. Castellaneta: Unit of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Bari, Italy.*

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*These authors contributed equally to this work.

Type I interferons (IFNs; IFN-$\alpha$ and IFN-$\beta$) are multifunctional cytokines, which activate or modulate immune responses.[4] Type I IFNs bind to type 1 IFN receptors (IFNAR) and activate Jak1 and Tyk2 that, in turn, phosphorylate signal transducer and activator of transcription (STAT)1 and STAT2 and promote the formation of the IFN-stimulated gene factor (ISGF) 3 complex. The ISGF3 complex binds to nuclear IFN-stimulated response elements (ISREs) and induces transcription of IFN-stimulated genes (ISGs)[4,5] that encode factors responsible for antiviral, antiproliferative, and immunoregulatory responses.[6] Due to their critical role in innate immunity, type I IFNs are used as therapeutic agents in viral hepatitis, leukemia, renal cell carcinoma, and multiple sclerosis.[7] On the other hand, type I IFNs can play pathogenic roles in some autoimmune diseases, such as psoriasis and systemic lupus erythematosus (SLE).[4]

Neutralization of IFN-$\alpha$ is a potential therapeutic approach in type I IFN-related diseases and therapeutic effects of IFN-$\alpha$ neutralizing antibody (Ab) have been reported in mouse models of human immunodeficiency virus (HIV) encephalitis and septic shock.[8,9] Recently, the safety of an anti-IFN-$\alpha$ monoclonal antibody (mAb) (MEDI-545) has been confirmed in a chronic psoriasis phase I trial.[10] Type I IFNs are regarded as critical factors in both warm and cold liver I/R injury. Thus, in mice, IFNAR knockout (KO) livers are protected from damage,[11,12] suggesting that IFN-$\alpha$ neutralization might be a therapeutic option for inhibition of liver I/R injury.

The transcription factor IFN regulatory factor (IRF)-1 is induced by cytokines (type I IFNs, IFN-$\gamma$, tumor necrosis factor-$\alpha$ [TNF-$\alpha$], interleukin [IL]-1), viruses, and retinoic acid.[13,14] It regulates cell proliferation, apoptosis, and immune responses. Overexpression of IRF-1 promotes apoptosis by up-regulation of Fas ligand (CD95L), while IRF-1-deficient hepatocytes are resistant to apoptosis.[15,16] IRF-1 is known to play a key role in liver I/R injury since high mobility group box 1 (HMGB-1) released from dying/dead cells up-regulates IRF-1 in hepatocytes through Toll-like receptor (TLR) 4 ligation and promotes liver injury.[17] Furthermore, IRF-1-deficient livers exhibit less I/R injury correlated with the reduced apoptosis due to diminished expression of death ligands, including Fas ligand and death receptor (DR) 5.[18,19]

Although type I IFNs are produced by almost all cells, IFN-$\alpha$ is produced mainly by plasmacytoid dendritic cells (pDCs), while IFN-$\beta$ is produced by fibroblasts, synoviocytes, and stromal cells.[20] pDCs are an unconventional subset of bone marrow-derived DCs and produce large amounts of IFN-$\alpha$ following TLR7 and TLR9 ligation.[21,22] Although rare cells, pDCs are recognized as important players in protecting the host from viral infection by producing IFN-$\alpha$. By contrast, pDC that sense self DNA through TLR9 and produce high amounts of IFN-$\alpha$, drive autoimmunity, and exacerbate SLE and psoriasis.[23] In addition to IFN-$\alpha$, pDC produce pro- (IL-6, IL-12, and TNF-$\alpha$) and antiinflammatory cytokines (IL-10) following TLR ligation.[24] Thus, like conventional DCs, pDCs play key roles in innate and adaptive immunity in the liver.[25] While conventional DCs have been implicated in the regulation of liver I/R injury,[26,27] to date, no data are available concerning the role of pDC in hepatic I/R injury.

In this study we hypothesized that IFN-$\alpha$ derived from pDC might be involved in the pathogenesis of liver I/R injury, and that IFN-$\alpha$ blockade might protect the liver from damage by inhibiting IRF-1 expression in hepatocytes and consequently reducing their apoptosis. Our data show that pDC are activated and produced IFN-$\alpha$ after liver I/R and that IFN-$\alpha$ promotes IRF-1 expression by hepatocytes *in vitro*. *In vivo*, IFN-$\alpha$ neutralization significantly reduces liver I/R-induced damage, with associated reduction in IRF-1, proinflammatory cytokine (IL-6 and TNF-$\alpha$), and death receptor (Fas and DR5) expression in the liver. Moreover, depletion of pDC *in vivo* reduces hepatic IFN-$\alpha$ and protects the liver from I/R injury.

## Materials and Methods

***Experimental Animals.*** Male C57BL/6J (B6; H-$2^b$) and IRF-1 KO (B6 background) mice, 8-12 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free central animal facility of the University of

*Address reprint requests to: Angus W. Thomson, Ph.D., D.Sc., Starzl Transplantation Institute, Department of Surgery, University of Pittsburgh School of Medicine, 200 Lothrop St., W1540 BST, Pittsburgh, PA 15621. E-mail: thomsonaw@upmc.edu; fax: 412-624-1172.*



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Pittsburgh School of Medicine. Experiments were conducted in accordance with the National Institutes of Health publication "Guide for the Care and Use of Laboratory Animals," under an Institutional Animal Care and Use Committee-approved protocol. Mice received Purina rodent chow (Ralston Purina, St. Louis, MO) and tap water *ad libitum*.

***Liver Ischemia and Reperfusion Injury Model.*** A nonlethal model of segmental (70%) hepatic warm ischemia was used, as described.[27] Mouse livers were exposed to 1-hour ischemia by clamping the portal triad. The animals were euthanized after 6 hours reperfusion.

***IFN-α Neutralization and pDC Depletion.*** For IFN-α neutralization experiments, anti-IFN-α mAb (clone F18; 50 μg/mouse intravenously, Abcam, Cambridge, MA) was injected 30 minutes before I/R injury. For *in vivo* pDC depletion, mice were injected for 3 consecutive days with anti-mPDCA-1 pure-functional grade mAb (500 μg/mouse, intraperitoneally; Miltenyi Biotec, Auburn, CA).

***Isolation and Stimulation of Liver pDC.*** mPDCA1$^+$pDC were isolated from livers of mice given the endogenous DC poietin fms-like tyrosine kinase 3 ligand (Flt3L; CHO cell derived recombinant human Flt3L; 10 μg/mouse/day i.p., for 10 days), as described.[28] Thus, bulk liver DC were enriched by density centrifugation using Nycodenz (Sigma, St. Louis, MO). For pDC purification (consistently >95%), mPDCA1$^+$ cells were positively selected from the DC-enriched fraction using immunomagnetic beads and a paramagnetic LS column (Miltenyi Biotec).

***Flow Cytometry (Cell Surface Staining).*** Liver pDC, liver nonparenchymal cells (NPC), and spleen cells were treated with FcγR-blocking rat antimouse CD16/32 mAb (2.4G2) to prevent nonspecific Ab binding. They were then incubated for 30 minutes with FITC-, PE-, APC-, PE-Cy5-, or PE-Cy7-conjugated mAbs to detect expression of CD11c (HL3), B220/CD45R (RA3-6B2), Siglec-H (eBio440c), I-A$^b$β-chain (25-9-17) (eBioscience, San Diego, CA), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), and B7-H1 (CD274) [MIH5] (BD Biosciences, San Diego, CA) and APC-mPDCA-1 (JF05-1C2.4.1; Miltenyi Biotec). All mAbs and appropriate Ig isotype controls were obtained from BD Pharmingen (San Diego, CA), unless specified. Flow analysis was performed using an LSR II flow cytometer (BD Bioscience) and results expressed as mean fluorescence intensity (MFI).

***RT-PCR.*** Messenger RNA (mRNA) expression was quantified by SYBER Green real-time reverse-transcription polymerase chain reaction (RT-PCR) with an ABI-Prism 7000 sequence detection system (PE Applied Biosystems, Foster City, CA) and primers specific for IFN-α (F: 5′-GCAACCCTCCTAGACTCATTCT-3′; R: 5′-CCAGCAGGGCGTCTTCCT-3′), TNF-α (F: 5′-CATCTTCTCAAAATTCGAGTGA-3′; R; 5′-TGGGAGTAGACAAGGTACAAC-3′), IL-6 (F: 5′-TCAATTCCAGAAACCGCTATGA-3′; R: 5′-CACCAGCATCAGTCCCAAGA-3′), IRF-1 (F: 5′-TTAGCCCGGACACTTTCTCTGATGG-3′; R: 5′-GTCCCCTCGAGGGCTGTCAATCTCT-3′), Fas (F: 5′-CTGCGATGAAGAGCATGGTTT-3′; R: 5′-CCATAGGCGATTTCTGGGAC-3′), FasL (F: 5′-TGAATTACCCATGTCCCCAG-3′; 5′-AAACTGACCCTGGAGGAGCC-3′), DR5 (F: 5′-TGACGGGGAAGAGGAACTGA-3′; R: 5′-GGCTTTGACCATTTGGATTTGA-3′), or β-actin (F: 5′-AGAGGGAAATCGTGCGTGAC-3′; R: 5′-CAATAGTGATGACCTGGCCGT-3′). The expression of each gene was normalized to that of β-actin mRNA, as described.[29]

***Hepatocyte Isolation and Culture.*** Hepatocytes (B6 mouse) were isolated by an *in situ* collagenase (type IV, Sigma) perfusion technique, as described.[17] Hepatocyte purity exceeded 98%, and viability exceeded 95% as determined by standard testing. Liver pDC from sham or I/R injury mice were incubated for 18 hours, without stimulation, to accumulate IFN-α in the supernatant. The pDC and their respective supernatants were then applied to hepatocyte cultures with/without anti-IFN-α mAb. Mouse IFN-α 4 (PBL Interferon Source, Piscataway, NJ) was used as a positive control. After a 3-hour coculture, the hepatocytes were harvested for RT-PCR.

***MTT Assay.*** Hepatocytes (B6 WT or IRF1KO) were cultured with or without IFN-α (1000 U/mL) for 4 hours. Hepatocyte viability were determined by MTT assay (Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions.

***Enzyme-Linked Immunosorbent Assay (ELISA).*** Levels of IFN-α in culture supernatants were determined using commercial ELISA kits from PBL Interferon Source following the manufacturer's instructions.

***Alanine Aminotransferase (ALT) Levels.*** Serum ALT levels were measured using the Opera Clinical Chemistry System (Bayer, Tarrytown, NY).

***Routine and Immunohistopathology.*** Liver tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin and eosin (H&E) and TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick end labeling) as described.[19]

***Statistical Analysis.*** Data are expressed as means ± 1 SD. Significances of differences between means were determined by the nonparametric Mann-Whitney $U$ test. $P < 0.05$ was considered significant.

