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Materials and Methods

SOM Text

Figs. S1 to S6

Tables S1 to S4

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Gender Disparity in Liver Cancer Due to Sex Differences in MyD88-Dependent IL-6 Production

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Hepatocellular carcinoma (HCC), the most common liver cancer, occurs mainly in men. Similar gender disparity is seen in mice given a chemical carcinogen, diethylnitrosamine (DEN). DEN administration caused greater increases in serum interleukin-6 (IL-6) concentration in males than it did in females. Furthermore, ablation of IL-6 abolished the gender differences in hepatocarcinogenesis in mice. DEN exposure promoted production of IL-6 in Kupffer cells (KCs) in a manner dependent on the Toll-like receptor adaptor protein MyD88, ablation of which also protected male mice from DEN-induced hepatocarcinogenesis. Estrogen inhibited secretion of IL-6 from KCs exposed to necrotic hepatocytes and reduced circulating concentrations of IL-6 in DEN-treated male mice. We propose that estrogen-mediated inhibition of IL-6 production by KCs reduces liver cancer risk in females, and these findings may be used to prevent HCC in males.

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is a dreaded complication of chronic liver disease that occurs in the setting of risk factors such as hepatitis B (HBV) and hepatitis C (HCV) viral infections, alcoholic liver disease, hemochromatosis, and nonalcoholic steatohepatitis (1). Most HCC appears in cirrhotic livers after years of chronic inflammation. The 5-year survival rate for patients with HCC, the increasing incidence of which is likely due to the spread of HCV (2), is only about 7%. Notably, men are about three to five times more likely to develop HCC than

women (3). A similar or even more pronounced gender disparity is seen in rodent HCC models (4, 5). Furthermore, administration of estrogens

to male mice inhibits development of chemically (DEN)-induced HCC (6). Nonetheless, the mechanisms that account for this gender disparity and the anticarcinogenic activity of estrogens are unknown.

Inflammation is a major contributing factor to carcinogenesis (7). HCC represents a classic case of inflammation-linked cancer (8), and chemically or genetically induced HCC depends on inflammatory signaling (5, 9, 10). To understand the mechanisms underlying gender disparity in HCC, we used the chemical carcinogen diethylnitrosamine (DEN), which causes HCC in 100% of male mice but only in 10 to 30% of female littermates (5, 6). The pathogenesis of HCC in this mouse model differs from that in humans and thus may not be directly comparable to human HCC. Nevertheless, the mouse model of DEN-induced HCC has a histology and genetic signature similar to that of human HCCs with poor prognosis (11) and recapitulates a dependence on inflammation and gender disparity seen in human HCC.

Interleukin-6 (IL-6) is a multifunctional cytokine largely responsible for the hepatic re-

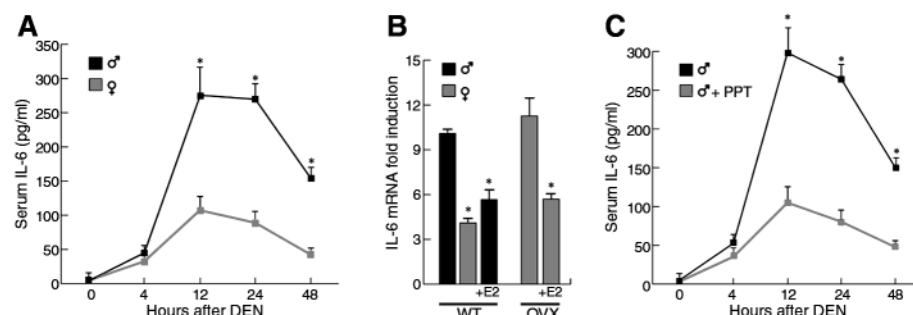
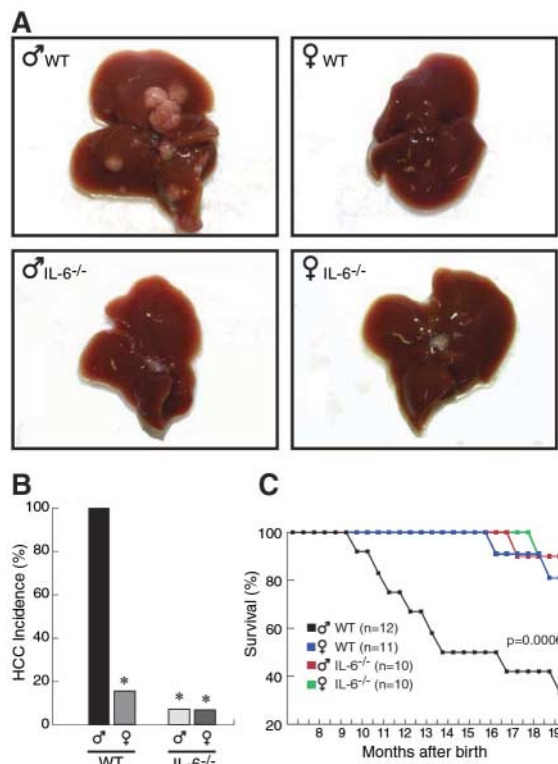


