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ORIGINAL ARTICLE

Hepatic overexpression of cAMP-responsive element modulator α induces a regulatory T-cell response in a murine model of chronic liver disease

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► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2015-311119).

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Received 18 November 2015
Accepted 17 August 2016
Published Online First 29 September 2016

Linked

► http://dx.doi.org/10.1136/gutjnl-2016-312900

CrossMark

To cite: Kuttkat N, Mohs A, Ohl K, *et al*. *Gut* 2017;**66**:908–919.

ABSTRACT

Objective Th17 cells are a subset of $CD4^+$ T-helper cells characterised by interleukin 17 (IL-17) production, a cytokine that plays a crucial role in inflammation-associated diseases. The cyclic AMP-responsive element modulator-α (CREMα) is a central mediator of T-cell pathogenesis, which contributes to increased IL-17 expression in patients with autoimmune disorders. Since an increased Th17 response is associated with a poor prognosis in patients with chronic liver injury, we investigated the relevance of Th17 cells for chronic liver disease (CLD) and hepatocarcinogenesis.

Design Transgenic mice overexpressing CREMα were crossed with hepatocyte-specific Nemo knockout mice ($Nemo^{\Delta hepa}$) to generate $Nemo^{\Delta hepa}$/$CREM\alpha^{Tg}$ mice. The impact of $CREM\alpha^{Tg}$ on CLD progression was examined. Additionally, soft agar colony formation assays, in vitro studies, adoptive transfer of bone marrow-derived cells (BMDCs) and T cells, and gene arrays in T cells were performed.

Results 8-week-old $Nemo^{\Delta hepa}$/$CREM\alpha^{Tg}$ mice presented significantly decreased transaminase levels, concomitant with reduced numbers of $CD11b^+$ dendritic cells and $CD8^+$ T cells. $CREM\alpha^{Tg}$ overexpression in $Nemo^{\Delta hepa}$ mice was associated with significantly reduced hepatic fibrogenesis and carcinogenesis at 52 weeks. Interestingly, hepatic stellate cell-derived retinoic acid induced a regulatory T-cell (Treg) phenotype in $CREM\alpha^{Tg}$ hepatic T cells. Moreover, simultaneous adoptive transfer of BMDCs and T cells from $CREM\alpha^{Tg}$ into $Nemo^{\Delta hepa}$ mice ameliorated markers of liver injury and hepatitis.

Conclusions Our results demonstrate that overexpression of CREMα in T cells changes the inflammatory milieu, attenuating initiation and progression of CLD. Unexpectedly, our study indicates that CREMα transgenic T cells shift chronic inflammation in $Nemo^{\Delta hepa}$ livers towards a protective Treg response.

Significance of this study

What is already known on this subject?

- Th17 cells are involved in the pathogenesis of different types of liver injury.
- Th17 cells are associated with a poor prognosis in hepatocellular carcinoma (HCC) patients.
- Mice overexpressing cyclic AMP-responsive element modulator α (CREMα) in T cells are characterised by enhanced production of interleukin 17 (IL-17), IL-21 and IL-22 as well as retinoid receptor-related orphan receptor gamma-t (RORγT), and have increased inflammatory response.

What are the new findings?

- Overexpression of CREMα in T cells attenuates chronic liver disease and HCC development.
- CREMα overexpression changed the inhibitory profile of hepatic T cells.
- Hepatic stellate cell-derived retinoic acid induces the differentiation of CREMα transgenic T cells towards a protective regulatory T-cell (Treg) response.
- $CREM\alpha^{Tg}$ T cells primed with Treg conditions attenuate hepatocyte damage.

How might it impact on clinical practice in the foreseeable future?

- To maintain a sufficient immune response, the timing and strength of immunosuppression have a major impact on inflammatory activity and end-stage cancer development. Therefore, our study demonstrates that the liver-specific inflammatory environment directs the response of regulatory T cells, which opens the basis for novel therapeutic strategies.