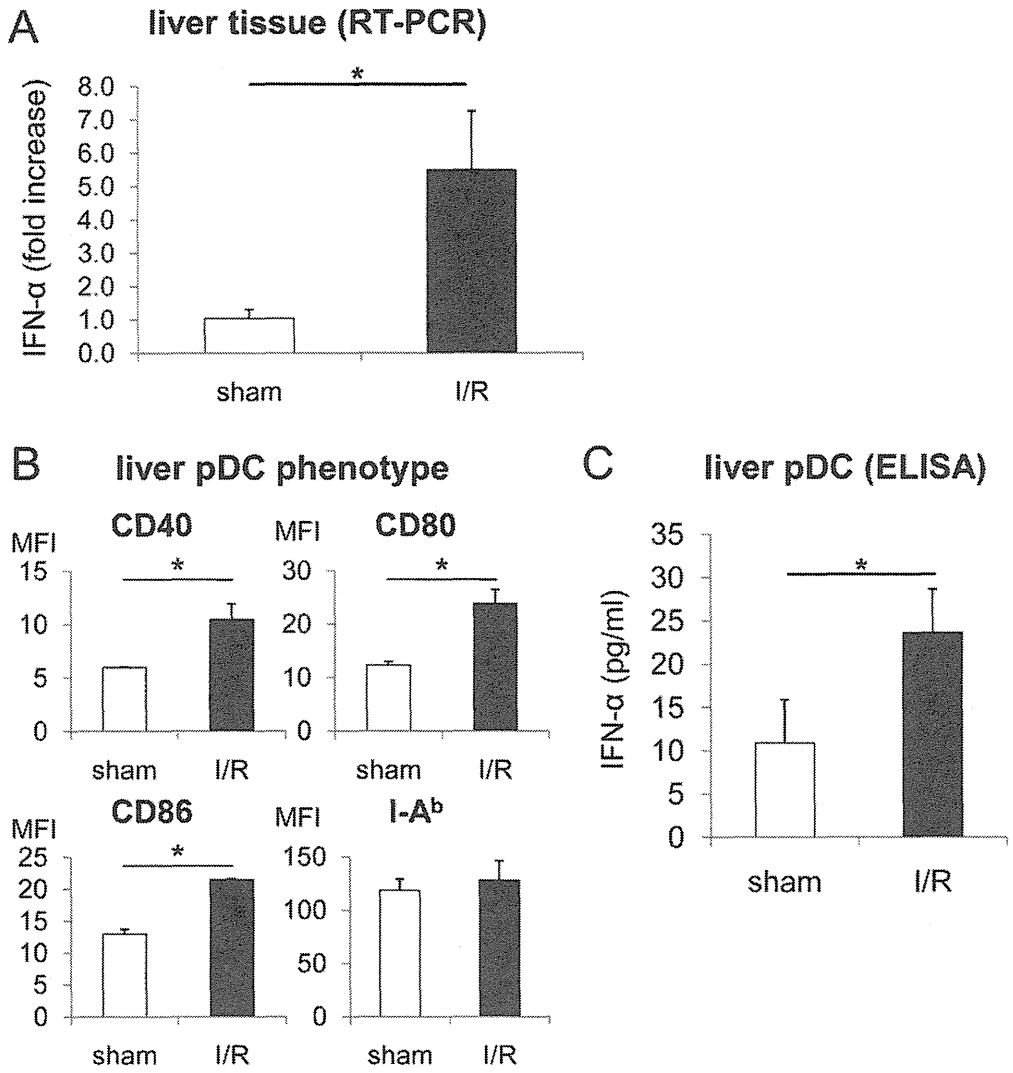


Fig. 1. Hepatic I/R injury stimulates phenotypic maturation and IFN-α production by liver interstitial pDC. B6 mice underwent I/R injury as described in the Materials and Methods. After 6 hours reperfusion, liver pDC were isolated by immunomagnetic bead sorting and phenotypic and functional analyses performed. (A) Analysis of whole liver tissue for IFN-α message by real-time RT-PCR. (B) Cell surface phenotype of liver pDC ($CD11c^{int}$ $B220^{+}$) from I/R injury and sham-operated control mice analyzed by mAb staining and flow cytometry. (C) IFN-α production by freshly isolated liver pDC isolated 6 hours after I/R injury measured by ELISA (n = 4 mice per group; $*P < 0.01$).

## Results

***IFN-α Production by Liver pDC Is Up-Regulated During Liver I/R Injury.*** To verify the level of IFN-α expression in B6 mouse liver following warm I/R, real-time RT-PCR was performed on whole liver tissue. As shown in Fig. 1A, IFN-α gene transcription was markedly and significantly up-regulated (~5-fold) after 6 hours I/R compared with sham-operated control mice. We also examined the phenotype and function of liver pDC freshly isolated after liver I/R. As shown in Fig. 1B, liver pDC from I/R injury mice displayed significant increases in the intensity of expression (MFI) of cell surface costimulatory molecules (CD40, CD80, CD86) compared with pDC from sham-operated controls, whereas similar levels of major histocompatibility complex (MHC) class II ($I\text{-}A^{b}$) were expressed by both groups. Moreover, freshly isolated liver pDC from I/R injury mice secreted enhanced quantities of IFN-α following 18 hours culture (Fig. 1C).

***IRF-1, Which Promotes Hepatocyte Apoptosis, Is Up-Regulated in Hepatocytes by IFN-α Secreted From pDC as the Result of I/R Injury.*** IRF-1 is induced in hepatocytes by stimulation with proinflammatory cytokines, such as type I IFNs, IFN-γ, TNF-α, and IL-1.[13,14] Therefore, we next examined whether

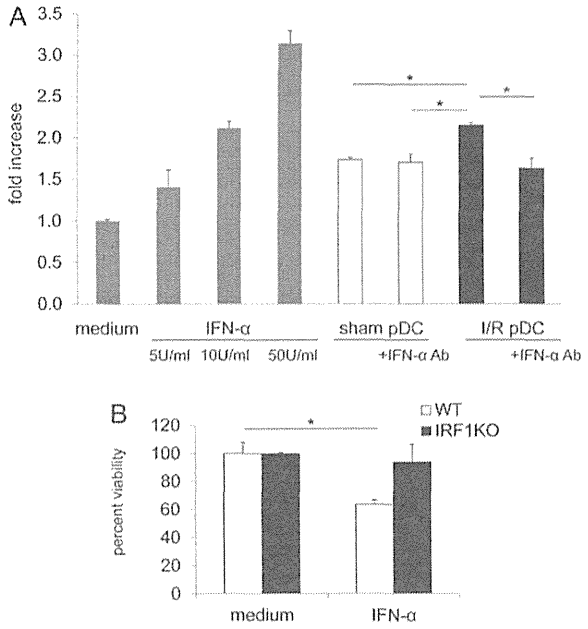


Fig. 2. During I/R injury, activated liver pDC promote IRF-1 production by hepatocytes. (A) Liver pDCs, isolated from sham-operated or I/R injury B6 mice, were incubated for 18 hours to accumulate IFN-α in the culture supernatant. Thereafter, pDC and their accompanying supernatants were transferred to normal mouse hepatocytes and cultured for 3 hours, with or without neutralizing anti-IFN-α mAb (5 μg/mL) added at the start of culture. IRF-1 production was assessed by RT-PCR. Mouse hepatocytes cultured with or without IFN-α were used as positive and negative controls, respectively. Overall analysis of n = 3 independent experiments. *$P < 0.01$. (B) Hepatocytes from WT or IRF-1 KO were cultured with or without IFN-α (1000 U/mL) for 4 hours. Hepatocyte viability was determined by MTT assay. *$P < 0.05$.

IFN-α released by liver pDC after liver I/R injury might up-regulate IRF-1 expression by cultured hepatocytes *in vitro*.[22,23,30] Liver pDC were isolated from sham-operated or I/R-injured mice and cultured overnight without stimulation. Liver pDC and their accompanying supernatants were transferred to mouse hepatocyte cultures for 3 hours, with or without addition of neutralizing anti-IFN-α mAb at the start of cocultures. IRF-1 production by hepatocytes was then measured by RT-PCR. As shown in Fig. 2A, liver pDC isolated from sham-operated mice, and to a significantly greater extent from I/R-injured mice, up-regulated IRF-1 production by mouse hepatocytes. Notably, neutralization of IFN-α significantly reduced the ability of liver pDC of I/R-injured mice to induce IRF-1 production by hepatocytes. To confirm the involvement of IRF-1 in IFN-α-induced hepatocyte apoptosis, the viability of hepatocytes from WT or IRF-1 KO mice in the presence of IFN-α was examined. Whereas the viability of WT hepatocytes was significantly decreased after a 4-hour incubation with IFN-α, that of IRF-1 KO hepatocytes was not affected (Fig. 2B).

**In Vivo *Blocking of IFN-α Reduces Liver I/R Injury, Associated With Reduced* In Situ *IL-6 and TNF-α Expression.*** As shown in Fig. 1, IFN-α is up-regulated during mouse liver I/R and is thought to be involved early in the pathogenesis of tissue injury.[11] To further evaluate the role of IFN-α in liver I/R, we injected mice with neutralizing anti-IFN-α mAb or isotype control Ig immediately before liver I/R injury. As is evident in Fig. 3A, serum ALT levels after 6 hours reperfusion were significantly lower (~50%) in the mAb-treated mice compared with the control group (Fig. 3A). Consistent with these reductions in ALT levels, the extent of tissue necrosis and the incidence of $TUNEL^+$ (apoptotic) cells (hepatocytes) in the anti-IFN-α-treated group were much lower than in the untreated I/R injury group (Fig. 3B-D). At the same time, gene transcripts for proinflammatory cytokines (IL-6 and TNF-α) in liver tissue were reduced significantly in the IFN neutralizing mAb-injected group (Fig. 3E).

**In Vivo *Blocking of IFN-α Down-Regulates IRF-1 Gene Transcription During Liver I/R Injury.*** IFN-α is a potent inducer of IRF-1[13] that promotes cell apoptosis in a Fas-related manner.[15] To ascertain whether the protective effect of IFN-α neutralization *in vivo* that was associated with reduced hepatocyte apoptosis was due to down-regulation of IRF-1 expression in liver tissue, we performed real-time RT-PCR for IRF-1, death ligand (FasL), and the death receptors Fas and DR5. As shown in Fig. 4A, IRF-1 gene transcription levels in livers of anti-IFN-α-mAb-treated mice were much lower (~80%) than in isotype-treated control mice. Consistent with the IRF-1 mRNA levels, Fas, DR5, and FasL gene transcription that is regulated by IRF-1 and determines the extent of apoptosis was also reduced significantly in anti-IFN-α-mAb-treated mouse livers (Fig. 4B). These data suggest that the protective effect of IFN-α neutralization in liver I/R injury is due to reduced apoptosis as the result of less IFN-α-mediated induction of IRF-1 and consequently less induction of both death receptors and death ligands that are regulated by IRF-1.

