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ORIGINAL ARTICLE

Lack of EGFR catalytic activity in hepatocytes improves liver regeneration following DDC-induced cholestatic injury by promoting a pro-restorative inflammatory response

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Abstract

Despite the well-known hepatoprotective role of the epidermal growth factor receptor (EGFR) pathway upon acute damage, its specific actions during chronic liver disease, particularly cholestatic injury, remain ambiguous and unresolved. Here, we analyzed the consequences of inactivating EGFR signaling in the liver on the regenerative response following cholestatic injury. For that, transgenic mice overexpressing a dominant negative mutant human EGFR lacking tyrosine kinase activity (ΔEGFR) in albumin-positive cells were submitted to liver damage induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), an experimental model resembling human primary sclerosing cholangitis. Our results show an early activation of EGFR after 1–2 days of a DDC-supplemented diet, followed by a signaling switch-off. Furthermore, ΔEGFR mice showed less liver damage and a more efficient regeneration following DDC injury. Analysis of the mechanisms driving this effect revealed an enhanced activation of mitogenic/survival signals, AKT and ERK1/2-MAPKs, and changes in cell turnover consistent with a quicker resolution of damage in response to DDC. These changes were concomitant with profound differences in the profile of intrahepatic immune cells, consisting of a shift in the M1/M2 balance towards M2 polarity, and the Cd4/Cd8 ratio in favor of Cd4 lymphocytes, overall supporting an immune cell switch into a pro-restorative phenotype. Interestingly, ΔEGFR livers also displayed an amplified ductular reaction, with increased expression of EPCAM and an increased number of CK19-positive ductular structures in portal areas, demonstrating an overexpansion of ductular progenitor cells. In summary, our work supports the notion that hepatocyte-specific EGFR activity acts as a key player in the crosstalk between parenchymal and non-parenchymal hepatic cells, promoting the pro-inflammatory response activated during cholestatic injury and therefore contributing to the pathogenesis of cholestatic liver disease.

Keywords: EGFR; transgenic mouse; DDC diet; cholestasis; liver disease; regeneration; inflammation; ductular reaction; hepatic progenitor cell

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No conflicts of interest were declared.

Introduction

The epidermal growth factor receptor (EGFR or ErbB1) signaling pathway is recognized as a key player in different pathophysiological contexts of liver biology. Although not crucial for liver development [1], a major role for this pathway has been described in different types and phases of liver injury responses. In fact, inactivation of EGFR by different means results in delayed liver regeneration and increased mortality after partial hepatectomy (PH) [2–4]. Similarly, deletion of specific EGFR ligands, such as amphiregulin (AREG), leads to impaired proliferative responses during liver regeneration [5,6]. *In vitro* studies have shone a light on the cellular mechanisms behind these effects, demonstrating that EGFR signaling is capable of promoting both proliferation and survival against different cellular insults in hepatocytes at different stages of differentiation [7,8]. However, despite the well-known hepatoprotective role of EGFR signaling in liver regeneration, its role in cholestatic liver diseases remains unresolved. Chronic cholestatic injury is caused by functional impairment of bile secretion and/or flow at the level of hepatocytes or cholangiocytes [9]. In consequence, bile acids accumulate locally, reaching toxic levels that cause hepatocellular, and hence liver, damage. Strikingly, both protective and detrimental profibrogenic effects have been reported for EGFR [10] in the context of cholestatic damage. EGFR deletion in hepatocytes of *Mdr2*$^{-/-}$ mice, a model of primary sclerosing cholangitis (PSC), aggravates liver damage, suggesting a protective role for EGFR during cholestatic damage [11]. In the same line of evidence, liver fibrosis after bile duct ligation (BDL) is exacerbated in heparin-binding EGF (HB-EGF) [12] and AREG [13] knockout mice compared with WT animals, demonstrating a protective role for these EGFR ligands against cholestatic liver fibrosis. Contrarily, EGFR inhibition by using erlotinib or neutralizing antibodies attenuates liver fibrosis induced by different insults, including BDL, in rodent models [14,15]. These observations, along with other evidence, highlight a rather complex role for EGFR signaling during chronic cholestatic injury.

The liver response to cholestatic damage involves processes shared by other types of liver injury, including inflammation, hepatocyte damage, and fibrosis, but it is also associated with the appearance of a ductular reaction (DR) in the portal tracts, a poorly understood process characterized by the proliferation of biliary epithelial cells and the appearance of reactive ductular structures, in which adult hepatic progenitor cells, known as oval cells in rodents (HPCs/OCs), are pivotal [16]. These bipotential progenitors constitute an alternative cellular source for liver repopulation when mature parenchymal cells are non-functional [17,18]. Despite such recognized regenerative potential, they may contribute to liver fibrosis and tumor development [19–21]. Indeed, the fate of HPCs/OCs is highly context-dependent and tightly regulated by components of the liver microenvironment [18,22], among them, the EGFR ligands [23–25]. Thus, infusion of EGF increases the expansion of ductal and periductal cells into liver acini [24]. Apart from triggering mitogenic signals, EGFR counteracts transforming growth factor-β (TGF-β)-induced apoptosis [23] and epithelial–mesenchymal transition in HPCs/OCs [25], and seems critical for induction of hepatic biliary lineage [26].

Here, we have used a novel transgenic mouse model overexpressing in the liver albumin-expressing cells a mutant human EGFR lacking tyrosine kinase activity that acts as a dominant negative mutant (ΔEGFR). This model has allowed us to decipher the molecular mechanisms of the EGFR pathway – specifically, the EGFR tyrosine kinase-dependent functions – in liver regeneration after a PH, and in liver carcinogenesis [2], but how these mice respond to a cholestatic injury has not yet been explored. To achieve this purpose, ΔEGFR mice were submitted to liver damage induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), which provokes ductular obstruction, leading to cholestatic damage resembling human PSC [27]. Our findings show that the absence of EGFR tyrosine kinase activity in albumin-expressing cells leads to reduced and delayed liver damage and more efficient regeneration upon DDC injury, concomitantly with a shift from a profibrotic to a restorative inflammatory response and an enhanced DR and expansion of HPCs/OCs.

Materials and methods

Animal model

Transgenic Alb-D654–1186huEGFR (ΔEGFR) mice were generated as described previously [2]. These mice express a truncated form of the human EGFR that lacks the intracellular catalytic domain (amino acids 654–1186) under the control of an albumin promoter, therefore achieving expression of the transgene specifically in hepatic albumin-positive cells. The ΔEGFR mouse line was maintained in a C57BL/6J background in heterozygosity, so WT and ΔEGFR mice belonged to the same strain. Mice were housed in the UCM animal facility, allowed food and water *ad libitum* in temperature-controlled rooms under a 12 h light/dark cycle, and routinely screened for pathogens in accordance with Federation of European Laboratory Animal Science Associations procedures. All animal procedures conformed to European Union Directive 86/609/EEC and Recommendation 2007/526/EC, enforced in Spanish law under RD 1201/2005. Animal protocols were approved by the Animal Experimentation Ethics Committee of the UCM and the Animal Welfare Division of the Environmental Affairs Council of the Government of Madrid (Proex 129/16). Six 8-week-old male mice were fed either a control diet or a diet containing the porphyrogenic compound DDC (0.1%) for up to 6 weeks, as described elsewhere [28,29] (DDC was from Cymit Quimica S.L., Barcelona, Spain and the diet from Envigo Laboratories, Barcelona, Spain).