Fig. 1. Differential IL-6 production after chemically induced liver injury. **(A)** Concentration of IL-6 in serum of male and female WT mice after injection of DEN (100 mg per kg of body weight; $n = 3$ mice per time point). **(B)** IL-6 mRNA levels in livers of male, female, or ovariectomized (OVX; ovariectomy was done 2 weeks before DEN administration) female mice 4 hours after DEN injection. E2 (50 μ g/kg) in corn oil was injected intraperitoneally 2 hours before DEN was administered. **(C)** Male B6 mice ($n = 3$) were injected with ER α -specific agonist propyl-pyrazole-trisphenol (PPT; 5 μ g/kg in corn oil) 2 hours before DEN injection, and serum IL-6 was measured at the indicated times after DEN injection. Results in (A) to (C) are means \pm SE. Asterisks indicate a significant ($P < 0.05$; Student's t test) difference relative to WT male mice.

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Fig. 2. Lower incidence of HCC tumors and longer survival of IL-6^{-/-} mice. **(A)** Livers of 8-month-old DEN-treated mice. Multiple HCCs are seen only in WT male liver. **(B)** Incidence of HCC (>0.5 mm) in WT male ($n = 14$), WT female ($n = 13$), IL-6^{-/-} male ($n = 14$), and IL-6^{-/-} female ($n = 15$) mice 8 months after DEN (25 mg/kg) injection. Asterisks indicate significant ($P < 0.05$; Student's t test) differences relative to WT male mice. **(C)** Survival curves of WT and IL-6^{-/-} mice injected with DEN (25 mg/kg) at 15 days of age ($P = 0.0006$; log-rank test for significance).



sponse to infections or systemic inflammation, often termed the “acute phase response.” Concentrations of IL-6 in serum are increased in situations of chronic liver inflammation including alcoholic hepatitis, HBV and HCV infections, and steatohepatitis, conditions that may lead to development of HCC (12). IL-6 concentrations are also increased in patients with HCC relative to normal subjects (13). Whether IL-6 is causal or contributory to HCC is unknown. However, IL-6 is thought to contribute to hepatocyte proliferation (14), and DEN administration to male mice results in IL-6 production that depends on I κ B kinase β (IKK β) in myeloid cells, most likely the resident liver macrophages called Kupffer cells (KCs). In addition to preventing IL-6 production, ablation of IKK β in myeloid cells prevents compensatory hepatocyte proliferation (5), a response triggered by hepatocyte death.