**In Vivo *pDC Depletion Reduces IFN-α Gene Transcription and Protects Livers From I/R Injury.*** pDCs are regarded as the main source of IFN-α in the body.[20] To further investigate their role in IFN-α production and liver I/R injury, we depleted pDC *in vivo* before surgery using anti-PDCA1 mAb. Mice were injected i.p. for 3 consecutive days with anti-mPDCA-1 pure-functional grade Ab (500 μg),

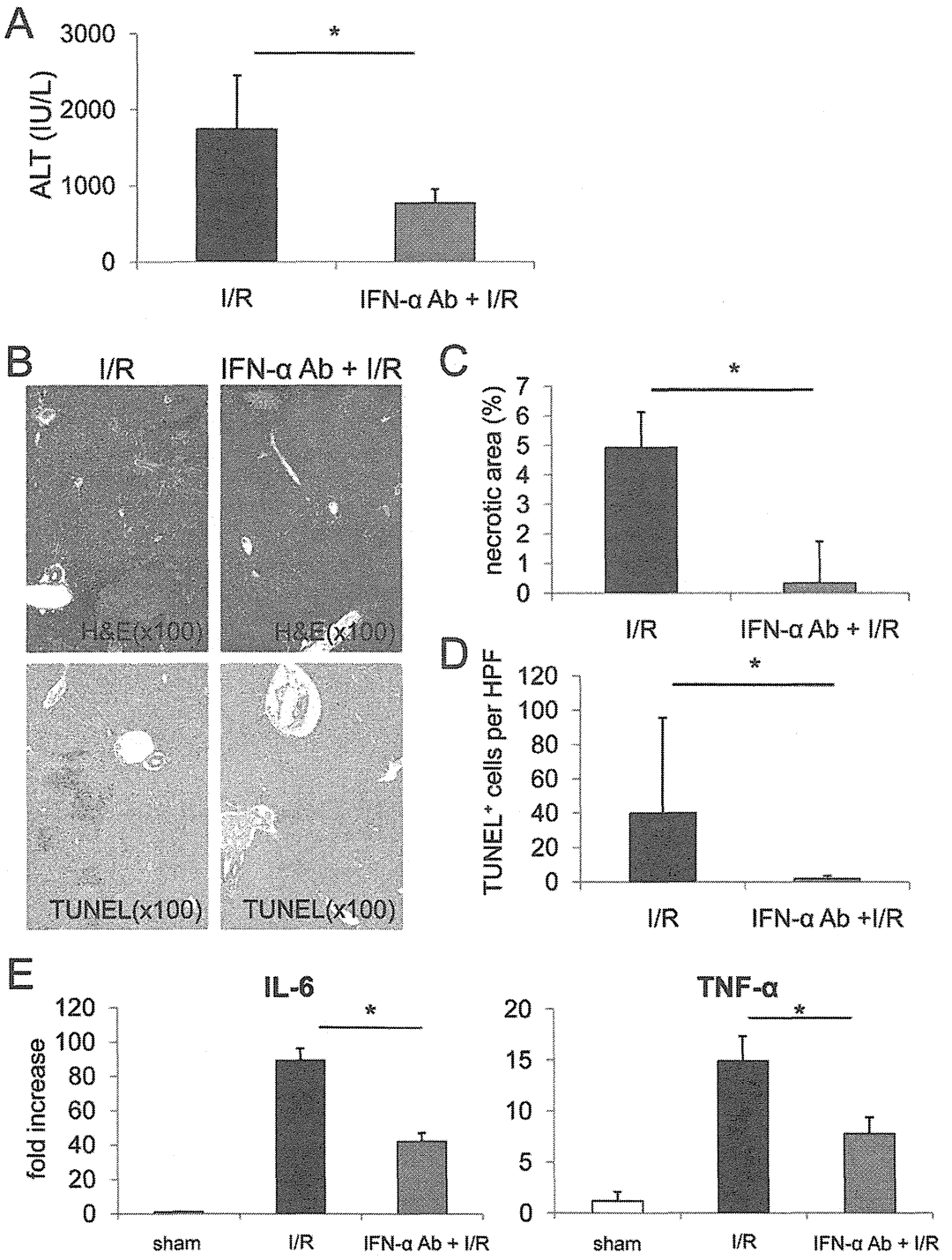


Fig. 3. *In vivo* neutralization of IFN-α reduces liver I/R injury, associated with reduced IL-6 and TNF-α gene expression. B6 mice were injected intravenously (i.v.) with neutralizing IFN-α mAb (50 μg/mouse; i.v.) or isotype control Ig, 30 minutes before being submitted to I/R injury. After 6 hours reperfusion, serum and livers were harvested. (A) Serum ALT levels; overall analysis of 4 mice per group; $*P < 0.05$. (B) Representative H&E- and TUNEL-stained sections of liver tissue. (C) Analysis of the extent of necrosis. (D) Incidence of apoptotic ($TUNEL^+$) cells per high-power field. (E) mRNA levels of IL-6 and TNF-α in whole liver tissue. (C-E) Overall analysis of n = 4 mice per group. $*P < 0.01$.

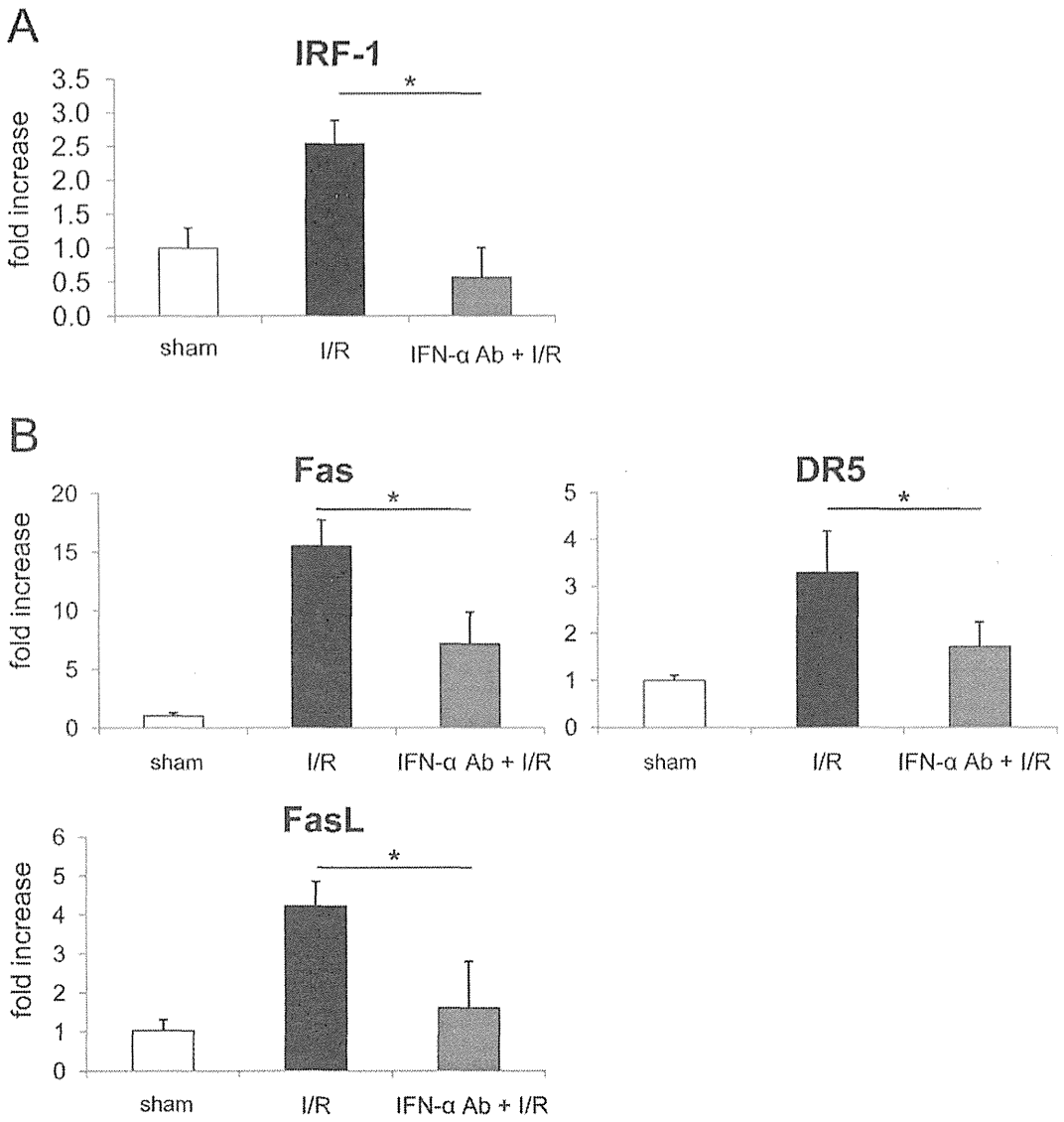


Fig. 4. The protective effect of IFN-α neutralization is associated with down-regulation of IRF-1 gene transcription and death molecule expression. B6 (n = 4 per group) mice were injected i.v. with IFN-α neutralizing mAb (50 μg/mouse; i.v.) or isotype control Ig, 30 minutes before being submitted to I/R injury. After 6 hours reperfusion, liver tissue was harvested. (A) IRF-1 and (B) death ligands (FasL) and death receptor (Fas and DR5) mRNA expression in liver tissue were evaluated by real-time RT-PCR. *$P < 0.01$.

and 24 hours after the last injection underwent I/R. Figure 5A shows the profound depletion of liver pDC (Siglec-H$^+$B220$^+$) achieved by mAb administration. IFN-α induction in injured livers was markedly inhibited (~70% reduction) by pDC depletion (Fig. 5B). As shown in Fig. 5C-F, the livers of pDC-depleted mice were protected from I/R injury, as evidenced by reductions in serum ALT (C) and tissue damage (areas of necrosis and numbers of TUNEL$^+$ cells; D-F). Taken together, these data suggest that liver pDCs are an important source of IFN-α during liver I/R injury and play a key role in the pathogenesis of I/R injury.

## Discussion

Type I IFNs (IFN-α/β) are pleiotropic cytokines produced by DC, macrophages, other immune cells, fibroblasts, and virally infected cells, which contribute both to viral clearance and disease pathogenesis. They have been shown recently to play a pathogenic role in warm and cold liver I/R injury. Thus, in mice, IFNAR KO livers are protected from such injury.[11,12] Specific cytokine (e.g., TNF-α and IL-6) neutralization has already proved an effective approach for disease therapy in the clinic[31,32] and an IFN-α neutralizing Ab