Isolation of hepatic non-parenchymal cells for analysis of immune cell populations

Hepatic non-parenchymal cells were isolated as described previously [30]. In brief, livers were collected and washed with PBS. They were transferred immediately to HBSS (Hank's Balanced Salt Solution; Gibco, Life Technologies, Carlsbad, CA, USA) at room temperature, disintegrated, and filtered through 100-µm cell strainers. Then homogenates were centrifuged at 500 × *g* for 5 min at room temperature and cell pellets were resuspended in 36% Percoll solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) containing 100 UI/ml heparin (HIBOR 5000 UI; ROVI, Madrid, Spain). After centrifugation at 800 × *g* without brake for 20 min at room temperature, supernatants were discarded and then erythrocytes were removed from cell pellets by using a red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). The resulting cell pellets were washed with cold HBSS, centrifuged at 500 × *g* for 5 min at 4 °C, and finally resuspended in cold HBSS for further analysis.

Flow cytometry analysis of immune cell populations

Isolated non-parenchymal liver cells were incubated, protected from light, with the antibodies listed in Supplementary materials and methods, or their corresponding isotype controls, for 20 min at room temperature. After washing steps, cells were resuspended with PBS. Flow cytometry data were acquired using the FACSCanto II system (BD Biosciences, Erembodegem, Belgium) and data analysis was performed using a Cytomics FC500 Flow Cytometer (Beckman Coulter Life Sciences, Miami, FL, USA) with the CXP program.

Statistical analysis

Statistical analysis was performed by paired Student's *t*-test analysis or one-way ANOVA to calculate *P* values once a normal distribution of data was verified using the Shapiro–Wilk test. For data not following a normal distribution, a Kruskal–Wallis test was used. $p \leq 0.05$ was considered statistically significant.

Results

Receptors and ligands of the EGFR family are regulated during cholestatic injury

The regulation of EGFR in cholestatic liver conditions has not been consistently evaluated, although evidence of EGFR downregulation was obtained in *Mdr2*$^{-/-}$ mice [31]. To have a clearer picture of the regulation of the EGFR signaling pathway during cholestatic injury, and to determine the functional role of EGFR signaling in this specific context, we submitted WT and ΔEGFR transgenic mice to the DDC diet model of biliary injury.

We first assessed the expression and activation of EGFR in liver extracts of WT and ΔEGFR mice under the DDC diet. EGFR protein levels were strongly downregulated after 1–6 weeks of DDC feeding (Figure 1A). The levels of active phosphorylated EGFR were similarly downregulated (Figure 1B). We also examined the expression of EGFR ligands, since increased expression of *Areg* has been reported in human [primary biliary cholangitis (PBC) and PSC] and experimental models of cholestasis [13]. Both *Areg* and *Hbegf*, but not transforming growth factor-alpha, *Tgfa*, were upregulated in the livers of mice fed the DDC diet (supplementary material, Figure S1). To elucidate whether changes in different EGFR ligands were also found in human cholestasis, we examined public GEO datasets (GSE61256) including PBC and PSC patient samples (supplementary material, Figure S2). Except for *TGFA*, the other EGFR ligand mRNAs, *HBEGF*, *EGF*, and *AREG*, show significantly elevated levels in PSC; however, in PBC, the levels of these ligands are more heterogeneous and no significant changes were seen in any case. Unlike the ligands, mRNA levels for the EGFR family receptors (*EGFR*, *ERBB2*, *ERBB3*, *ERBB4*) did not show differences compared with control samples. The data show that not only *AREG* but also other EGFR ligands are significantly upregulated in PSC. Nevertheless, upregulation of ligands by itself does not prove activation of EGFR signaling. In fact, the data shown in Figure 1B did not support such activation, at least following 2 and 6 weeks of DDC diet treatment. To clarify this issue, we analyzed EGFR activation at earlier time points after the DDC diet was started (Figure 1C). Interestingly, the levels of phosphorylated EGFR were enhanced during the first 2 days and declined afterwards, results that indeed demonstrate early activation of the EGFR pathway during cholestatic injury that is followed by signaling exhaustion or switch off.

Inactivation of EGFR in hepatocytes ameliorates DDC-induced liver damage and favors regeneration

In an attempt to understand the role played by EGFR signaling during DDC-induced cholestatic injury, we next examined the consequences of inactivating EGFR signaling on the hepatic response to DDC injury. Analysis of serum markers of hepatic damage revealed increased levels of alanine aminotransferase, alkaline phosphatase (ALP), and total bilirubin after 1 week of the DDC diet, albeit differences in kinetics were seen between different parameters (Figure 2A). Importantly, ΔEGFR mice showed lower levels of all these markers as well as a faster and sharper decline in comparison with WT mice. Differences were particularly clear for ALP and bilirubin, the latter showing a recovery of basal levels after 6 weeks of the diet. Additionally, we measured the liver-to-body weight ratio, a parameter commonly used to evaluate liver damage and predict the regenerative response [32]. The results showed an increased ratio in both WT and ΔEGFR mice under the DDC diet, the increase being higher in ΔEGFR mice, particularly at long term (6 weeks), although differences did not reach