INTRODUCTION

Sustained inflammation is a pathological hallmark of chronic liver disease (CLD) and promotes liver fibrosis and the growth of hepatocellular carcinoma (HCC). Th17 cells are a recently discovered subset of $CD4^+$ T-helper cells characterised by the production of their signature cytokine interleukin 17 (IL-17). Several lines of evidence have shown that Th17 cells are involved in the pathogenesis of different types of liver injury including alcoholic liver disease (ALD), non-alcoholic steatohepatitis, viral hepatitis and HCC development.[1]

The cyclic AMP (cAMP)-responsive element modulator (CREM) belongs to the family of basic leucine zipper transcription factors. The α-isoform (CREMα) is the most abundant isoform in T cells and serves, due to the lack of a transactivation domain, as a transcriptional repressor. Together with the cAMP response element-binding protein,

CREMα is a central mediator of T-cell proliferation by regulating IL-2 expression.[2 3] Indeed, repression of IL-2 production associated with increased CREMα mRNA and protein expression has been observed in T cells of patients with systemic lupus erythematosus.[3 4] Concomitantly, transgenic (Tg) mice overexpressing CREMα specifically in T cells (CREMα^{Tg}) are characterised by an increased binding of CREMα to the IL-2 promoter, leading to decreased IL-2 production and T-cell proliferation.[5]

In experimental in vivo models of contact dermatitis and acute lung injury, as well as in vitro, CREMα-overexpressing mice cells display a Th17 phenotype, characterised by enhanced production of IL-17, IL-21 and IL-22 as well as retinoid receptor-related orphan receptor gamma-t (RORγt), promoting tissue inflammation and the recruitment of leukocytes, mainly neutrophils.[5–7]

Aberrant nuclear factor-κB signalling plays a fundamental role in a wide range of inflammation-mediated diseases including rheumatoid arthritis, asthma and human cancers such as Hodgkin's lymphoma and colitis-associated cancer.[8] Knockout mice for the IKK complex members, IKKβ or NEMO, die *in utero* as a result of tumor necrosis factor (TNF)-mediated cell death of hepatocytes. Moreover, we have recently shown that hepatocyte-specific Nemo knockout (Nemo$^{\Delta hepa}$) mice are viable but develop chronic liver injury characterised by TNF-dependent inflammation and scar formation leading to liver fibrosis, hepatitis and HCC within 1 year of age.[9] Hence, disease progression in this experimental animal model mimics the progression of human CLD.

In the current study we hypothesised that an increased Th17 response, caused by overexpression of CREMα, would exacerbate liver injury in Nemo$^{\Delta hepa}$ mice. However, we found ameliorated liver injury and reduced carcinogenesis. We, thus, analysed the underlying changes in disease progression and T-cell differentiation.

MATERIALS AND METHODS

Generation of nemo$^{\Delta hepa}$/CREMα^{Tg} mice

Hepatocyte-specific IKK/Nemo knockout mice (Nemo$^{\Delta hepa}$)[10] were crossed to CD2-CREMα transgene-expressing mice (CREMα^{Tg} mice) to generate Nemo$^{\Delta hepa}$/CREMα^{Tg} mice. CREMα^{Tg} mice were crossed to non-CREMα^{Tg} littermates for four generations. Cre littermates served as controls. Animals were housed under specific pathogen-free conditions in the animal facility of University Hospital Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen. To investigate disease progression, male mice were sacrificed at 8, 13 and 52 weeks. All experiments were in line with the criteria of the authority for environment conservation and consumer protection of the state North Rhine-Westphalia (LANUV, Germany).

For details on methodology, please see online supplementary material.

RESULTS

CREMα ameliorates the onset of CLD in Nemo$^{\Delta hepa}$ mice

Since earlier studies suggested that activated Th17 cells and Th17-related cytokines play a prominent role in hepatic inflammation in human liver disease, we studied the impact of Th17 cells on initiation and progression of CLD. Therefore, we generated Nemo$^{\Delta hepa}$/CREMα^{Tg} animals by crossing Nemo$^{\Delta hepa}$ with Tg mice overexpressing CREMα specifically in T cells (CREMα^{Tg}) (see online supplementary figure S1A).