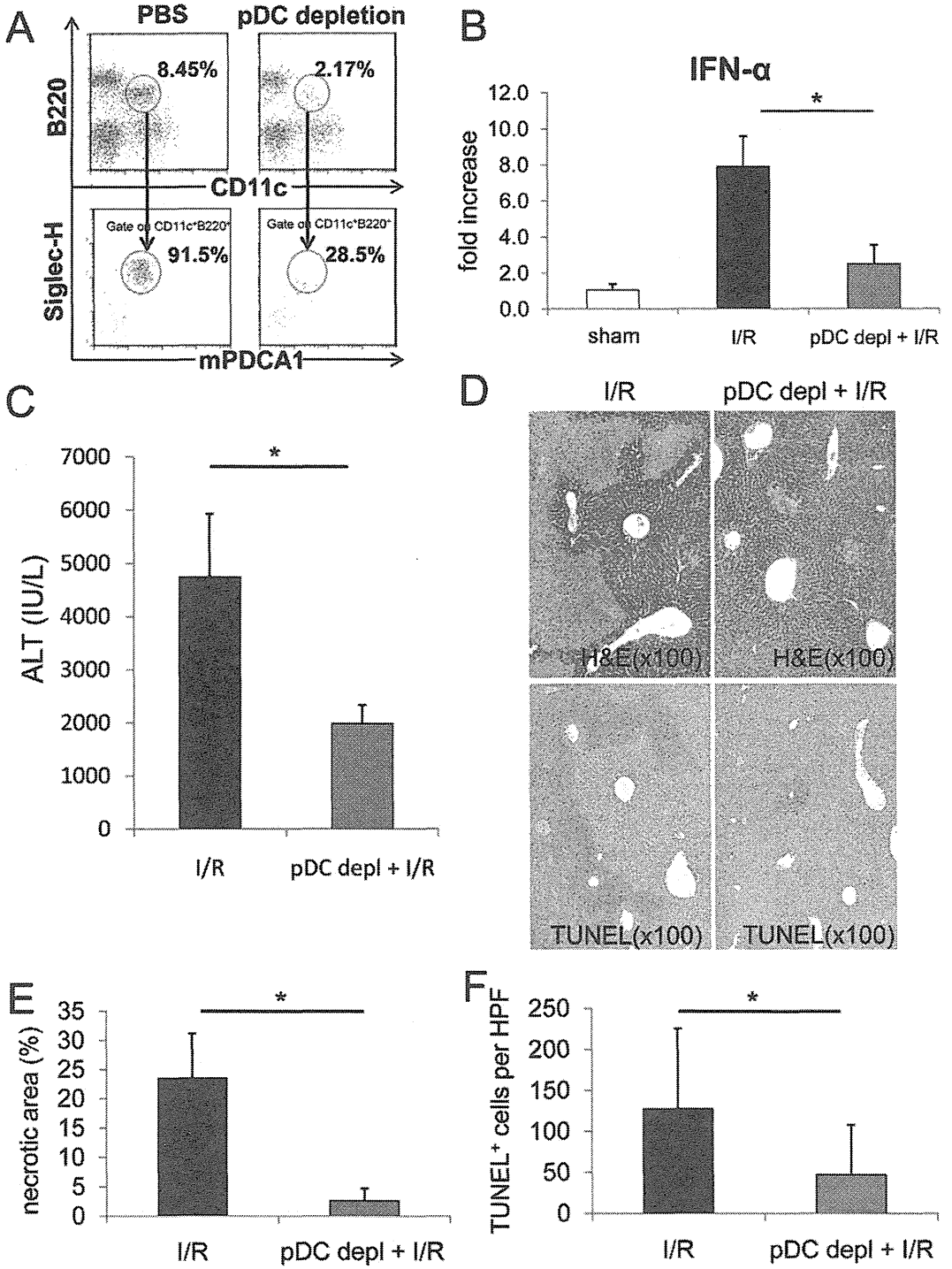


Fig. 5. *In vivo* pDC depletion protects the liver from I/R injury. B6 mice were injected for 3 consecutive days with anti-mPDCA-1 pure-functional grade mAb (500 $\mu$g, i.p.) or isotype control Ig. (A) To assess the depleting efficiency of the Ab, freshly isolated liver nonparenchymal cells were stained for CD11c, B220, Siglec-H, and mPDCA-1 and analyzed by flow cytometry. $CD11c^{int}B220^{+}$Siglec-$H^{+}$m$PDCA\text{-}1^{+}$ cells (pDC) were gated and their frequency calculated. Data are representative of 3 mice per group. (B-F) Twenty-four hours after the final mAb injection, the vasculature supplying the left and median lobes of the liver was occluded for 60 minutes. The liver was then reperfused for 6 hours. (B) IFN-$\alpha$ mRNA expression was measured by real-time RT-PCR. (C) Serum ALT levels ($n = 5$; $^{*}P < 0.05$). (D) H&E and TUNEL staining were performed to assess the extent of liver damage and parenchymal cell apoptosis. Representative histopathological images of ischemic lobes are shown. (E) Extent of necrotic area and (F) TUNEL-positive cell number per high-power field (HPF) were analyzed in liver tissue. depl = depletion.

(MEDI-5) is already in phase I clinical trials in psoriasis that have confirmed its safety.[10] Here we show a marked reduction in murine liver warm I/R injury either by selective depletion of pDC or IFN-α neutralization and a possible underlying protective mechanism of impaired production of IRF-1, a key downstream molecule of IFN-α signaling[13] in the liver.

IRF-1 is a transcription factor induced by viruses, double-stranded RNA, retinoic acid, and cytokines, including IFN-α. It regulates cell proliferation, apoptosis, and immune responses.[33] We show in this study that IFN-α neutralization reduces IRF-1 induction by hepatocytes *in vitro* and in the liver after I/R injury. IRF-1 is known to induce apoptosis by increasing expression of FasL.[15,19] We also found that IFN-α neutralization reduced hepatocyte apoptosis after liver I/R injury, consistent with lower expression of death ligand (FasL) and death receptors (Fas and DR5) within the liver. These results suggest that IFN-α neutralization attenuates the induction of IRF-1 in hepatocytes and consequently Fas-FasL and DR5 expression, resulting in reduced apoptosis following liver I/R injury.

Recently, other IRF members have been shown to contribute to the pathogenesis of liver warm I/R injury. IRF-2 is structurally similar to IRF-1 and competes with IRF-1 as an antagonist. Klune et al.[34] have reported that overexpression of IRF-2 protects the liver from I/R injury by an IRF-1-dependent mechanism. IRF-3 is another transcription factor that is involved in type-I IFN and other proinflammatory cytokine production after TLR stimulation. Interestingly, IRF-3-deficient mice exhibit severe warm liver injury due to greater IL-17 induction and enhanced neutrophil infiltration.[35]

Type I IFNs have immunostimulatory capacity and can activate immune cells, including T cells, natural killer (NK) cells, and DCs.[36] IFN-α-primed DCs produce enhanced levels of proinflammatory cytokines, such as IL-1β, IL-6, IL-12, IFN-γ, and TNF-α, and promote T helper type-1 cell responses.[37] In this study, we have shown that IFN-α neutralization decreases key proinflammatory cytokines, IL-6 and TNF-α, after liver I/R injury. This finding is consistent with recent observations using IFNAR KO mice.[11,12] Although we did not identify the sources of these proinflammatory cytokines, our data suggest that IFN-α is involved in promotion of proinflammatory cytokine production during liver I/R injury.

pDCs are comparatively abundant in the liver compared with secondary lymphoid tissue[38] and regarded as the main source of IFN-α.[20,39] These nonconventional DCs predominantly express TLR7/TLR9 and produce high amounts of IFN-α in response to their ligation. Although these TLR receptors recognize single-stranded RNA and double-stranded DNA as pathogen-associated molecular patterns during viral infection,[40] TLR7/TLR8/TLR9 on pDC can sense self-RNA (TLR7/8)[41] and self-DNA (TLR9)[23] as damage-associated molecular patterns and produce IFN-α. In this study, we found that hepatic warm I/R injury rapidly up-regulated IFN-α gene transcription in the liver after 6 hours reperfusion. This finding is consistent with an earlier report that type I, but not type II IFN receptor KO mice are protected from warm liver I/R damage.[11] Moreover, very recently, using a clinically relevant mouse model of extended hepatic cold preservation followed by orthotropic liver transplantation (OLT), Shen et al.[12] have shown that liver graft but not recipient type I IFN receptor KO deficiency is required to ameliorate I/R injury in OLT.

Notably, in the present study, mice profoundly depleted of pDC exhibited less tissue damage after liver I/R compared with control mice. The decreased liver injury in pDC-depleted mice was associated with a significant reduction in IFN-α (Fig. 5B) in hepatic tissue. Interestingly, after 6 hours reperfusion, the extent of hepatic IFN-α message in pDC-depleted animals was >70% lower than in controls, suggesting that pDCs are the main source of type I-IFNs in the liver. We did not compare IFN-α production by liver pDC and Kupffer cells that are much more abundant in the liver than pDC and play a role in regulation of hepatic IRI.[42,43] However, since pDC depletion resulted in such profound reduction in hepatic IFN-α expression, it may be concluded that liver macrophages are not a major source of IFN-α. Since IFN-β, another type I IFN, may be produced by macrophages during liver I/R injury,[11] and shares downstream STAT1 and IRF-1 signaling pathways with IFN-α, it would be of interest in future studies to address the *in vivo* function of both IFNs in this model.

Here we also show that, following reperfusion, liver pDC acquire a more mature phenotype and secrete enhanced amounts of IFN-α that are associated with higher levels of IFN-α expression in the liver. Liver pDC isolated after warm I/R (activated pDC) secreted IFN-α that contributed to enhanced IRF-1 production by hepatocytes. Taken together, these observations suggest that during I/R, liver pDC may be activated by self-DNA to produce IFN-α that plays a key role in the pathogenesis of I/R injury. A schematic outline of the proposed mechanisms suggested by our findings is shown in Fig. 6.

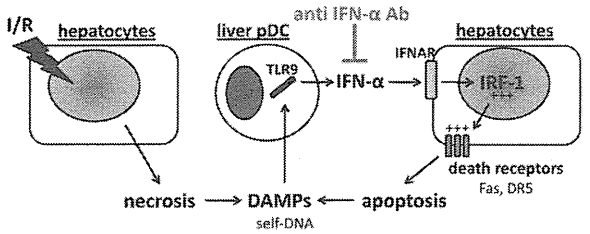


Fig. 6. Schematic representation of the proposed role of pDC and type I IFN in hepatic I/R. Vascular occlusion involving 70% of the liver for 1 hour followed by 6 hours reperfusion induces hepatocyte necrosis and release of damage-associated molecular patterns (DAMPs), such as the TLR9 ligand self-DNA. Self-DNA engages TLR9 in hepatic pDC that, in turn, are activated and release comparatively large amounts of type I IFN (IFN-α). Thereafter, IFN-α engages its receptor (IFNAR) on hepatocytes and induces transcription of IRF-1, a key proinflammatory factor involved in the pathogenesis of I/R injury, able to perpetuate liver damage and release new DAMPs. Blocking of type I IFN using neutralizing anti-IFN-α mAb down-regulates hepatocyte IRF-1 with a concomitant reduction in hepatocyte apoptosis (decreased levels of Fas and DR5) and proinflammatory cytokines, resulting in reduced liver damage. +++Elevated expression.

Conventional myeloid DC that are important regulators of innate and adaptive immunity, have been implicated previously in the regulation of inflammation and tissue injury after liver I/R,[26,27,37,44] with both inhibitory and enhancing effects reported. Our recent observations[27] suggest that the local liver microenvironment plays an important role in determining DC function during I/R injury. However, no previous reports have focused on the role of pDC in hepatic I/R injury. pDCs are believed to exhibit immunoregulatory properties due to their poor immunostimulatory function, Treg-inducing ability, and IL-10 production.[45,46] Depletion of these cells *in vivo* exacerbates viral infection,[47] asthma,[48] and transplant rejection.[49] On the other hand, the present study shows that pDC depletion can protect the liver from warm I/R injury and reveals an underlying pathological role of these cells. Similar to our results, pDC have been shown to play a pathological role in experimental allergic encephalomyelitis (EAE) in mice, with pDC depletion reducing disease activity.[50] Type I IFNs derived from pDC are important for inhibition of viral replication and clearance,[21] but type I IFNs are also pathogenic in the early phase of EAE[50] and in our liver warm I/R injury model. Overall, these observations suggest that the specific role of type I IFNs in each experimental model may determine the role of pDC (protective or pathogenic).

Taken together, our novel findings demonstrate that IFNα secreted by activated pDC is involved in the promotion of hepatic I/R injury and in modulating the expression of proinflammatory cytokines such as IL-6 and TNF-α and, most important, IRF-1 which regulates hepatocyte apoptosis. The data provide significant new insight into mechanisms that promote liver I/R injury by way of a pDC-IFN-α-IRF-1 pathway and support the therapeutic potential of IFN-α neutralization for amelioration of liver I/R injury.