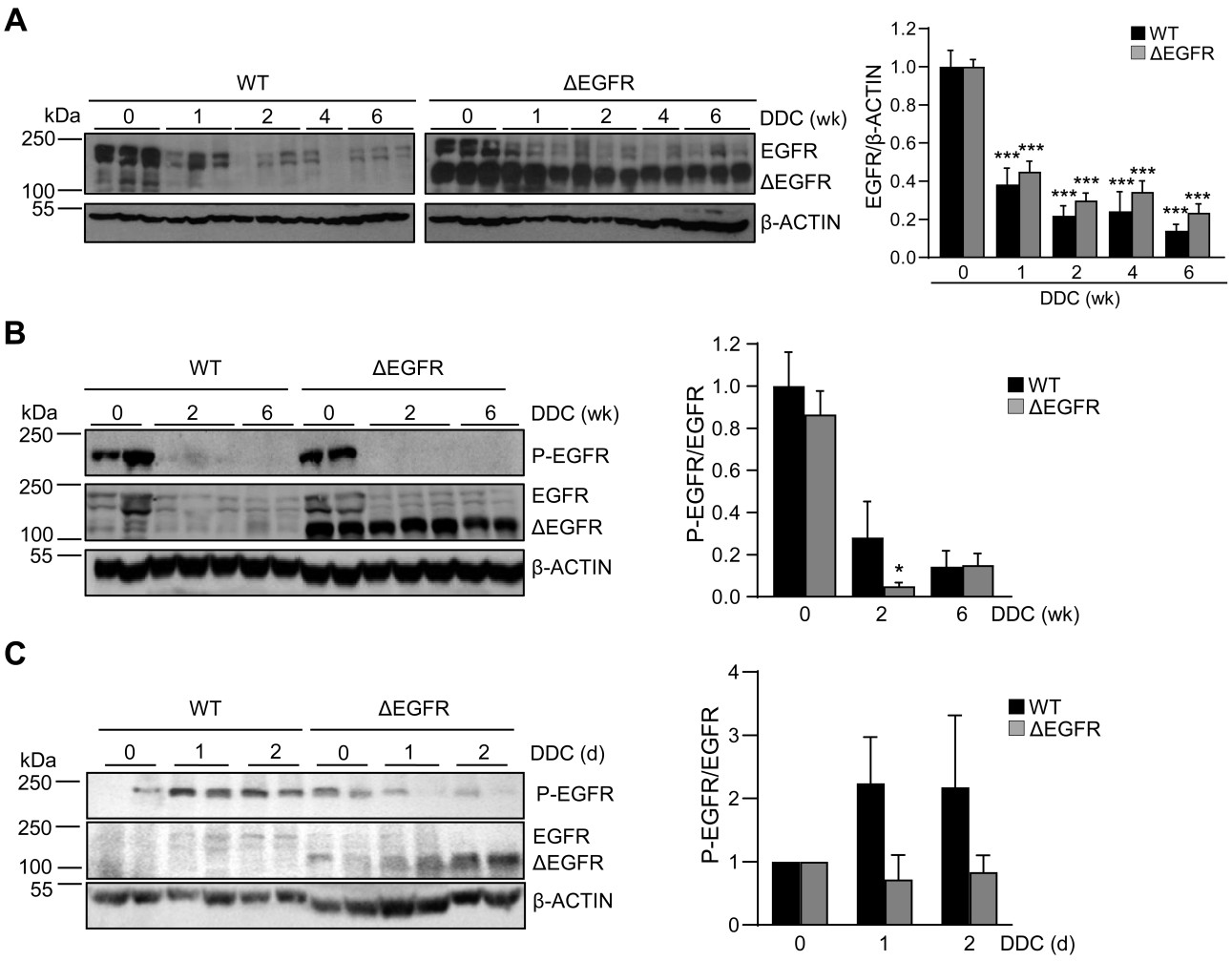

Figure 1. Analysis of EGFR in the livers of mice subjected to a DDC diet. (A) Western blot analysis of EGFR levels in the liver. One representative experiment is shown (left panel). Optical density values are mean ± SEM of 4–6 animals per group (right panel). (B, C) Western blot analysis of phosphorylated and total EGFR levels in the livers of mice fed the DDC diet for 2–6 weeks (B) or 1 or 2 days (C). One representative experiment is shown (left panel). Optical density values are mean ± SEM of 4–9 animals per group (right panel). Data were compared with the untreated group (mice fed a standard chow diet). $^{*}p < 0.05$, $^{***}p < 0.001$.

statistical significance (Figure 2B). Differences were clearer when we looked at changes in liver weight. While WT mice displayed a stable loss of liver weight up to the end of treatment, ΔEGFR livers showed a transient loss after 2 weeks and an increase afterwards (Figure 2C), data consistent with a better regenerative response in these mice. Finally, we analyzed the expression levels of a set of fibrosis markers. As expected, all of them were significantly induced under the DDC diet, an experimental model known to be associated with biliary fibrosis [27], but this induction was reduced and delayed in ΔEGFR livers (Figure 2D). Although no significant differences were found between WT and ΔEGFR livers in collagen fiber deposition measured by Picro-Sirius Red staining (data not shown), the mRNA levels of *Loxl1* and *Loxl2*, lysyl oxidase enzymes that catalyze the covalent crosslinking of collagen I fibers, essential for its stabilization, were upregulated in WT but not in ΔEGFR mice after 6 weeks of DDC feeding (supplementary material, Figure S3). Taken together, these results may support the existence of differences in extracellular matrix properties and therefore in the hepatic regenerative microenvironment between WT and ΔEGFR mice that could affect the long-term ECM remodeling capacity, an issue that deserves further analysis. Collectively, the results support an attenuation of damage and an improved regenerative response in ΔEGFR mice in response to the DDC diet.

To evaluate the mechanisms involved in the enhanced liver regenerative capacity of ΔEGFR mice, we analyzed the activation of ERK1/2–MAPK and AKT, two signaling pathways known to play an important role during liver regeneration, stimulating cell proliferation and cell survival [33,34]. ΔEGFR mice showed stronger and prolonged activation of these signals in the liver in response to the DDC diet (Figure 3A). In parallel, we analyzed the cell labeling index and caspase-3 activation in these livers. No significant differences were found in the number of Ki67-positive parenchymal cells between WT and ΔEGFR mice, although proliferation showed a