Compensatory proliferation appears to have a critical role in DEN-induced hepatocarcinogenesis (5, 10), and IL-6 is necessary for normal liver regeneration (14), so we examined gender effects on DEN-induced IL-6 production (15). Administration of DEN resulted in higher amounts of circulating IL-6 in males than in females (Fig. 1A). A similar gender bias was seen for accumulation of IL-6 mRNA in liver (Fig. 1B). Ad-

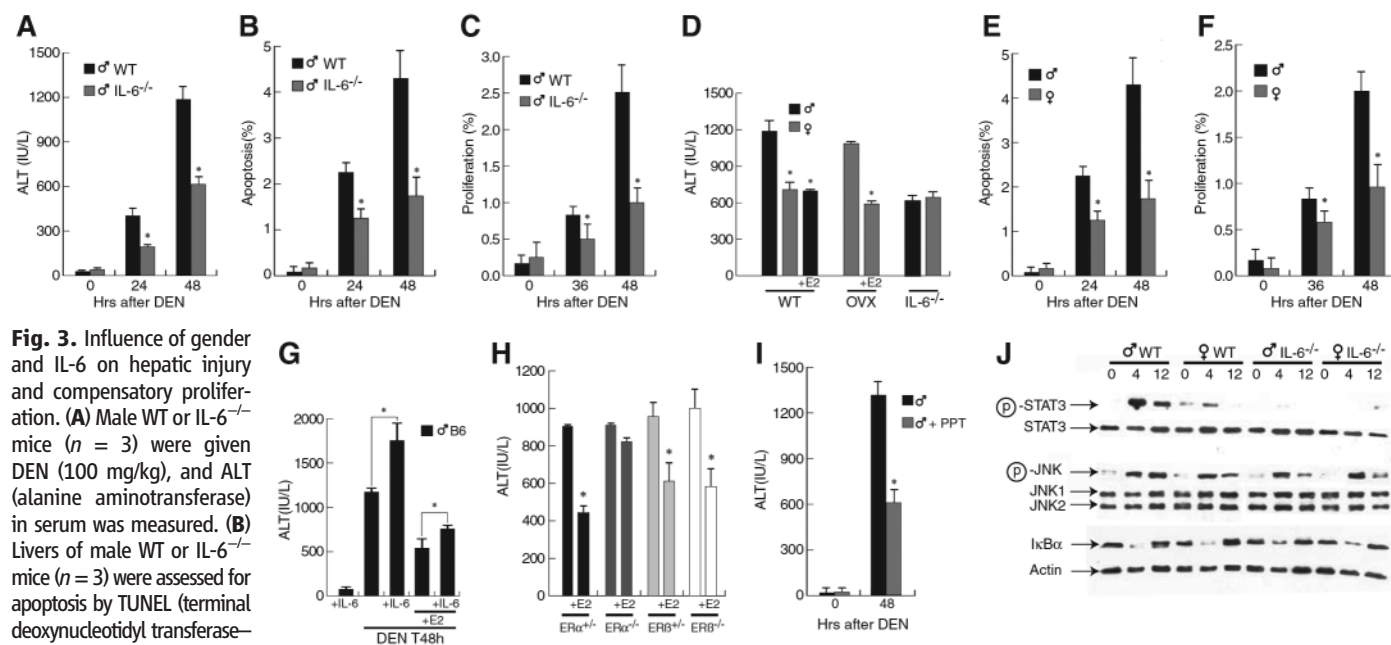


Fig. 3. Influence of gender and IL-6 on hepatic injury and compensatory proliferation. **(A)** Male WT or IL-6^{-/-} mice ($n = 3$) were given DEN (100 mg/kg), and ALT (alanine aminotransferase) in serum was measured. **(B)** Livers of male WT or IL-6^{-/-} mice ($n = 3$) were assessed for apoptosis by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining after DEN injection. **(C)** Hepatocyte proliferation in livers of DEN-injected male WT or IL-6^{-/-} mice ($n = 3$) was assessed by injecting mice with bromodeoxyuridine (BrdU) (1 mg per mouse) 2 hours before the liver was removed. BrdU-positive cells were identified by immunostaining. **(D)** Serum ALT was measured 48 hours after DEN injection ($n = 3$ per group). OVX: female mice ovariectomized 2 weeks before DEN administration. E2 (50 μ g/kg) in corn oil was injected 2 hours before DEN. Similar studies assessing differences between male and female mice ($n = 3$) were done for apoptosis **(E)** and proliferation **(F)**. **(G)** Six-week-old male B6 mice ($n = 3$) were given E2 or vehicle (corn oil) 2 hours before DEN injection. Recombinant IL-6 (10 μ g) or sham buffer (phosphate-buffered saline) was given subcutaneously at the time of DEN administration. Serum ALT was measured 48 hours later. **(H)** Male ER α ^{-/-}