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# DAP12 Deficiency in Liver Allografts Results in Enhanced Donor DC Migration, Augmented Effector T Cell Responses and Abrogation of Transplant Tolerance

**O. Yoshida[1], S. Kimura[1], L. Dou[1,2], B. M. Matta[1], S. Yokota[1], M. A. Ross[3], D. A. Geller[1] and A. W. Thomson[1,4,*]**

[1]*Department of Surgery, Starzl Transplantation Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA*
[2]*Hepatic Surgery Center, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China*
[3]*Center for Biologic Imaging, University of Pittsburgh School of Medicine, Pittsburgh, PA*
[4]*Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA*
**Corresponding author: Angus W. Thomson, thomsonaw@upmc.edu*

**Liver interstitial dendritic cells (DC) have been implicated in immune regulation and tolerance induction. We found that the transmembrane immunoadaptor DNAX-activating protein of 12 kDa (DAP12) negatively regulated conventional liver myeloid (m) DC maturation and their *in vivo* migratory and T cell allostimulatory ability. Livers were transplanted from C57BL/6($H2^b$) (B6) WT or $DAP12^{-/-}$ mice into WT C3H ($H2^k$) recipients. Donor mDC ($H2\text{-}K^{b+}CD11c^+$) were quantified in spleens by flow cytometry. Anti-donor T cell reactivity was evaluated by *ex vivo* carboxyfluorescein diacetate succinimidyl ester-mixed leukocyte reaction and delayed-type hypersensitivity responses, while T effector and regulatory T cells were determined by flow analysis. A threefold to fourfold increase in donor-derived DC was detected in spleens of $DAP12^{-/-}$ liver recipients compared with those given WT grafts. Moreover, pro-inflammatory cytokine gene expression in the graft, interferon gamma (IFNγ) production by graft-infiltrating $CD8^+$ T cells and systemic levels of IFNγ were all elevated significantly in $DAP12^{-/-}$ liver recipients. $DAP12^{-/-}$ grafts also exhibited reduced incidences of $CD4^+Foxp3^+$ cells and enhanced $CD8^+$ T cell IFNγ secretion in response to donor antigen challenge. Unlike WT grafts, $DAP12^{-/-}$ livers failed to induce tolerance and were rejected acutely. Thus, DAP12 expression in liver grafts regulates donor mDC migration to host lymphoid tissue, alloreactive T cell responses and transplant tolerance.**

**Keywords: DAP12, dendritic cells, liver transplant, T cells, tolerance**

**Abbreviations: Ab, antibody; Ag, antigen; ALT, alanine aminotransferase; APC, antigen-presenting cell(s); B7-H1, B7 homologue 1; BM, bone marrow; CFSE, carboxyfluorescein diacetate succinimidyl ester; DAP12, DNAX-activating protein of 12 kDa; DC, dendritic cell(s); DTH, delayed-type hypersensitivity; Flt3L, fms-like tyrosine kinase 3 ligand; IFNγ, interferon gamma; IRAK, interleukin-1 receptor-associated kinase; ITAM, immunoreceptor tyrosine-based activation motif; LN, lymph node; LPS, lipopolysaccharide; m, myeloid; MLR, mixed leukocyte reaction; MST, mean survival times; NK, natural killer; NPC, nonparenchymal cell(s); p, plasmacytoid; siRNA, small interfering RNA; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cell(s); TREM, triggering receptor expressed on myeloid cells**

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## Introduction

Liver grafts between MHC-mismatched mice (1), certain rat strains (2,3) and outbred pigs (4) are accepted without immunosuppressive therapy. Whereas in mice, liver allografts induce robust, donor-specific tolerance (1), in humans, the liver is generally regarded as the most tolerogenic of transplanted whole organs (5,6). Mechanisms underlying liver transplant tolerance are not well understood, but production of soluble MHC Class I by the graft and regulatory functions of donor-derived hematopoietic cells have been proposed (3,7,8). The liver is an immune organ, with a unique consistency of both nonparenchymal cells (NPC) and parenchymal cells that have been implicated in tolerance induction (9–11). These include professional and nonprofessional antigen (Ag)-presenting cells (APC), that is, dendritic cells (DC) and hepatic macrophages (Kupffer cells) (12,13), sinusoid-lining endothelial cells (14,15), hepatic stellate cells (16,17) and hepatocytes (18). There is strong evidence that these hepatic APC play important roles in immune regulation and tolerance induction (10). DC are uniquely well-equipped APC that promote self-tolerance in the steady state (19) and regulate immunity (20,21). Liver-resident DC comprise

several subsets (10,12). In addition to conventional $CD11b^+$ $CD11c^+$ myeloid (m)DC, other DC subsets found in the mouse liver include rarer, nonconventional plasmacytoid (p) DC ($CD11b^-$ $CD11c^{lo}$ $B220^+$ plasmacytoid DC $Ag^+$ [PDCA]-$1^+$) (22). They are characterized by their immaturity and resistance to maturation (23,24) and display tolerogenic properties (8,25,26). Liver DC can attenuate hepatic inflammation and fibrosis (27–29) and regulate liver warm and transplant-induced ischemia-reperfusion injury (30,31). Moreover, liver DC can subvert T cell responses (12,25,26,32–34) and prolong allograft survival (35).

Molecular mechanisms whereby hepatic APC regulate/inhibit T cell responses include the expression of B7 homologue 1 (B7-H1) (14,34,36,37), IL-10 (38,39), FasL (40,41), the Notch ligand Jagged 1 (18), CD39 (31) and DNAX-activating protein of 12 kDa (DAP12) (42). DAP12 is a homodimeric immunoreceptor tyrosine-based activation motif (ITAM)-bearing transmembrane adaptor protein that is highly expressed in lymphoid tissues and the lung and, to a much lesser degree, in whole liver tissue (43,44). It is expressed by DC, macrophages and natural killer (NK) cells and integrates signals through multiple receptors, including triggering receptor expressed on myeloid cells (TREM)-1 and -2, NKG2D, Ly49, myeloid DAP12-associating lectin-1 and CD200R (45–48). By associating with distinct receptors, DAP12 can potentiate or inhibit leukocyte activation, with the outcome determined by the avidity between the DAP12-associated receptor and its ligand (49). Macrophages from $DAP12^{-/-}$ mice have increased phagocytic capacity (50) and DAP12/TREM-1/2 activation modulates phagocytosis (51,52).

Conventional mDC propagated from the bone marrow (BM) of $DAP12^{-/-}$ mice exhibit a more mature phenotype than those from WT controls (53), while DAP12-deficient lung $CD11c^+$ APC enhance Ag-specific T cell responses *in vivo* (54). Recently, using small interfering RNA (siRNA) to silence DAP12, we found (42) that DAP12 promoted the expression of IL-1 receptor-associated kinase (IRAK)-M, a negative regulator of Toll-like receptor (TLR) signaling and the production of IL-10 by liver mDC. Consequently, DAP12 restrained their T cell allostimulatory activity. In this study, we examined the role of DAP12 in determining the *in vivo* migrational function of liver-derived mDC, the survival of liver transplants from $DAP12^{-/-}$ mice and underlying effects on T cell responses to the allograft. Our data suggest that DAP12 expression in liver grafts regulates the migration of donor mDC to host secondary lymphoid tissue, Th1 cell-mediated alloimmune responses and the induction of transplant tolerance.

## Materials and Methods

### *Mice*

Male C57BL/6 (B6;$H2^b$), BALB/c ($H2^d$) and C3H ($H2^k$) mice (8- to 12-week-old) were purchased from the Jackson Laboratory (Bar Harbor, ME). $DAP12^{-/-}$ mice (55), generated initially in the 129/SvJ and B6 hybrid background as described (56), were backcrossed onto the B6 background and kindly provided by Dr. Marco Colonna, Washington University School of Medicine, St. Louis, MO. Animals were maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh School of Medicine. Experiments were conducted under an Institutional Animal Care and Use Committee-approved protocol and in accordance with criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. Animals were fed a diet of Purina rodent chow (Ralston Purina, St. Louis, MO) and received tap water *ad libitum*.

### *Reagents*

Complete culture medium comprised RPMI-1640 (BioWhittaker, Walkersville, MD) supplemented with 10% (v/v) fetal calf serum (Nalgene, Miami, FL), nonessential amino acids, L-glutamine, sodium pyruvate, penicillin–streptomycin and 2-mercaptoethanol (all from Life Technologies, Gaithersburg, MD). Lipopolysaccharide (LPS) and CpG A; ODN 1585 were purchased from InvivoGen (San Diego, CA). Chinese hamster ovary cell-derived recombinant human fms-like tyrosine kinase 3 ligand (Flt3L) was obtained from Amgen (Seattle, WA).

### *Isolation of mouse liver and spleen DC*

DC were isolated and purified as described in detail (24). Briefly, livers and spleens were recovered from mice given the endogenous DC poietin Flt3L (10 μg/day intraperitoneally; 10 days) and digested in collagenase (Sigma, St. Louis, MO). Bulk DC were enriched by density gradient centrifugation using Histodenz (Sigma). For pDC purification (>95%), $PDCA\text{-}1^+$ cells were positively selected from the DC-enriched fraction using immunomagnetic beads and a paramagnetic LS column (Miltenyi Biotec, San Diego, CA) (22). mDC ($CD11b^+CD11c^+PDCA\text{-}1^-$) were isolated from the pDC-depleted, DC-enriched fraction using anti-CD11c immunomagnetic beads (Miltenyi Biotec) as described (22). The purity of mDC consistently exceeded 95%.

### *Flow cytometry*

Liver DC, hepatic NPC and spleen cells were treated with FcγR-blocking rat anti-mouse CD16/32 mAb (2.4G2) to prevent nonspecific antibody (Ab) binding. They were then incubated for 30 min with fluorescein isothiocyanate-, phycoerythrin (PE)-, APC-, PE-cyanin (Cy)5- or PE-Cy7-conjugated mAbs to detect expression of CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11c (HL3), B220/CD45R (RA3-6B2), $I\text{-}A^b$ β-chain (25-9-17) (all eBioscience, San Diego, CA), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), $H2\text{-}K^b$ (AF6-88.5) and B7 H1 (CD274) (MIH5) (BD Biosciences, San Diego, CA). For intracellular cytokine staining, cells were fixed with 4% paraformaldehyde and permeabilized using 0.1% saponin, then stained with anti-mouse interferon gamma (IFNγ) Ab (XMG1.2) (BioLegend, San Diego, CA). For Foxp3 staining, cells were fixed and permeabilized using Foxp3 Fix Permkit (eBioscience) and stained with anti-Foxp3 mAb (FJK-16s) (eBioscience). Appropriate Ig isotype controls were obtained from BD PharMingen (San Diego, CA). Flow analysis was performed using an LSR Fortessa flow cytometer (BD Biosciences). Results are expressed as percent positive cells and mean fluorescence intensity.

### *T cell purification*

Bulk splenocyte suspensions were incubated with an mAb cocktail consisting of anti-CD45R/B220 (RA3-6B2), anti-CD16/CD32 (2.4G2), anti-TER-119, anti-CD11b (M1/70) and anti-Ly6G (RB-8C5) obtained from BD PharMingen and non-T cells eliminated by immunomagnetic negative selection using Dynabeads (Invitrogen, Grand Island, NY) following the manufacturer's instructions.

### *Mixed leukocyte reaction*

To assess the T cell allostimulatory activity of liver mDC or pDC, freshly isolated, unstimulated or stimulated DC were used as stimulators of allogeneic BALB/c T cells ($2 \times 10^5$/well) in 72 h mixed leukocyte reaction (MLR) using 96-well, round-bottom plates. For *ex vivo* T cell re-stimulation or measurement of anti-donor responses, bulk splenocytes of sensitized or transplanted mice were used as responders, and T cell–depleted (CD3ε Microbeads kit; Miltenyi Biotec) B6 splenocytes as stimulators. For the final 18 h of culture, 1 μCi of [$^3$H]-thymidine (Perkin Elmer, Waltham, MA) was added to each well. Radioisotope incorporation was determined using a beta scintillation counter (Perkin Elmer) and results were expressed as mean cpm ± 1 SD of triplicate wells. Alternatively, T cell proliferation was determined by carboxyfluorescein diacetate succinimidyl ester (CFSE)-MLR using responder T cells labeled with CFSE (Invitrogen) as described (42).

### *Cytokine assays*

Cytokine levels in culture supernatants or serum samples were determined by cytometric bead array flex sets (BD Biosciences) (IL-6, tumor necrosis factor [TNF]α and IFNγ) or by ELISA (IL-12p40 and IFNα) (BioLegend and PBL Biomedical Labs, Piscataway, NJ, respectively), following the supplier's instructions.