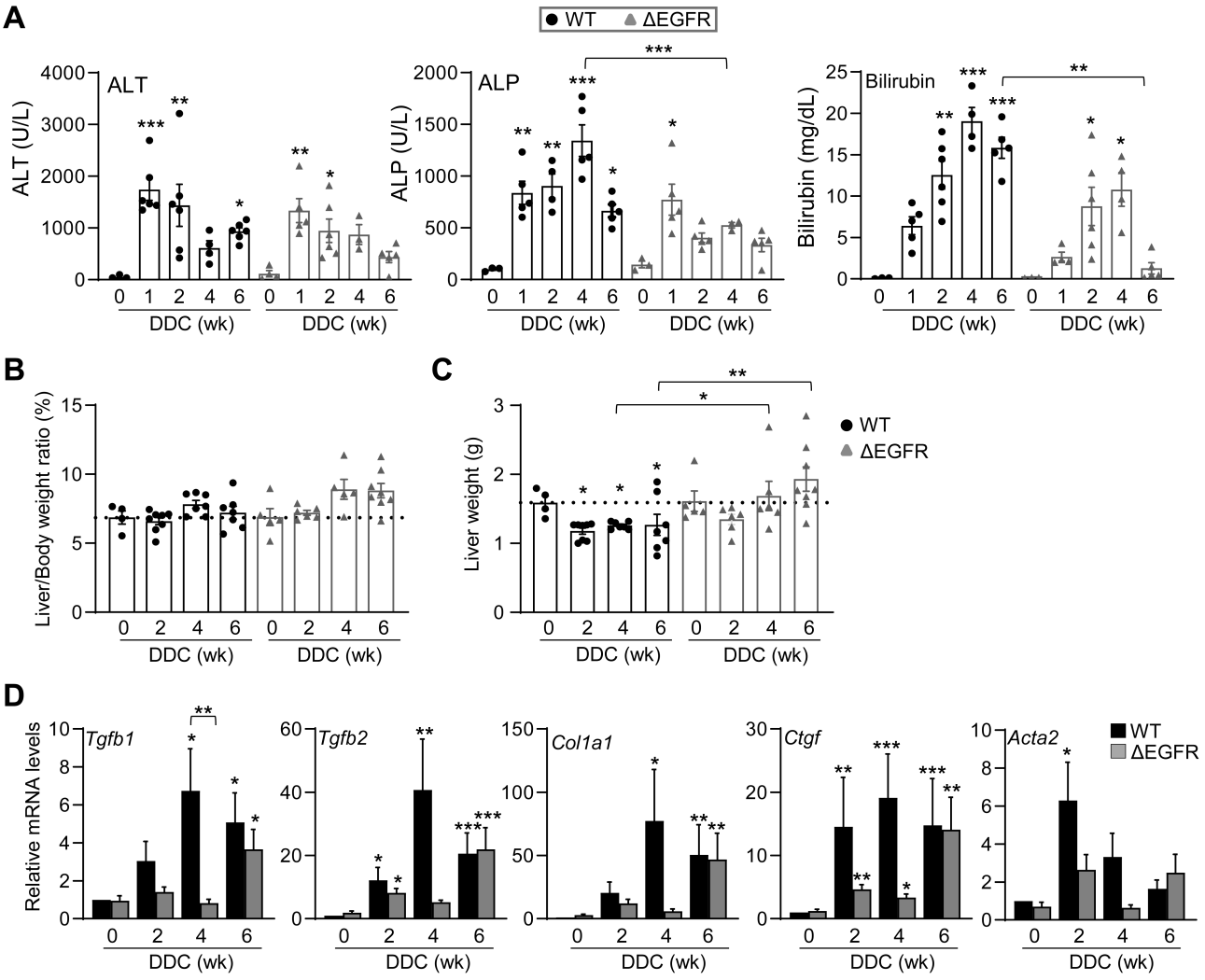

Figure 2. Reduced liver damage in ΔEGFR mice under a DDC diet. (A) Serum levels of alanine aminotransferase (ALT), ALP, and total bilirubin. Data are mean ± SEM of 3–6 animals per group. (B) Liver-to-body weight ratio. Data are mean ± SEM of 4–8 animals per group. (C) Liver weight (g). Data are mean ± SEM of 4–8 animals per group. (D) RT-qPCR analysis of *Tgfb1*, *Tgfb2*, *Col1a1*, *Ctgf*, and *Acta2* mRNA levels in the liver. Data are mean ± SEM of 4–11 animals per group. Data were compared with the untreated group or as indicated; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

tendency to be higher in WT at 6 weeks (Figure 3B). To analyze caspase-3 activation, we used both western blot analysis (Figure 3C) and fluorometric assays (supplementary material, Figure S4A), which rendered similar data: that is, the pattern of cleaved caspase-3 was quite different in ΔEGFR mice, showing a transient increase after 2 weeks of diet that decreased at 6 weeks, whereas WT mice showed a progressive increase in the levels, which reached maximal levels at 6 weeks, suggesting a persistent cell death effect, and thus stronger hepatocellular damage, in WT mice. Immunohistochemical analysis to detect cleaved caspase-3 showed that both hepatocytes and cells within the ductular reaction stained positive, showing that parenchymal and non-parenchymal cells undergo apoptosis in response to DDC-induced damage (supplementary material, Figure S4B). Collectively, our data demonstrate changes in cell turnover in ΔEGFR mice consistent with quicker damage resolution in response to DDC injury.

ΔEGFR livers show enhanced but modified portal inflammation in response to a DDC diet

A role for EGFR in liver inflammation during regeneration or hepatocarcinogenesis has been proposed [2,5], and because inflammation is an important component of the cholestatic injury induced by DDC [27], we next aimed to compare the inflammatory response in WT and ΔEGFR mice fed a DDC-supplemented diet. Histopathological examination revealed greater portal inflammation in the livers from ΔEGFR mice after 2 weeks (Table 1 and supplementary material, Figure S5). Additionally, we isolated and analyzed by flow cytometry the intrahepatic immune cell populations. An increase in both intrahepatic myeloid ($Cd45^{+}$ $Cd11b^{+}$) and resident macrophage ($Cd45^{+}$ $Cd11b^{+}$ $F4/80^{+}$) subsets was