and ER β ^{-/-} mice and littermate heterozygote controls ($n = 3$) were injected with E2 (50 μ g) in corn oil or vehicle 2 hours before DEN injection, and serum ALT was measured 50 hours later. **(I)** Male mice ($n = 3$ per point) were injected with PPT (5 mg/kg in corn oil) or vehicle 2 hours before DEN injection, and serum ALT was measured 50 hours later. All results for (A) to (I) are means \pm SE, and asterisks indicate $P < 0.05$ (Student's t test). **(J)** Cells from livers of male, female, and IL-6^{-/-} mice were lysed at the indicated times after DEN injection. STAT3 and JNK activation, and I κ B α degradation, were assessed by separating with SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies to the indicated proteins. Phosphorylation (P) of STAT3 and JNK indicates activation. Phospho-STAT3 and STAT3 were from one gel, as were Phospho-JNK and JNK1/2, and I κ B α and actin.

ministration of estradiol (E2) to male mice reduced IL-6 mRNA abundance, whereas ovariectomy augmented accumulation of IL-6 mRNA in females. The latter was largely prevented by E2 administration (Fig. 1B), as well as by the estrogen receptor α (ER α) agonist propylpyrazole-trisphenol (PPT) (Fig. 1C). No gender differences were seen in IL-6 expression or hepatocyte proliferation after partial hepatectomy (fig. S1, A and B).

Pronounced gender-specific differences in IL-6 production were also seen in mice treated with carbon tetrachloride (CCl₄), a promoter of HCC development (fig. S2) (16). The cytotoxic effects of DEN and CCl₄ are dependent on their metabolic activation within the hepatocyte by cytochrome P450 2E1 (CYP 2E1) (17). Expression of CYP 2E1 did not differ between males and female mice treated with DEN (fig. S3A). Once activated, DEN forms DNA adducts (18). DEN-induced DNA modification and damage should lead to activation of the p53-mediated genomic surveillance response. Indeed, DEN administration led to rapid increase in expression of the p53 target genes p21 and Mdm2, but the response was practically identical in males and females (fig. S3B).

To determine whether the gender bias in IL-6 production accounts for the sex difference in HCC development, we examined DEN-induced hepatocarcinogenesis in male and female IL-6 knockout (IL-6^{-/-}) mice and wild-type (WT) controls. All male WT mice developed HCC, as did 13% of WT females (Fig. 2, A and B). A marked reduction in HCC incidence was seen in IL-6^{-/-} males, whereas no difference was seen between WT and IL-6^{-/-} females. In a cohort of mice monitored for survival, WT male mice exhibited shorter mean survival times than IL-6^{-/-} males or females of either genotype (Fig. 2C).

We examined the role of IL-6 in gender differences in short-term responses elicited by DEN. Compared to WT animals, IL-6^{-/-} males displayed significantly less hepatic injury after DEN administration as evidenced by reduced alanine aminotransferase (ALT) release (Fig. 3A), less apoptosis (Fig. 3B), and less necrosis (fig. S4A).

Differences in compensatory proliferation matched the degree of injury, such that IL-6^{-/-} males exhibited fewer proliferating hepatocytes than WT counterparts at 36 and 48 hours after DEN administration (Fig. 3C). Treatment of male mice with an antagonistic antibody that blocks IL-6 receptor signaling also provided protection from DEN-induced liver injury (fig. S5).