### *Real-time reverse-transcription polymerase chain reaction*

Messenger RNA (mRNA) expression was quantified by SYBR Green real-time reverse-transcription polymerase chain reaction using an ABI-Prism 7000 sequence detection system (PE Applied Biosystems, Foster City, CA) and primers specific for IFNγ (F: 5′-CACGGCACAGTCATTGAAAG-3′; R: 5′-TTTTGCCAGTTCCTCCAGAT-3′), TNFα (F: 5′-CATCTTCTCAAAATTC-GAGTGA-3′; R: 5′-TGGGAGTAGACAAGGTACAAC-3′), IL-6 (F: 5′-TCAATTC-CAGAAACCGCTATGA-3′; R: 5′-CACCAGCATCAGTCCCAAGA-3′), IL-12 (F: 5′-AACCATCTCCTGGTTTGCCA-3′; R: 5′-CGGGAGTCCAGTCCACCTC-3′), granzyme B (F: 5′-CGATCAAGGATCAGCAGCC-3′; R: 5′-CTGGGTCTTCTC-CTGTTCT-3′), perforin (F: 5′-GAAGACCTATCAGGACCAGTACAACTT-3′; R: 5′-CAAGGTGGAGTGGAGGTTTTG-3′), Foxp3 (F: 5′-CACCTATGCCACCCT-TATCC-3′; R: 5′-CGAACATGCGAGTAAACCAA-3′) or β-actin (F: 5′-AGAGG-GAAATCGTGCGTGAC-3′; R: 5′-CAATAGTGATGACCTGGCCGT-3′). The expression of each gene was normalized to the expression of β-actin mRNA using the comparative cycle threshold method (57).

### *Delayed-type hypersensitivity*

BALB/c mice were immunized by subcutaneous (s.c.) injection at the base of the tail with $10.10^6$ purified WT B6 or DAP12$^{-/-}$ liver mDC. Seven days later, the mice were challenged s.c. in the hind footpad with $10.10^6$ B6 splenocytes. Phosphate-buffered saline (PBS) alone was injected into the contralateral hind footpad as a control. Footpad thickness was measured as described (34) at time 0, and at 24 and 48 h after challenge, using Quick Mini Series 700 digital calipers (Mitutoyo, Kawasaki, Japan).

### *Liver transplantation and histopathology*

Liver recovering and orthotopic liver transplantation without hepatic artery reconstruction were performed as described initially by Qian et al (1,58) with minor modifications. Liver grafts (WT B6 or DAP12$^{-/-}$) were perfused with University of Wisconsin solution via the portal vein, then transplanted orthotopically into normal C3H recipients by anastomosis of the supra-hepatic vena cava with a running 10-0 suture and by anastomosis of the portal vein and inferior vena cava using the cuff technique. The bile duct was connected via ligation over the stent. Liver enzyme (alanine aminotransferase [ALT]) levels were quantified in serum as described (59). Allograft survival was determined by host survival and rejection assessed histologically. Hematoxylin and eosin stained tissue sections were graded in a "blinded" fashion by a transplant pathologist (AJD) using the Banff schema for acute liver rejection (60).

### *Immunohistochemistry*

Immunofluorescence staining for Foxp3$^+$ cells in cryostat sections of liver tissue was performed as described (61).

### *DC trafficking*

CFSE-labeled, purified liver mDC ($10.10^6$) were injected s.c. into one hind footpad of normal, allogeneic recipients and CFSE$^+$CD11c$^+$ cells determined in the draining popliteal lymph node (LN), 24 h later by flow cytometry. Following liver transplantation, donor-derived DC (H2-K$^{b+}$CD11c$^+$) were quantified similarly in host spleen cell suspensions.

### *Statistical analyses*

The significances of differences between means were ascertained using the unpaired Student's t-test, two-way analysis of variance or log-rank test using Prism version 5.00 (Graphpad Software, San Diego, CA). $p < 0.05$ was considered significant.

## Results

### *DAP12$^{-/-}$ conventional liver mDC exhibit a more mature phenotype, enhanced production of pro-inflammatory cytokines and greater T cell allostimulatory ability*

We first examined the cell surface phenotype of conventional liver mDC freshly isolated from WT B6 and DAP12$^{-/-}$ animals. Previous studies (24,32,38,62,63) have described in detail the comparative immaturity and maturation-resistance of normal mouse liver DC compared with those in other parenchymal organs and secondary lymphoid tissues. Using flow cytometry, we observed that, typically for mouse steady-state liver mDC, moderate levels of MHC Class II (IA$^b$), but very low levels of co-stimulatory and co-regulatory molecules (CD40, CD80, CD86, B7-H1) were expressed on unstimulated cells. These levels were increased significantly on both WT and DAP12KO$^{-/-}$ liver mDC after TLR4 ligand (LPS) stimulation (Figure 1A). The expression levels of MHC II, co-stimulatory and co-regulatory molecules were enhanced by DAP12 deficiency, both in the steady state and following DC activation (Figure 1A and B). In addition, secretion of pro-inflammatory cytokines (IL-6, TNFα and especially IL-12p40, which was increased fourfold) by DAP12$^{-/-}$ liver mDC was enhanced significantly following LPS stimulation compared to WT controls (Figure 1C).

Consistent with their low levels of cell surface co-stimulatory molecules, unstimulated WT liver mDC elicited low levels of allogeneic T cell proliferation in MLR. As anticipated, they displayed greater allostimulatory activity before and after LPS stimulation (Figure 2A and B). In addition, DAP12$^{-/-}$ liver mDC enhanced significantly (threefold) the production of IFNγ by the responder T cell population compared with WT DC (Figure 2C). Taken together, these data confirm and extend our earlier findings using siRNA (42) to silence DAP12, which showed that this

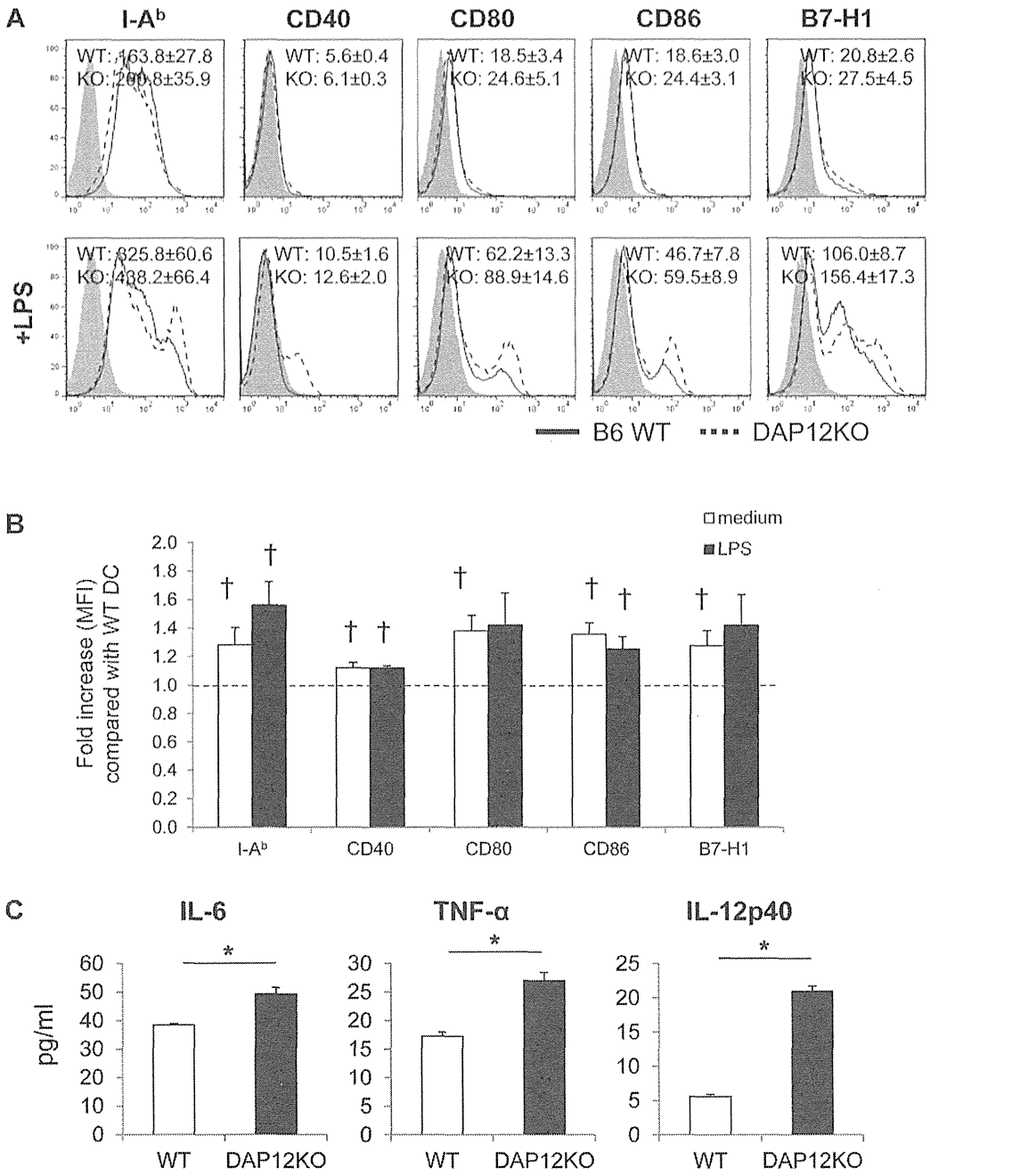


**Figure 1: DAP12$^{-/-}$ mouse (C57BL/6; B6) liver myeloid (m) dendritic cells (DC) express elevated levels of cell surface MHC Class II (I-A$^b$), co-stimulatory and co-regulatory molecules and secrete increased levels of pro-inflammatory cytokines.** (A) Flow cytometric analyses of mAb-stained cells WT or DAP12$^{-/-}$ liver mDC cultured overnight in the absence or presence of LPS. Gray profiles indicate isotype controls. Representative data are shown, together with the mean fluorescence intensity (MFI) ± 1 SD for each molecule. (B) Fold increase in MFI for each molecule expressed by DAP12$^{-/-}$ compared with WT liver mDC; †$p < 0.05$. (C) Concentrations of IL-6, TNFα and IL-12p40 in culture supernatants of unstimulated or LPS-stimulated WT and DAP12$^{-/-}$ liver mDC. Data shown are means ± 1 SD obtained from n = 4 independent experiments; *$p < 0.01$. DAP12, DNAX-activating protein of 12 kDa; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