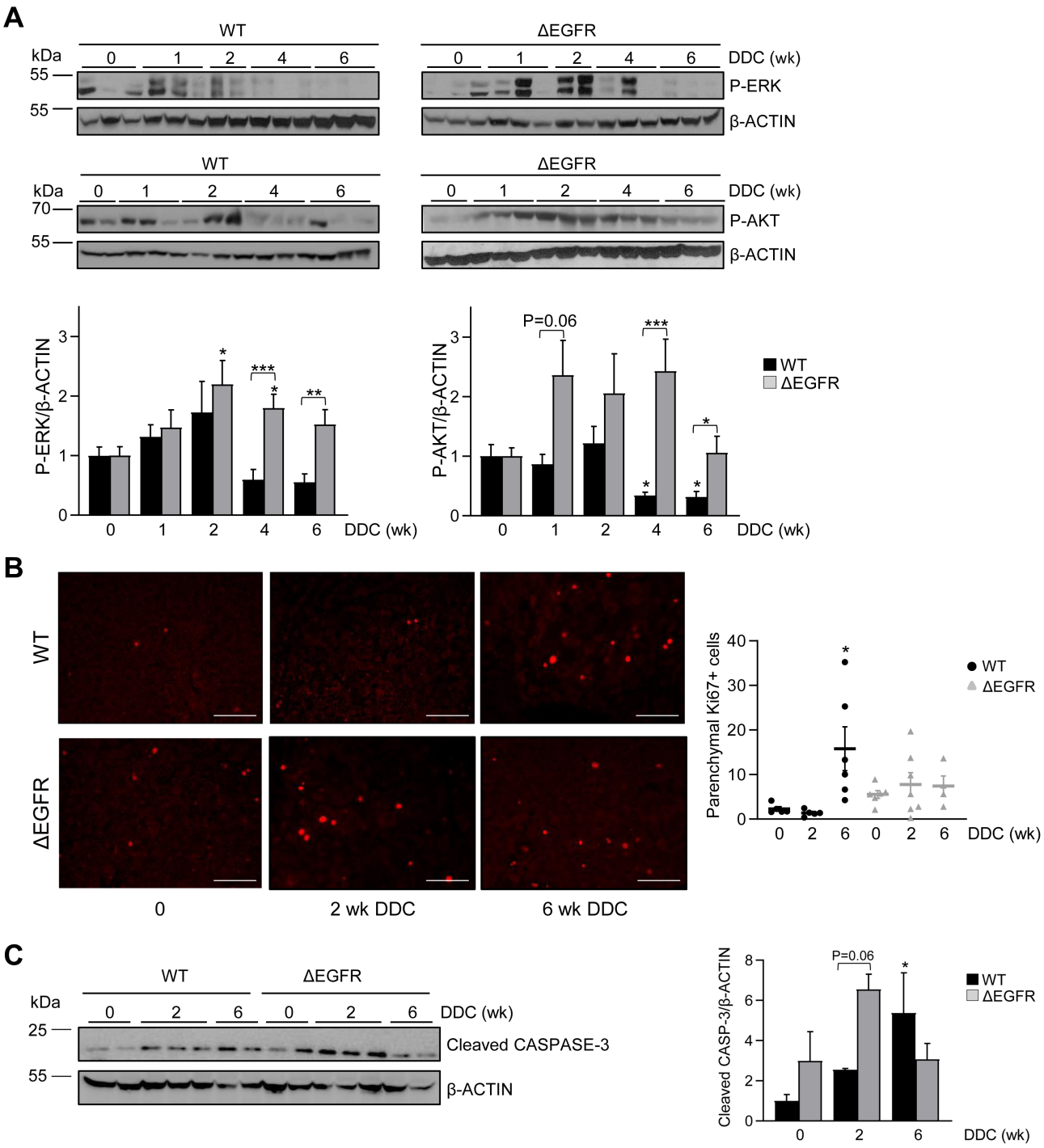

Figure 3. Inactivation of EGFR in hepatocytes favors liver regeneration during DDC-induced cholestatic injury. (A) Western blot analysis of phosphorylated AKT (P-AKT) and ERK1/2-MAPKs (P-ERK) levels in the liver. One representative experiment is shown (upper panel). Optical density values are mean ± SEM of 4–9 animals per group (lower panel). (B) Immunofluorescence analysis for Ki67 in liver tissue sections. Representative images are shown in the left panel and quantification of Ki67-positive cells in the right panel. Data are mean ± SEM of 4–7 animals per group. Scale bar: 50 μm. (C) Western blot analysis for cleaved caspase-3 levels in the liver. One representative experiment is shown (left panel). Optical density values are mean ± SEM of 3 animals per group (right panel). Data were compared with the untreated group or as indicated; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

found after the DDC diet intervention, indicating an infiltration of leukocytes (defined as $Cd45^+$ cells) after 1 and 4 weeks (Figure 4A,B) in both genotypes. While no differences were detected between WT and ΔEGFR mice in the $Cd45^+$ $Cd11b^+$ myeloid population (Figure 4A), ΔEGFR mice had a lower percentage of $Cd45^+$ $Cd11b^+$ $F4/80^+$ resident macrophages in the liver after 4 weeks of DDC injury (Figure 4B). Furthermore, a shift in the M1/M2 balance towards M2 polarity was detected in ΔEGFR livers (Figure 4C,D and supplementary material, Figure S6A,B) consistent with enhanced expression of the anti-inflammatory cytokines

Table 1. Histopathological analysis of inflammatory parameters in the livers of ΔEGFR mice fed the DCC diet.

	Parameter	Portal inflammation (%)				Lobular inflammation (%)			
	Score	0–1	2	3	4	0–1	2	3	4
WT	Untreated	100	–	–	–	100	–	–	–
	DDC 2 weeks	11.1	66.7	22.2	–	88.9	11.1	–	–
	DDC 6 weeks	–	20	80	–	20	60	20	–
ΔEGFR	Untreated	100	–	–	–	60	40	–	–
	DDC 2 weeks	–	44.4[†]	55.6	–	77.8	22.2	–	–
	DDC 6 weeks	–	33.3	66.7	–	16.7	50	33.3	–

Histopathological analysis of portal and lobular inflammation in liver tissue sections. Percentage of animals assigned a specific score is shown. Four to nine animals per group were analyzed.
[†] $p = 0.07$ data compared with Mann–Whitney *U*-test followed by Monte Carlo simulation.

Il10 and *Il6* that promote M2 polarization, and decreased expression of the pro-inflammatory cytokine *Tnfa* characteristic of M1 macrophages (Figure 4E and supplementary material, Figure S6C). Regarding lymphocyte populations, although no differences between the groups were found in $Cd45^+$ $Cd3^+$ cells (supplementary material, Figure S6D), a shift in the Cd4/Cd8 ratio was observed in favor of Cd4 lymphocytes in ΔEGFR livers (Figure 4F,G and supplementary material, Figure S6E, F). Moreover, this response in ΔEGFR livers concurred with lower levels of memory Th1 $Cd4^+$ lymphocytes ($Cd62L^+$ $Cd44^+$ gated from $Cd45^+$ $Cd3^+$ $Cd4^+$) (Figure 4H), an increase in the Treg cell subpopulation ($Cd25^+$ $Cd127^-$ gated from $Cd45^+$ $Cd3^+$ $Cd4^+$) (Figure 4I and supplementary material, Figure S6G), a significant decrease in Il-17-producing lymphocytes ($Cd3^+$ $Cd4^+$ $IL17^+$) (Figure 4J,K), and an enhancement of effector memory T cells ($Cd62L^-$ $Cd44^+$ gated from $Cd45^+$ $Cd3^+$ $Cd4^+$) (Figure 4L), suggesting higher differentiation of Cd4 lymphocytes towards Th2 cells in ΔEGFR livers. Considered together, these data are consistent with a profound alteration in the inflammatory response accompanying the DDC-induced hepatocellular damage in livers from ΔEGFR mice, which show an immune cell switch into a pro-restorative phenotype. Interestingly, stronger activation of signal transducer and activator of transcription (STAT)-3 is seen in ΔEGFR livers upon DDC treatment (supplementary material, Figure S6H), demonstrating correlated changes in the activation of key inflammatory-associated signaling pathways.

ΔEGFR livers show an enhanced ductular reaction in response to the DDC diet

Given that the DDC diet model is associated with a strong ductular reaction (DR) and has proved to be a good model to study the activation and expansion of ductular progenitor cells in chronic liver disease [16,35], we next examined whether ΔEGFR mice showed alterations in this process that could directly contribute to the ameliorated damage. Histological analysis of H&E-stained liver sections showed a typical DR in both WT and ΔEGFR mice, with small basophilic cells expanding from periportal tracts and increased bile ductular structures (Figure 5A); however, DR was amplified in ΔEGFR livers, particularly at later stages (4 and 6 weeks of treatment). The levels of EPCAM and CK19, markers of ductular cells, were also elevated in ΔEGFR livers (Figure 5B and supplementary material, Figure S7). To examine the DR in more detail, we immunostained for CK19, and counted mature and immature ductular structures, including single or clustered positive cells (Figure 5C). ΔEGFR mice showed a significant increase in both mature and immature ductular structures after 2 weeks of the diet, this difference being maintained for up to 6 weeks in the case of immature structures, consistent with a persistently activated DR. These observations were further supported by an increased number of proliferating ductular cells in portal areas of ΔEGFR livers, measured by means of a specific histological scoring system (supplementary material, Table S2) and a quantitative analysis of Ki67/CK19 double-positive cells (Figure 5D). Considered together, these data demonstrate that lack of EGFR catalytic activity in liver albumin-positive cells leads to overexpansion of ductular progenitor cells. We have previously demonstrated that EGFR signaling promotes the proliferation of HPCs/OCs [23], so a plausible explanation for this overexpansion in the absence of active EGFR signaling could be the compensatory overactivation of pro-regenerative signals, as shown before (Figure 3A). Nevertheless, we checked for the pattern of expression of the ΔEGFR transgene in liver cells to clarify whether expanding ductular HPCs/OCs express the ΔEGFR transgene. While strong expression was detected in parenchymal hepatocytes, expression in DR cells was much lower or undetected; nevertheless, positive cells were identified and their number increased with time, likely as a consequence of the appearance of small hepatocytes [36] due to progenitor cell differentiation (supplementary material, Figure S8A). To verify the expression of the ΔEGFR transgene in ductular HPCs/OCs, we isolated them from WT and ΔEGFR livers, using a protocol established previously in our laboratory [36], established them in culture, and validated them (supplementary material, Figure S8B–D). ΔEGFR-OCs showed low expression of the transgene and moderately but significantly decreased EGFR-induced signaling (supplementary material, Figure S8E,F). These results show that EGFR signaling is at least partly active in the population of ductular HPCs/OCs expanding in liver parenchyma in response to DDC injury, and so it could also contribute to their expansion in the liver.