Consistent with previous publications (19, 20), DEN-induced liver injury was reduced in females or males given E2 2 hours before DEN administration (Fig. 3D). Injury was increased in ovariectomized females and reduced after E2 administration. Absence of IL-6 eliminated gender-related differences by reducing the extent of injury in males (Fig. 3D). DEN-induced apoptosis (Fig. 3E), necrosis (fig. S4B), and compensatory proliferation (Fig. 3F) were greater in male than in female mice. Administration of exogenous IL-6 augmented DEN-induced damage in both untreated and E2-treated male mice (Fig. 3G). The reduction of injury by E2 in IL-6-treated mice suggests that E2 may also attenuate downstream IL-6 signaling. Similar gender-related differences in liver injury were seen after administration of CCl₄ (fig. S2).

Using mice deficient in either ER α or ER β , we found that ER α is the receptor responsible for the protective effect of E2 (Fig. 3H), which was confirmed by decreased liver injury in male mice pretreated with the ER α -specific agonist PPT (Fig. 3I).

Another estrogen analog, tamoxifen, was found to act as a weak antagonist in this system and increase liver injury (fig. S6A). Absence of ER α also increased DEN-induced injury in females (fig. S6A).

IL-6 activates the transcription factor STAT3 (21). The activated form of STAT3 was absent in livers of IL-6^{-/-} mice, and WT female mice exhibited less STAT3 activation than males after DEN administration (Fig. 3J). Female mice or IL-6^{-/-} mice of both genders also exhibited reduced activation of the mitogen-activated protein kinase JNK (c-Jun N-terminal kinase) at 12 hours after DEN administration, whereas little if any difference was seen in DEN-induced I κ B α degradation. Sustained activation of JNK is required

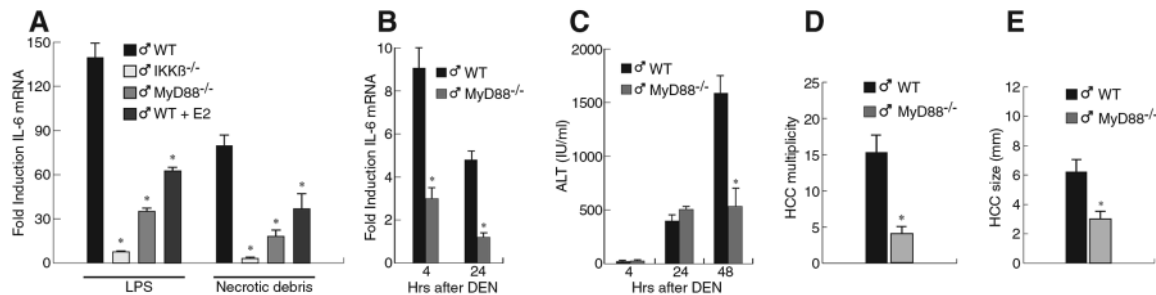
for DEN-induced liver injury as well as hepatocarcinogenesis (5, 10).

Estrogens inhibit IL-6 promoter activity by decreasing the activity of the transcription factors nuclear factor κ B (NF- κ B) and C/EBP β (22). KCs from male mice produced IL-6 when incubated with either bacterial lipopolysaccharide (LPS) or cellular debris released by necrotic hepatocytes (Fig. 4A). Both responses were strongly dependent on IKK β or the Toll-like receptor (TLR) adaptor protein MyD88 and were inhibited if the KCs were first incubated with E2 (Fig. 4A). We speculated that necrotic debris released by DEN-injured hepatocytes triggers cytokine production and compensatory proliferation (5). The TLR adaptor MyD88, which was required for IL-6 induction by necrotic debris, was also required for DEN-induced production of IL-6 in vivo (Fig. 4B) and for induction of liver injury (Fig. 4C). MyD88 was also required for optimal CCl₄-induced accumulation of IL-6 mRNA (fig. S7). MyD88 is also required for induction of liver injury in response to hypoxia (23) and LPS (24). Furthermore, MyD88 ablation suppressed DEN-induced hepatocarcinogenesis. MyD88^{-/-} male mice developed fewer (Fig. 4D) and smaller (Fig. 4E) HCC tumors than WT male mice.