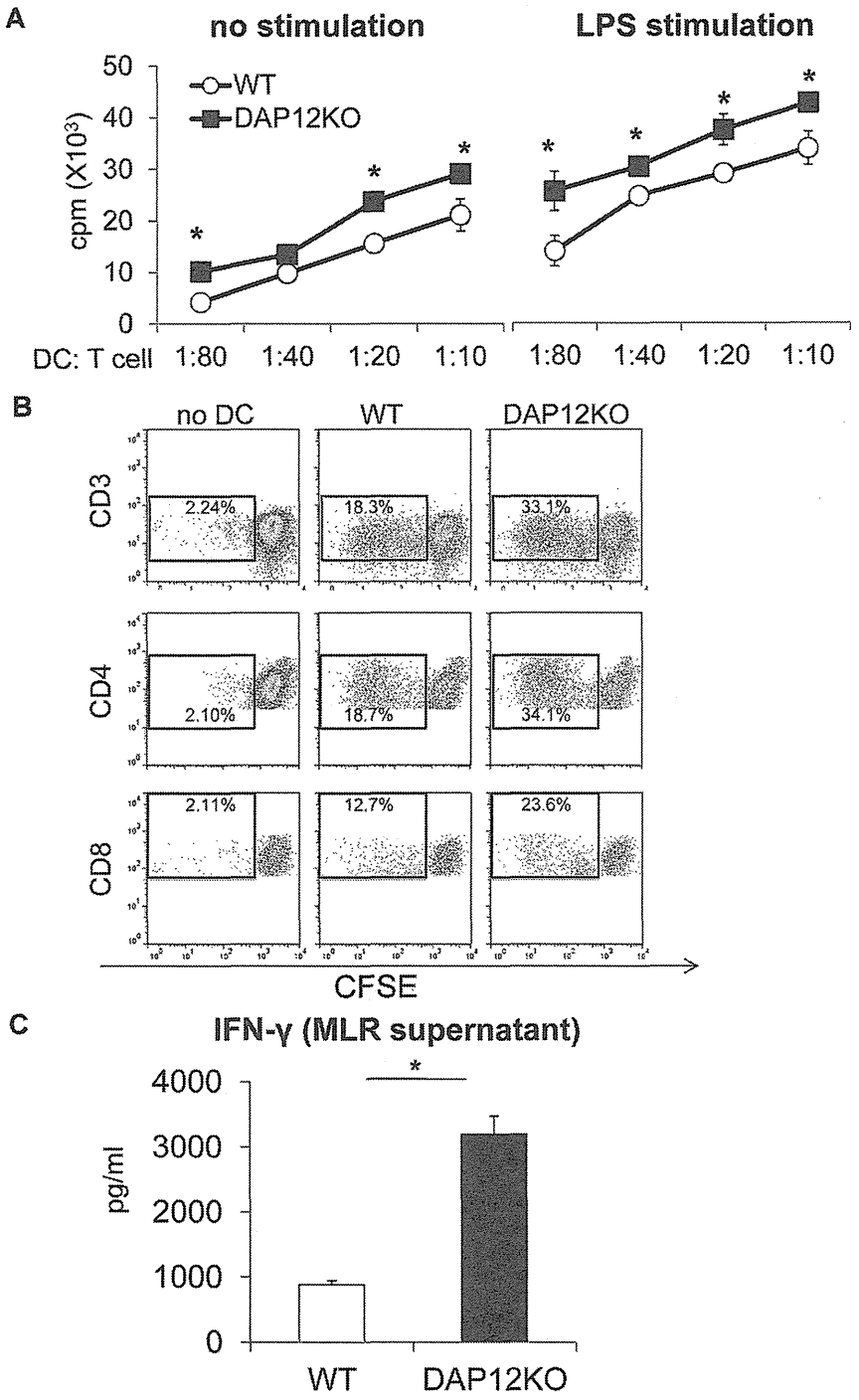


**Figure 2: Enhanced *in vitro* T cell allostimulatory activity of DAP12$^{-/-}$ compared with WT liver mDC.** Liver mDC were cultured with normal bulk allogeneic BALB/c T cells for 72 h as described in the "Materials and Methods" section. (A) Extent of T cell proliferation induced by unstimulated or LPS-stimulated DC at various DC:T cell ratios determined by thymidine incorporation; *$p < 0.05$. (B) Extent of CD4 and CD8 T cell proliferation induced by WT or DAP12$^{-/-}$ liver mDC at a DC:T cell ratio of 1:10 determined by CFSE-MLR. (C) Levels of IFNγ detected in MLR supernatants following T cell stimulation by WT or DAP12$^{-/-}$ liver mDC; *$p < 0.01$. Data are from $n = 4$ independent experiments. CFSE, carboxyfluorescein diacetate succinimidyl ester; DAP12, DNAX-activating protein of 12 kDa; IFNγ, interferon gamma; LPS, lipopolysaccharide; mDC, myeloid dendritic cells; MLR, mixed leukocyte reaction.

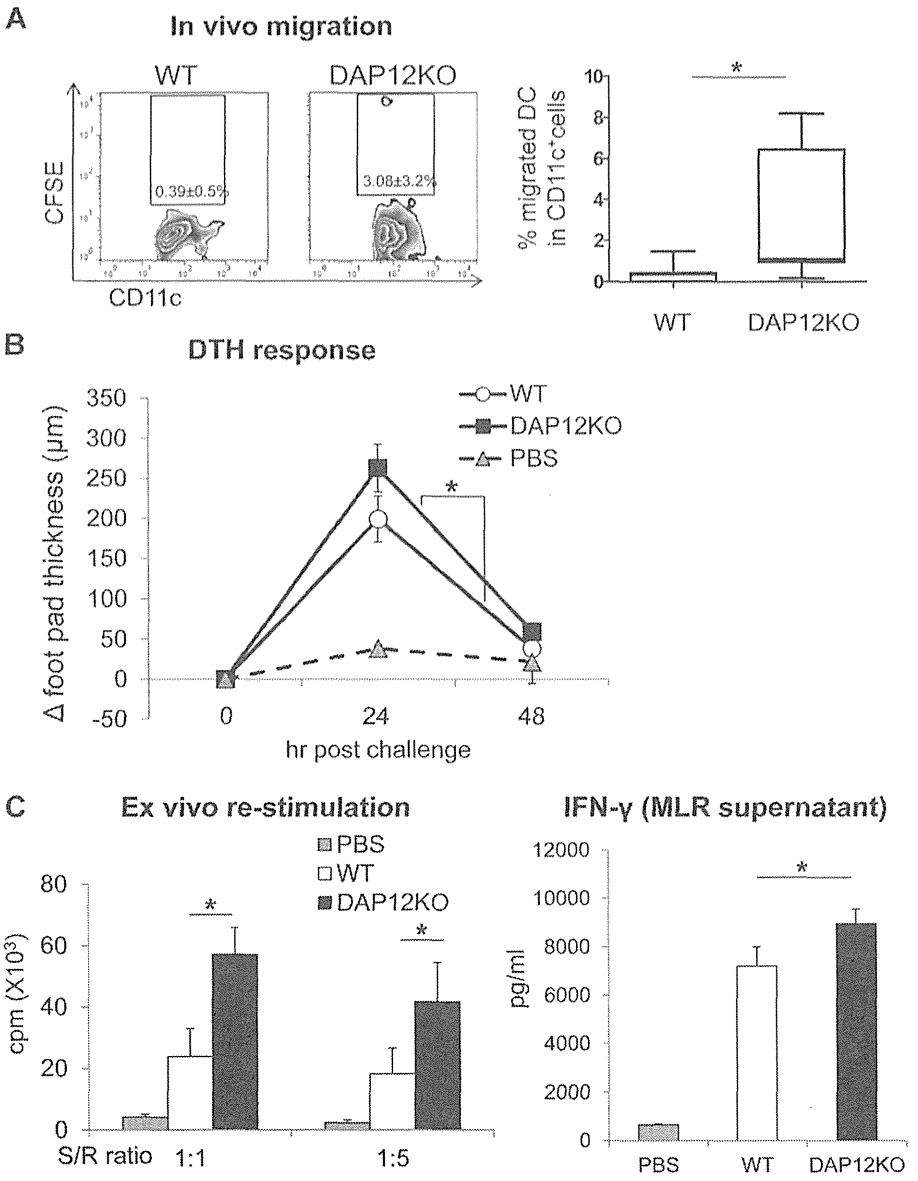


**Figure 3: Enhanced *in vivo* migratory ability of DAP12$^{-/-}$ liver mDC to secondary lymphoid tissue of allogeneic recipients and their increased capacity to induce delayed-type hypersensitivity (DTH) responses.** (A) B6 CFSE-labeled WT or DAP12$^{-/-}$ liver mDC ($10.10^6$) were injected subcutaneously (s.c.) into one hind footpad of normal BALB/c recipients. CFSE CD11c$^+$ (donor) DC were enumerated 24 h later in popliteal lymph nodes by flow cytometry. Representative data are shown on the left, and means ± 1 SD obtained from n = 7 separate experiments are shown on the right; *$p < 0.05$. (B) Groups of six BALB/c mice were sensitized by sc injection at the base of the tail of $10.10^6$ DC and DTH responses elicited 7 days later. Increases in footpad thickness over the ensuing 48 h are shown; *$p < 0.05$. (C) *Ex vivo* proliferative responses and IFNγ production by splenic T cells from mice immunized with WT or DAP12$^{-/-}$ liver mDC; *$p < 0.05$. CFSE, carboxyfluorescein diacetate succinimidyl ester; DAP12, DNAX-activating protein of 12 kDa; IFNγ, interferon gamma; mDC, myeloid dendritic cells.

transmembrane adaptor protein plays a significant role in negative regulation of mouse liver mDC maturation and T cell stimulatory function.

We also examined the phenotype and function of DAP12$^{-/-}$ liver pDC. As shown in Figure S1, although there was no marked change in their surface expression of MHC Class II and co-regulatory molecules, DAP12$^{-/-}$ liver pDC secreted increased amounts of IFNα in response to CpG stimulation and exhibited increased T cell allostimulatory activity compared with WT liver pDC.

### *DAP12$^{-/-}$ liver mDC exhibit enhanced ability to migrate to host secondary lymphoid tissue and to prime alloreactive T cells* in vivo

We next examined the impact of DAP12 deficiency on the *in vivo* migratory and T cell allostimulatory abilities of liver mDC. Previously, migration of allogeneic liver DC from the periphery to host secondary lymphoid tissue has been documented, both in normal WT mice following cell infusion (23,64) and in recipients of liver allografts that develop donor-specific tolerance (1). Following local (s.c.) injection of $10.10^6$ CFSE-labeled B6 WT liver mDC into one hind footpad of BALB/c mice, very few cells (<0.5%) trafficked to the draining popliteal LN within 24 h (Figure 3A). By contrast, significantly greater numbers of

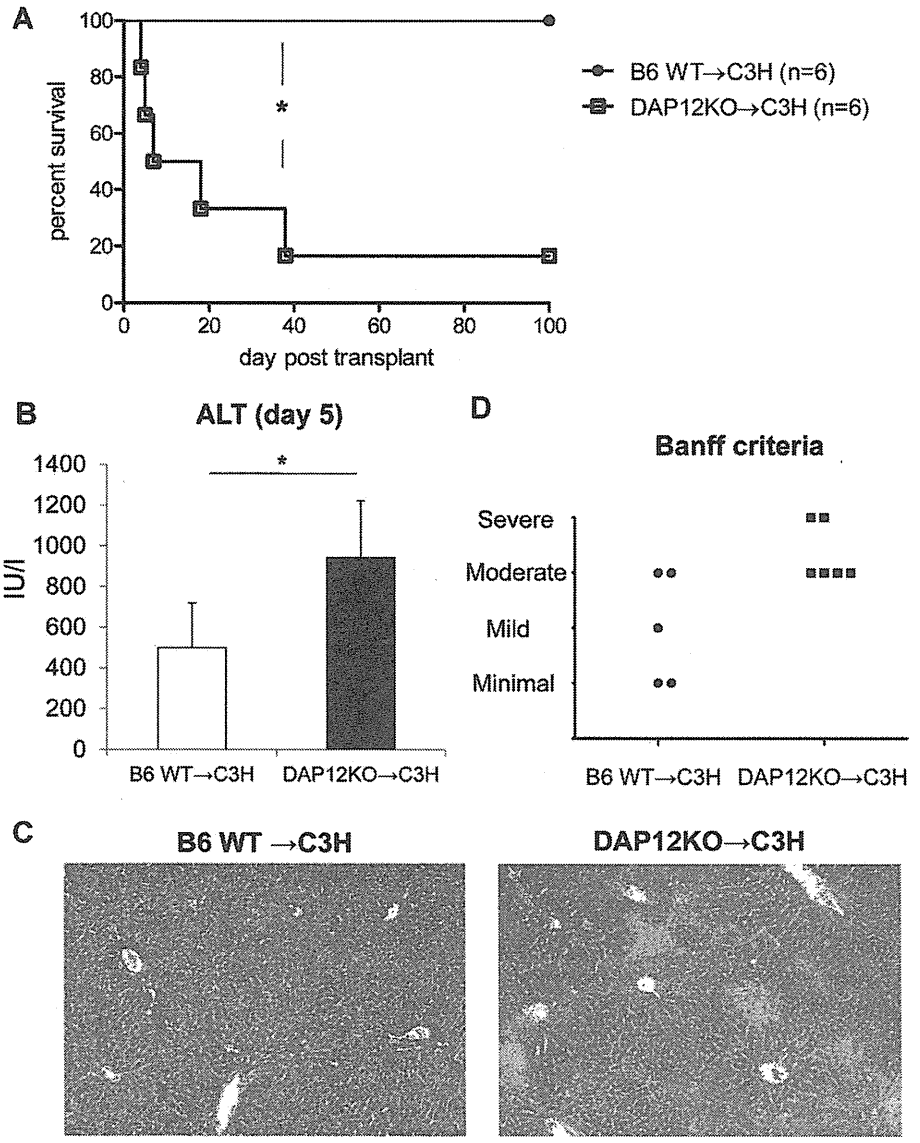


**Figure 4: DAP12$^{-/-}$ liver allografts are rejected acutely.** (A) Whereas normal WT B6 livers transplanted into C3H recipients were accepted indefinitely (>100 days), those from DAP12$^{-/-}$ donors were rejected acutely. Actuarial graft survival curve (n = 6 transplants per group); *$p < 0.001$. (B) Serum ALT levels 5 days posttransplant; *$p < 0.01$. (C) Histopathological appearance of the allografts showing enhanced inflammation and necrosis in DAP12$^{-/-}$ grafts and (D) Banff criteria assessment of rejection. ALT, alanine aminotransferase; DAP12, DNAX-activating protein of 12 kDa.

injected DAP12$^{-/-}$ liver DC were detected in the draining lymphoid tissue under the same experimental conditions.