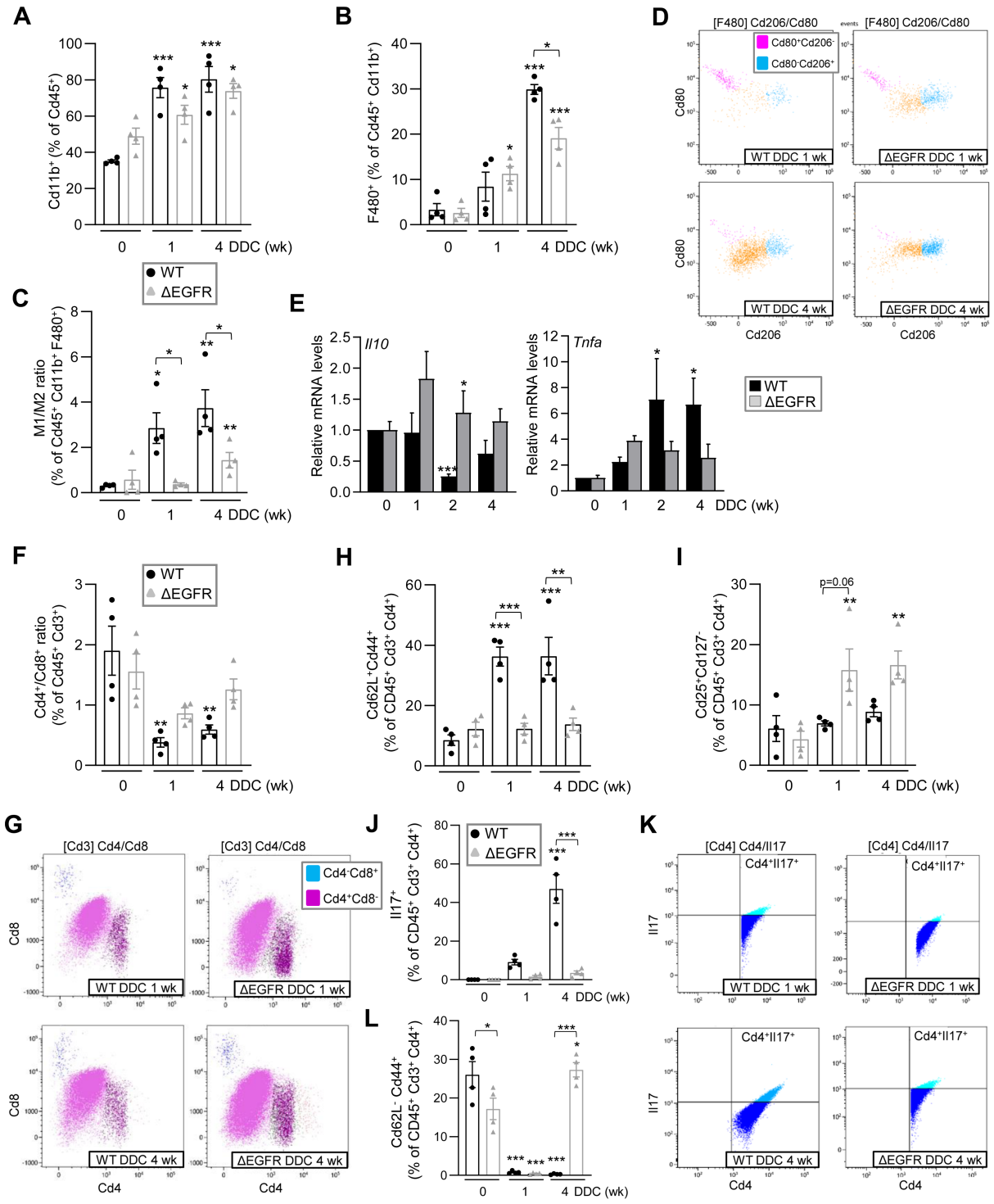

Figure 4. ΔEGFR livers of mice under a DDC diet present an altered inflammatory response profile. (A–D) Non-parenchymal liver cells were isolated and analyzed by flow cytometry. Data are mean ± SEM of 4 animals per group. (A) Analysis of myeloid lineage cells (percentage of $Cd11b^+$ cells pre-gated on $Cd45^+$ cells). (B) Analysis of active resident macrophages (percentage of $F4/80^+$ cells pre-gated on $Cd45^+$ $Cd11b^+$ cells). (C) M1/M2 macrophage ratio was calculated by analyzing M1 cells (percentage of $Cd80^+$ $Cd206^-$ from $Cd45^+$ Cd11b $F480^+$ cells) and M2 cells (percentage of $Cd80^-$ $Cd206^+$ from $Cd45^+$ Cd11b $F480^+$ cells). (D) Representative images of flow cytometry analysis. (E) RT-qPCR analysis of *Il10* and *Tnfa* mRNA levels in the liver. Data are mean ± SEM of 4–10 animals per group. (F–L) Non-parenchymal liver cells were isolated and analyzed by flow cytometry of 4 animals per group. (F) Cd4/Cd8 cell ratio was calculated by analyzing $Cd4^+$ cells (percentage of $Cd4^+$ cells pre-gated on $Cd45^+$ $Cd3^+$ cells) and $Cd8^+$ cells (percentage of $Cd8^+$ cells pre-gated on $Cd45^+$ $Cd3^+$ cells). (G) Representative images of flow cytometry analysis. (H) Analysis of naïve $Cd4^+$ lymphocytes (percentage of $Cd62L^+Cd44^-$ cells pre-gated on $Cd45^+$ $Cd3^+$ $Cd4^+$ cells). (I) Analysis of T-reg lymphocytes (percentage of $Cd25^+Cd127^-$ cells pre-gated on $Cd45^+$ $Cd3^+$ $Cd4^+$ cells). (J) Analysis of Th17 lymphocytes (percentage of $Il17^+$ cells pre-gated from $Cd3^+Cd4^+$). (K) Representative images of flow cytometry analysis. (L) Analysis of Cd4 effector cells (percentage of $Cd62L^-$ $Cd44^+$ cells pre-gated on $Cd45^+$ $Cd3^+$ $Cd4^+$). In all cases, data were compared with the untreated group or as indicated; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