Administration of DEN also leads to modest accumulation of tumor necrosis factor- α (TNF- α) mRNA (5), another proinflammatory cytokine thought to be involved in liver regeneration (25). However, TNF- α expression did not exhibit gender-dependent differences (fig. S8A), and ablation of TNF- α or its type 1 receptor (TNFR1) had little if any effect on production of IL-6 in response to DEN (fig. S8B). Thus, IL-6 induction and liver injury are dependent on signaling via MyD88 but not through TNFR1. Accordingly, ablation of TNFR1 had no significant effect on DEN-induced hepatocarcinogenesis (fig. S8, C and D).

Our results explain why females are less prone to liver cancer than males. This study and others (5, 10) show a strong correlation between the amount of liver damage during acute toxicity and inflammation and the extent of HCC development. We found that both liver injury and

Fig. 4. Requirement of MyD88 for IL-6 production, injury, and hepatocarcinogenesis after DEN treatment. (A) Accumulation of IL-6 mRNA was measured by real-time polymerase chain reaction in KCs from male WT, IKK β ^{-/-}, or MyD88^{-/-} mice ($n = 3$ experiments per time point) after incubation with LPS (10 ng/ml) or necrotic debris prepared by cycles of freeze-thawing of primary hepatocytes. Where indicated, cells were incubated with E2 (10 ng/ml) 30 min before stimulation. (B and C) Male WT and MyD88^{-/-} mice were injected with DEN, and liver IL-6 mRNA (B) or serum ALT (C) was measured.



(D and E) Number of HCCs (D) and sizes (E) in livers of WT and MyD88^{-/-} male mice 8 months after DEN (25 mg/kg) administration. Results in (A) to (E) are means \pm SE. Asterisks indicate a significant ($P < 0.05$; Student's t test) difference relative to WT males.

compensatory proliferation were strongly dependent on IL-6 and that the absence of this tumor-promoting cytokine resulted in almost complete inhibition of DEN-induced hepatocarcinogenesis. IL-6 production by KCs was largely dependent on MyD88, an adaptor molecule that acts downstream of TLRs as well as IL-1 receptor (26). Because DEN is not a direct macrophage or KC activator (27), hepatocyte necrosis may be an intermediate in the pathway through which DEN or CCl₄ exposure results in IL-6 production. Various macromolecules released by necrotic cells activate macrophages through TLRs, the receptors which in turn activate MyD88 (28, 29). TRIF, another TLR adaptor protein (26), is not required for DEN- or CCl₄-induced IL-6 production and liver injury (27). MyD88 signaling, but not TNFR1 signaling, was required for optimal DEN-induced hepatocarcinogenesis in male mice. DEN-induced hepatocarcinogenesis appears to depend on an inflammatory response, triggered by hepatocyte necrosis, that leads to production of IL-6. Estrogens, at concentrations present in females but not in males, suppress IL-6 production and therefore inhibit chemically induced liver carcinogenesis. A similar mechanism could account for the gender bias in liver cancer in humans. If so, estrogen-mimetic compounds capa-

ble of inhibiting excessive IL-6 production might be used to prevent progression of chronic liver disease to HCC in men.

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- In experiments performed in our laboratory, incubation of KCs or bone marrow-derived macrophages with DEN did not induce expression of IL-6 mRNA. DEN administration to TRIF^{-/-} mice resulted in as much IL-6 induction as seen in WT mice.
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- We thank J. Feramisco for help with image capture and analysis and E. Karin for help with the surgical models. Supported by National Institute of Diabetes and Digestive and Kidney Diseases grant DK007202 (W.E.N.); Japan Society for the Promotion of Science (T.S.); Human Frontier Science Program (S.K.); and NIH grants ES004151 and ES006376, CA118165, and the Superfund Basic Research Program (M.K.). M.K. is an American Cancer Society Professor.

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Materials and Methods

Figs. S1 to S8
References

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Regulation of Spontaneous Intestinal Tumorigenesis Through the Adaptor Protein MyD88

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Inflammation is increasingly recognized as an important component of tumorigenesis, although the mechanisms and pathways involved are not well understood. Tumor development is regulated by products of several modifier genes, but instructions for their tumor-specific expression are currently unknown. We show that the signaling through the adaptor protein MyD88 has a critical role in spontaneous tumor development in mice with heterozygous mutation in the adenomatous polyposis coli (APC) gene. We found that MyD88-dependent signaling controls the expression of several key modifier genes of intestinal tumorigenesis and has a critical role in both spontaneous and carcinogen-induced tumor development. This study thus reveals the important role of an innate immune signaling pathway in intestinal tumorigenesis.