When DAP12$^{-/-}$ compared with WT B6 liver mDC were injected s.c. (base of tail) into fully allogeneic BALB/c recipients that were challenged 7 days later by local (footpad) injection of donor-strain (B6) splenocytes, significantly increased T cell–mediated delayed-type hypersensitivity (DTH) responses were observed (Figure 3B), indicating enhanced ability of DAP12$^{-/-}$ liver mDC to prime allogeneic T cells *in vivo*. Moreover, *ex vivo* re-stimulation of host T cells with donor APC revealed markedly enhanced anti-donor T cell proliferative responses (Figure 3C) and significantly increased IFNγ levels in MLR supernatants (Figure 3D) of mice immunized with DAP12$^{-/-}$ liver DC. Thus, DAP12 deficiency augments the ability of liver mDC to migrate to allogeneic host secondary lymphoid tissue, prime allogeneic T cells and elicit anti-donor inflammatory responses *in vivo*.

### *DAP12 deficiency in donor livers increases graft inflammation and abrogates transplant tolerance*

To examine the role of DAP12 deficiency in orthotopic liver transplantation, WT or DAP12$^{-/-}$ B6 (H2$^{b}$) livers were transplanted into normal C3H (H2$^{k}$) recipients without immunosuppressive therapy. Whereas in keeping with previous reports (1,65,66), WT liver allografts survived indefinitely (mean survival times [MST]: >100 days; n = 6), all DAP12$^{-/-}$ grafts were rejected acutely (MST: 13 days; n = 6; $p < 0.005$) (Figure 4A). To evaluate rejection, we euthanized graft recipients and evaluated liver injury by serum ALT and histological examination using Banff criteria, on Day 5 after transplantation. Serum ALT levels were elevated significantly in recipients of DAP12$^{-/-}$ livers compared with those given WT grafts (Figure 4B). Consistent with serum ALT levels, more severe rejection was observed in DAP12$^{-/-}$ allografts compared with WT grafts (Figure 4C and D).

### *DAP12 deficiency enhances donor liver mDC migration to host lymphoid tissue*

Liver transplantation in mice is associated with the migration of immature donor interstitial DC and their precursors to host lymphoid tissues (32,64), an event that has been implicated in the induction of liver transplant tolerance (32,67). There was no influence of DAP12 deficiency on the yield of mDC from BM precursors *in vitro* (Figure S2). To examine the migration

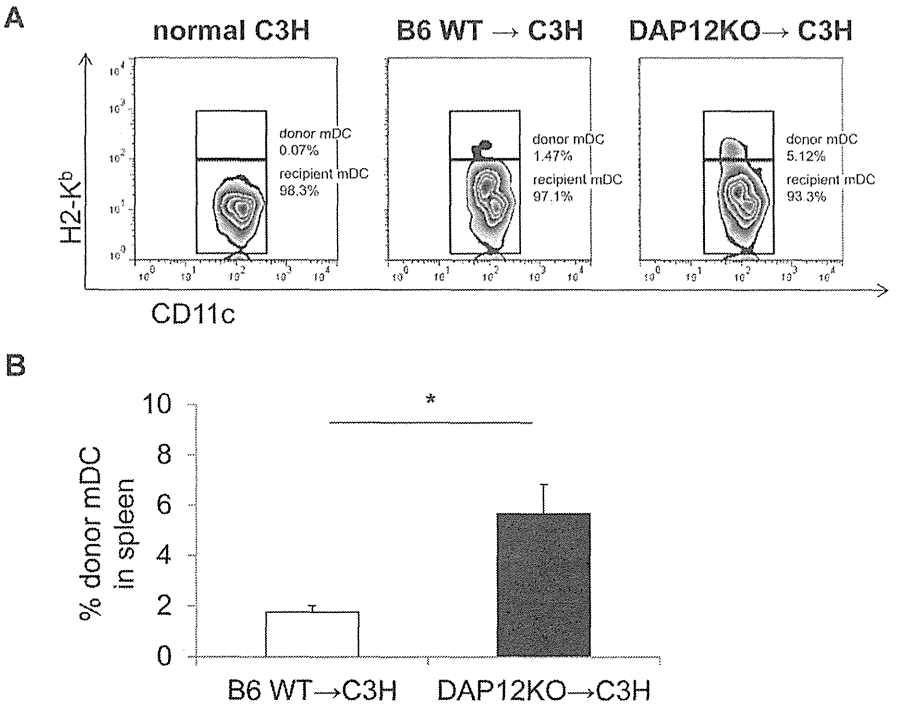


**Figure 5: Increased numbers of donor mDC migrate from DAP12$^{-/-}$ compared with WT liver allografts.** Livers were transplanted orthotopically from B6 WT or DAP12$^{-/-}$ donors to WT C3H recipients without immunosuppression and the incidences of donor-derived (H2-K$^{b+}$) CD11c$^{+}$ DC determined in host spleens 24 h later by flow cytometry. Representative data (n = 4 transplants) are shown in the upper panel (A) and means ± 1 SD in the lower panel (B); *$p < 0.01$. DAP12, DNAX-activating protein of 12 kDa; mDC, myeloid dendritic cells.

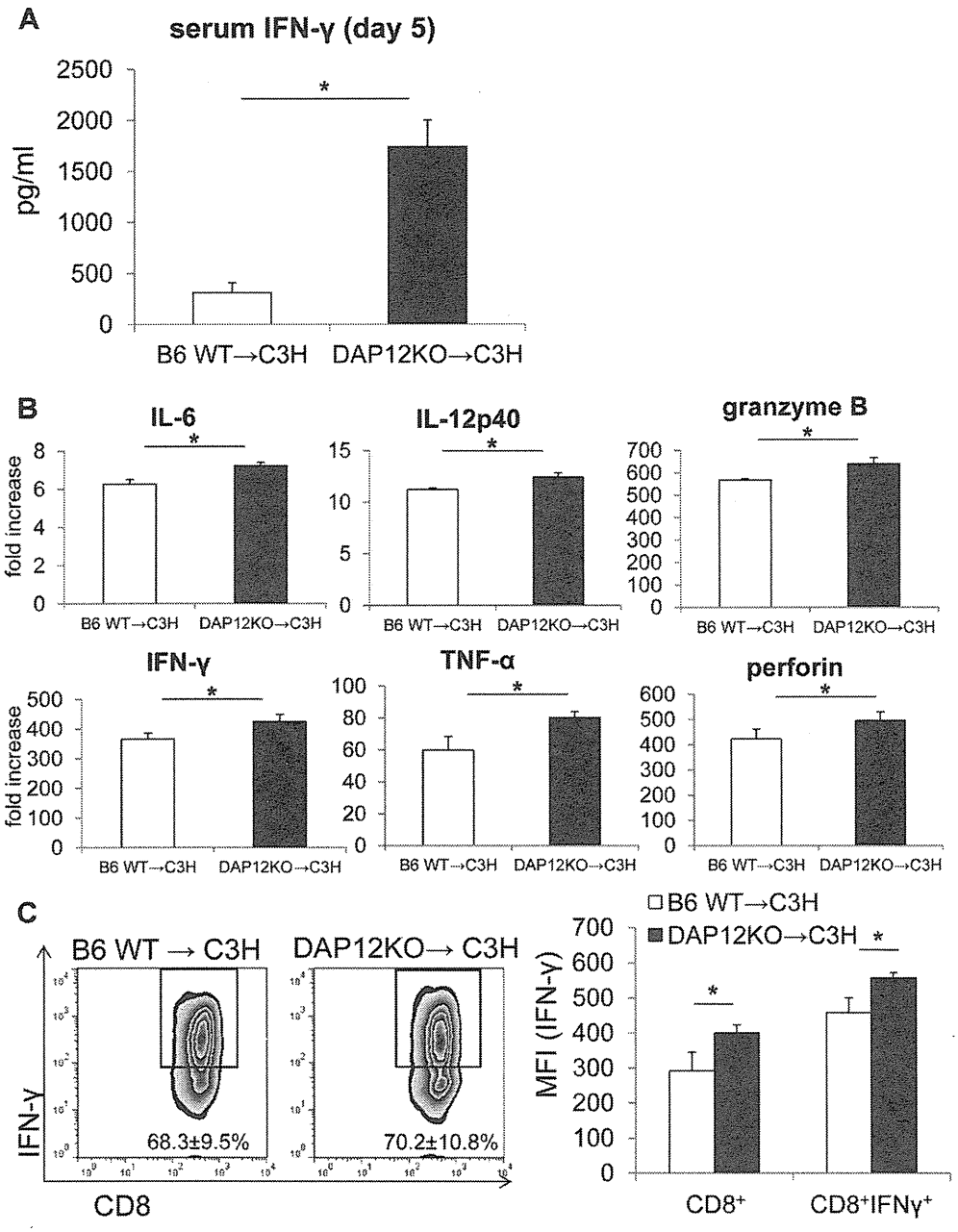


**Figure 6: Systemic levels of IFNγ and intra-graft expression of pro-inflammatory cytokines are increased following transplantation of DAP12$^{-/-}$ compared with WT liver allografts.** (A) Serum IFNγ levels 5 days posttransplant in recipients of either B6 WT or DAP12$^{-/-}$ liver grafts; *$p < 0.001$. (B) Intragraft pro-inflammatory cytokine, granzyme B and perforin levels on Day 5 posttransplant; *$p < 0.05$. (C) Expression of IFNγ by graft CD8$^+$ T cells. Results (means ± 1 SD) were obtained from groups of three transplanted animals; *$p < 0.05$. DAP12, DNAX-activating protein of 12 kDa; IFNγ, interferon gamma.

of donor liver mDC to spleens of allogeneic recipients, WT or DAP12$^{-/-}$ B6 (H2$^b$) livers were transplanted into normal C3H (H2$^k$) recipients without immunosuppressive therapy. As shown in Figure 5A, 24 h after WT liver transplantation, a small proportion (<2%; 1.76 ± 0.25%) of spleen CD11c$^+$ cells were of donor origin, whereas a significantly higher (approx. threefold) incidence (5.67 ± 1.17%; $p < 0.01$) of donor DC was observed in the recipients of DAP12$^{-/-}$ livers (Figure 5B).