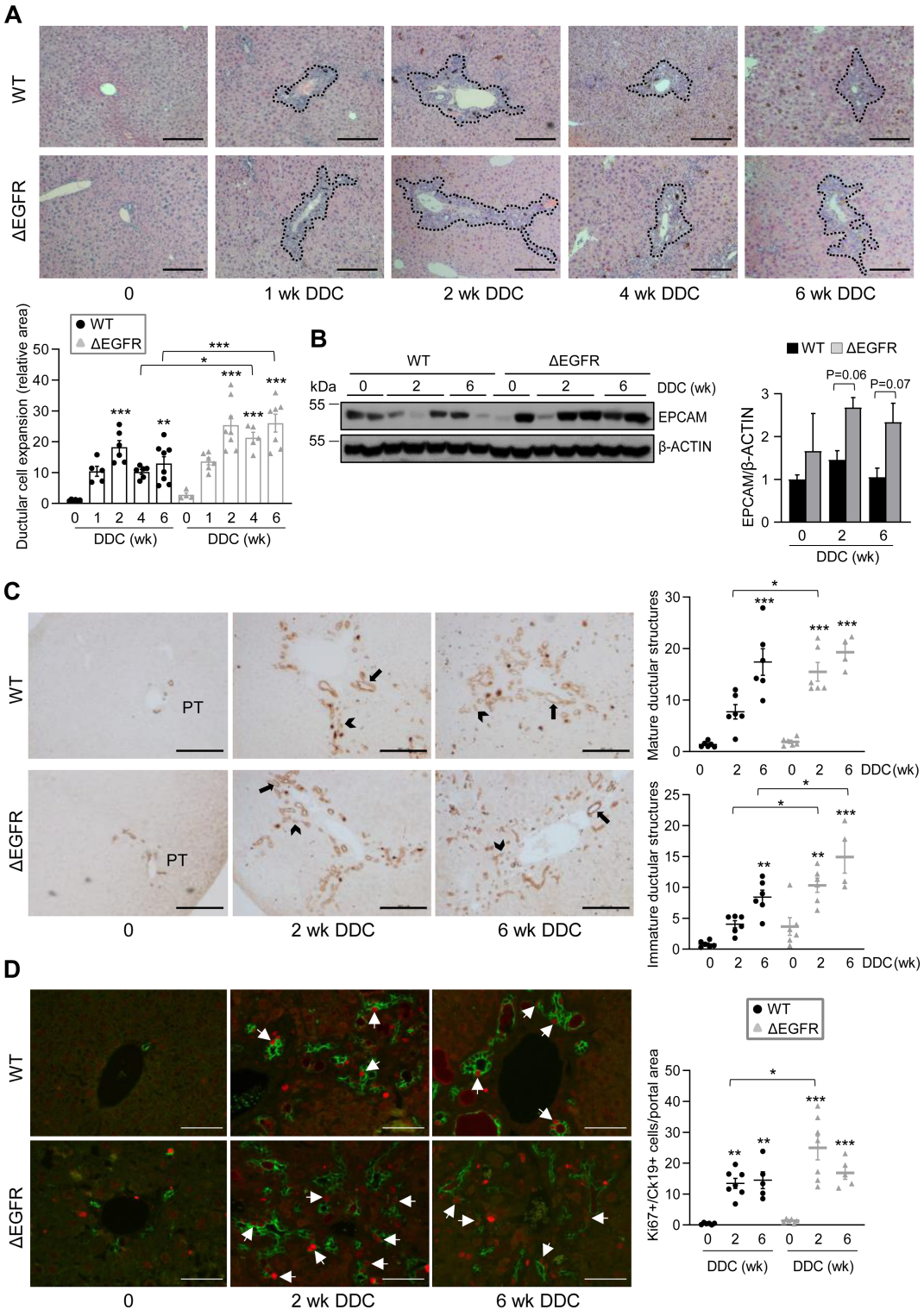

Figure 5. Stronger ductular response in ΔEGFR livers under a DDC diet. (A) Representative images of H&E staining in liver tissues after DDC treatment. Scale bar: 100 μm. Dotted lines mark the edges of the area of cells' expansion in the portal tracts. Quantitative morphometric analysis of the cells' expansion by measuring areas from ten periportal regions of each animal (4–8 animals per group) is shown. (B) Western blot analysis of EPCAM levels in the liver. One representative experiment is shown (left panel). Optical density values are mean ± SEM of 4–12 animals per group (right panel). (C) Representative images of CK19 IHC staining in liver tissues of mice under a DDC diet. Scale bar: 100 μm (left panel). Quantitative analysis of mature and immature ductular structures by measuring areas from ten portal regions of each animal (4–8 animals per group) is shown (right panel). Black arrows indicate mature ductular structures; black arrowheads indicate immature ductular structures. PT, portal triad. (D) Representative images of CK19 and Ki67 double immunofluorescence staining in liver tissues of mice under a DDC diet. Scale bar: 50 μm (left panel). Quantitative analysis of Ki67/CK19-positive cells by counting in ten portal areas of each animal (5–7 animals per group) is shown in the right panel. White arrows indicate double-positive cells. In all cases, data were compared with the untreated group or as indicated; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

Understanding the signaling pathways and molecular regulation of liver regenerative responses is key to providing a basis for modulating and improving these responses with therapeutic purposes in patients suffering from chronic liver diseases. Contradictory findings reported with regard to the role of EGFR in different chronic injuries suggest differential roles for this signaling pathway depending on the injury context, while demonstrating multifaceted and unconventional actions for EGFR in liver regeneration.

The data shown here provide evidence for regulation of the EGFR pathway following DDC-induced liver damage: specifically, upregulation of ligands and early activation of EGFR followed by receptor downregulation, suggesting signaling exhaustion or switch off (Figure 1). It is not clear whether this event is a consequence of cellular damage or a critical event for regulation of the regenerative process. EGFR signaling is part of a complex signaling network in which different regulatory loops finely and dynamically regulate receptor signaling [37], so some of these mechanisms could operate in the context of a cholestatic injury.

More importantly, we have demonstrated that lack of EGFR kinase activity in hepatocytes leads to an attenuation of liver damage following consumption of the DDC diet. Thus, ΔEGFR mice show a smaller increase and a faster recovery of liver damage-associated serum markers, especially those correlated with cholestatic injury, in parallel to an attenuated induction of profibrotic signals and markers (Figure 2). The stronger and prolonged activation of ERK1/2–MAPK and AKT in ΔEGFR mice (Figure 3A) suggests a potential activation of compensatory signals, a common phenomenon in mutant mouse models during liver regeneration. In fact, ΔEGFR mice submitted to partial hepatectomy showed overactivation of the HGF/c-Met pathway that partially compensated for EGFR inactivation [2]. Specific data on cell death and proliferation are consistent with quicker damage resolution in livers of ΔEGFR mice, a fact supported by a faster decline of cell death (Figure 3C). The lowest cell death level observed after 6 weeks of the diet in ΔEGFR mice when compared with WT mice could explain the lower compensatory proliferation in parenchymal cells, since these two processes are known to be associated (Figure 3B) [38,39]. Additionally, cellular recovery in ΔEGFR livers could also be related to the clear shift observed in the inflammatory response that accompanies the DDC-induced liver injury in these livers towards a restorative and anti-inflammatory profile.