Inflammatory responses contribute to carcinogenesis through multiple mechanisms (1–3). Activation of the transcription factor nuclear factor κ B (NF- κ B), a key mediator of inflammation, has a critical role in the regulation of tumor development resulting from chronic inflammation or exogenous mutagens (4, 5). NF- κ B is activated by multiple stimuli (6), and it is currently unknown which pathway is critically in-

involved in cancer-associated inflammation and the tissue repair response (7). The role of inflammatory and tissue repair responses in spontaneous carcinogenesis, independent of chronic inflammation or administration of exogenous carcinogens, has not yet been characterized. However, signaling through Toll-like receptors (TLRs) of the innate immune system to MyD88 (a signaling adaptor of TLRs) has a critical role in the control of tissue renewal responses (8–11).

A link between intestinal tissue renewal and tumorigenesis was established when the genetic basis of familial associated polyposis (FAP) was mapped to the APC gene (12). Germline and sporadic mutations in APC occur in >85% of

FAP and >80% of sporadic colorectal tumors (12). A mouse model of spontaneous intestinal tumorigenesis was discovered in a forward genetic screen (13). These mice, designated Apc^{Min/+}, have a mutation in the APC gene and develop 60 to 80 intestinal adenomas, mostly at the distal small intestine (14). Given the role of the MyD88 signaling in intestinal tissue renewal and repair, we investigated the role of this pathway in spontaneous intestinal carcinogenesis in Apc^{Min/+} mice.

To examine a potential role of the MyD88 signaling pathway in spontaneous intestinal tumorigenesis, we generated Apc^{Min/+} mice on MyD88-sufficient and -deficient backgrounds and analyzed sex- and age-matched cohorts. On average, Apc^{Min/+} mice die within 6 months of age from complications of intestinal tumors (13). In contrast, mortality of Apc^{Min/+} MyD88^{-/-} mice was dramatically reduced as compared with Apc^{Min/+} littermate controls (Fig. 1A). We investigated the red blood cell (RBC) status of these mice, a marker of intestinal tumorigenesis, and found that the anemia observed in Apc^{Min/+} mice was significantly ameliorated in Apc^{Min/+} MyD88^{-/-} mice (Fig. 1B). MyD88-dependent signaling therefore contributes substantially to the severe mortality and morbidity caused by inactivation of APC.

The number of visible polyps (≥ 0.5 mm in diameter) was next quantified by stereoscopic microscopy. The number of macroadenomas in the small intestines or colon of Apc^{Min/+} MyD88^{-/-} mice was reduced compared with that in Apc^{Min/+} controls (Fig. 2A and fig. S1). Loss of MyD88 decreased the number of small intestinal tumors

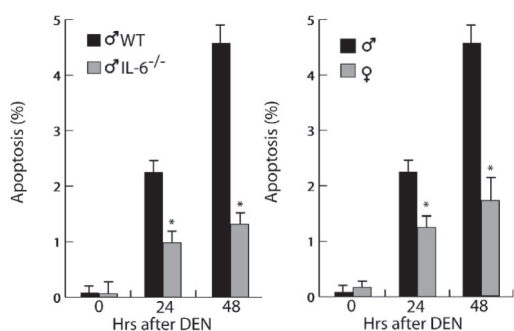
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ERRATUM

Post date 4 December 2009

Reports: "Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production" by W. E. Naugler *et al.* (6 July 2007, p. 121). The originally published Fig. 3B inadvertently duplicated Fig. 3E. The corrected 3B, along with originally published 3E, is provided here.



Corrected Figure 3B

Original (and correct) Figure 3E

Gender Disparity in Liver Cancer Due to Sex Differences in MyD88-Dependent IL-6 Production

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