Hepatic macrophages include both tissue-resident Kupffer cells and macrophages recruited from the circulating bone marrow-derived monocytes. They are key players in liver disease and have been reported to contribute to cholestatic injury [40]. However, there is a substantial heterogeneity within the hepatic macrophage population that sustains their divergent role in liver disease [41]. A clear phenotypic switch from M1 to M2 subtype was observed in the intrahepatic macrophages in ΔEGFR livers compared with WT livers, as early as 1 week after the diet (Figure 4 and supplementary material, Figure S6). This switch is further supported by a change in the intrahepatic cytokine expression profile between WT and ΔEGFR mice, with predominance of anti-inflammatory members (*Il10*, *Il6*) in ΔEGFR, as opposed to pro-inflammatory ones (*Tnf*) in WT (Figure 4 and supplementary material, Figure S6) [42,43]. M2 macrophages display a regulatory phenotype and can promote tissue repair. Among other mechanisms, they have the ability to induce Treg cells and are involved in the phagocytosis of apoptotic cells [41]. Consistent with this, we also see an increase in Treg cells, as well as a decrease in Th17 cells, the latter being elevated during cholestatic injury and having profibrogenic properties [44,45]. Interestingly, the recently achieved single-cell atlas in PSC has identified naïve-like $CD4^+$ T cells that tend to acquire Th17 effector functions as a likely contributor to the PSC pathogenesis [46]. Such a process is impaired in ΔEGFR livers.

Consideration of our results together supports the notion that the profound changes taking place in liver immune cells in ΔEGFR mice could contribute to balance inflammation in the DDC-injured liver and to restore homeostasis. These results are of unquestionable interest. Evidence exists supporting a role for EGFR signaling in liver inflammation. In fact, EGFR is activated in liver macrophages after CCl_4-induced injury, and EGFR inhibition has been linked to decreased expression of inflammatory mediators and decreased infiltration of inflammatory cells during CCl_4- or HFD-induced injury [47,48]. However, how EGFR acts to regulate inflammation during chronic liver injury is not known. Our data suggest a scenario in which hepatocyte EGFR catalytic activity would contribute to the pathogenesis of cholestatic injury by inducing specific changes in the liver immune cell phenotype that promote an imbalanced inflammation. In this line of thought, previous work has shown that EGFR induces the expression of inflammatory mediators in liver macrophages and hepatocarcinoma cells, this having a tumor-promoting effect [49,50]. Activation of EGFR in human cholangiocytes is also required for a robust inflammatory response [51]. This evidence upholds our hypothesis, but future research will help us to clarify the EGFR-dependent mechanisms operating in hepatocytes to modulate the outcome of the regenerative response. We have explored several signaling pathways directly or indirectly associated with the EGFR pathway. The data so far have failed to show differences in Notch signaling components (Notch receptors, Jag ligands, target gene *Hes1* or regulators Numb and Nrarp) or the Hippo effector YAP (Yes-associated protein) between WT and ΔEGFR livers upon DDC treatment (data not shown), suggesting that hepatocyte-specific inactivation of EGFR is not sufficient to disrupt these signaling axes. However, ΔEGFR livers display stronger activation of STAT3 (supplementary material, Figure S6H). This

could be related to the altered cytokine profile, specifically the higher expression of *Il6* (supplementary material, Figure S6C), although additional studies are needed to define the specific stimulatory signals. Based on studies demonstrating hepatoprotective and pro-regenerative actions of STAT3 [52,53], it is feasible to hypothesize that it likely contributes to the differences in regenerative response between WT and ΔEGFR livers.

Inflammatory signals are also involved in HPC expansion [54,55]. Thus, removal of macrophages during the regenerative response induced by 2-acetylaminofluorene (AAF)/PH significantly decreases liver IL6 expression, STAT3 activation, and HPC/OC expansion, compromising liver regeneration [56]. In this line, an enhanced inflammatory response is evidenced in the portal tracts of ΔEGFR livers at 2 weeks (Table 1 and supplementary material, Figure S5), coinciding with enhanced proliferation of ductular cells (Figure 5). The stronger activation of mitogenic and survival signals, ERK1/2–MAPKs, and AKT, seen in ΔEGFR livers, fits well with a pro-regenerative milieu. Although the source of this enhanced signaling is not revealed, it could also be associated with the inflammatory signals that are being secreted in ΔEGFR livers, as cytokines (IL6, IL10) can trigger their activation [57,58].

Data are scarce regarding the consequences of inhibiting the EGFR pathway in the liver on the fate of HPCs/OCs. A study using Mx1-driven EGFR conditional knockout mice described how the EGFR pathway via NOTCH1 promotes biliary lineage specification while suppressing the hepatocytic lineage, and proposed an improved differentiation into hepatocytes as the mechanism responsible for enhancing HPC/OC-driven regeneration upon DDC treatment in the absence of EGFR [26]. Our model provides a different scenario and new perspectives on the actions of this receptor during the hepatic response to DDC injury. In our model, EGFR is inactivated only in hepatocytes, and partially in the HPC/OC population, the latter being likely due to weaker promoter expression in these cells. Consequently, direct EGFR-dependent proliferative activity on HPCs/OCs can still take place to some degree, regardless of the effect of inflammatory or other potential signals. In any event, whether or not the enhanced HPC/OC proliferation plays a critical role in the improved restorative response is not known, but based on previous results from our group and others showing the critical role of these cells in regeneration upon DDC injury [28,59], it is likely the case.

In conclusion, our work points to novel actions of EGFR, particularly a key role for hepatocyte EGFR catalytic activity in the regulation of the inflammatory response during cholestatic injury. Specifically, our data point to a scenario where EGFR activity in hepatocytes critically contributes to the pro-inflammatory response activated during liver injury and therefore to the pathogenesis of cholestatic liver disease, acting as a key player in the crosstalk between parenchymal and non-parenchymal hepatic cells. This study opens paths to explore therapeutic approaches aimed at inactivating EGFR in hepatocytes to promote a restorative inflammatory response to facilitate liver regeneration and prevent disease progression.

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Author contributions statement

NL, JGS, CGC, CR, JS, CMR, PV, LA, SCP, AF, PB, CG and AMV were involved in data acquisition, analysis, and/or interpretation. AMP, MGB, AF, LM, JCS, IF and AS were responsible for generation of the animal model. AMV, IF, BH and AS acquired funding. BH, IF and AS conceived and designed the study. BH and AS wrote the manuscript. IF and AMV reviewed and edited the manuscript. All the authors reviewed and approved the final version of the manuscript.

Data availability statement

Data are available upon request from the authors.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Expression of EGFR family ligands in the livers of mice fed the DDC diet

Figure S2. Expression of mRNAs for ligands and receptors of the EGFR family in the livers of patients with primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC)

Figure S3. Analysis of lysyl oxidase-like 1 and 2 mRNA expression in ΔEGFR livers of mice fed the DDC diet

Figure S4. Analysis of caspase-3 activation in ΔEGFR livers of mice fed the DDC diet

Figure S5. Representative H&E images for visualization of inflammatory cells in the portal areas of WT and ΔEGFR livers of mice fed the DDC diet

Figure S6. Analysis of the inflammatory response in ΔEGFR livers of mice fed the DDC diet

Figure S7. Analysis of the ductular reaction in the livers of mice fed the DDC diet

Figure S8. Analysis of the expression of the ΔEGFR transgene and EGFR signaling activation in oval cells

Table S1. Primer sequences used in quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) (referred to in Supplementary materials and methods)

Table S2. Analysis of ductal proliferation in the livers of mice fed the DDC diet