To analyse the onset of CLD, we first assessed liver injury in 8-week-old mice. Nemo$^{\Delta hepa}$ mice are characterised by high levels of serum alanine (ALT) and aspartate transaminases (AST). In contrast, Nemo$^{\Delta hepa}$/CREMα^{Tg} animals exhibited significantly reduced ALT and AST levels, indicating reduced liver injury in 8-week-old animals (figure 1A). H&E staining revealed only mild differences in hepatic damage between Nemo$^{\Delta hepa}$/CREMα^{Tg} and Nemo$^{\Delta hepa}$ mice (figure 1B, C), associated with a significantly decreased non-alcoholic fatty liver disease activity (NAS) score (figure 1D).

The pathogenesis of CLD in Nemo$^{\Delta hepa}$ mice is associated with increased hepatocyte apoptosis and compensatory proliferation.[9 11] Thus, we analysed the effect of CREMα overexpression in T cells using markers of cell death and proliferation in Nemo$^{\Delta hepa}$/CREMα^{Tg} livers. Immunostaining against cleaved caspase-3 (see figure 1E and online supplementary figure S1B) and Ki-67 (see figure 1F and online supplementary figure S1C) did not show any difference in apoptosis or compensatory proliferation in 8-week-old mice. Additionally, gene expression analysis revealed no significant difference in the expression of the proapoptotic proteins *Bak* or *Bim*, with exception of *Bax*, a proapoptotic protein that is activated by cleavage of caspases, significantly highly expressed in Nemo$^{\Delta hepa}$ in contrast with Nemo$^{\Delta hepa}$/CREMα^{Tg} livers (see online supplementary figure S1D). In accordance, gene expression of the antiapoptotic proteins *Bcl-2* or *Bcl-XL* was not altered in Nemo$^{\Delta hepa}$ compared with Nemo$^{\Delta hepa}$/CREMα^{Tg} livers (see online supplementary figure S1D). However, the mRNA expression of proliferating cell nuclear antigen *(Pcna)*, a specific S-phase cell-cycle marker,[12] was significantly decreased in 8-week-old Nemo$^{\Delta hepa}$/CREMα^{Tg} compared with Nemo$^{\Delta hepa}$ livers (see online supplementary figure S1E). Altogether, these results suggest that overexpression of CREMα in T cells improves the onset of CLD in NEMO$^{\Delta hepa}$ mice.

Overexpression of CREMα in T cells reduces hepatic inflammation in NEMO$^{\Delta hepa}$ mice

Inflammatory responses upon liver injury comprise resident as well as infiltrating immune cells. Both the innate and the adaptive immune system are important triggers of liver inflammation. Therefore, we examined the amount and the composition of infiltrating immune cells by fluorescence-activated cell sorting (FACS) analysis and immunofluorescence staining. Eight-week-old Nemo$^{\Delta hepa}$/CREMα^{Tg} compared with Nemo$^{\Delta hepa}$ livers revealed reduced numbers of CD11b^{+} dendritic cells (DCs) and CD8^{+} T cells (see figure 2A–C and online supplementary figure S2A). Moreover, immune cell populations such as granulocytes (see online supplementary figure S2A, B), inflammatory monocytes (see online supplementary figure S2A–D), CD4^{+} T cells (see online supplementary figure S3A, B) and natural killer T (NKT) cells (see online supplementary figure S3A, C) were decreased in Nemo$^{\Delta hepa}$/CREMα^{Tg} livers, while B cells were enhanced compared with Nemo$^{\Delta hepa}$ animals (see online supplementary figure S3A, D).

Since chronic liver inflammation triggers liver fibrogenesis, we studied changes in hepatic collagen deposition and desmin expression as markers of hepatic stellate cell (HSC) activation. Collagen IA1 and desmin immunofluorescence and quantification and Sirius red staining (not shown) revealed significantly decreased markers of hepatic fibrogenesis in livers of 13-week-old Nemo$^{\Delta hepa}$/CREMα^{Tg} compared with Nemo$^{\Delta hepa}$ mice (figure 2D–F). Thus, overexpression of CREMα in T cells exerts an antifibrogenic effect by reducing the inflammatory milieu and extracellular matrix deposition in Nemo$^{\Delta hepa}$ livers.

Nemo$^{\delta hepa}$/CREMα^{Tg} mice display reduced carcinogenesis

Nemo$^{\Delta hepa}$ mice spontaneously develop HCC within 1 year.[9] Therefore, we next focused on studying the impact of CREMα overexpression in T cells on tumour initiation and progression.

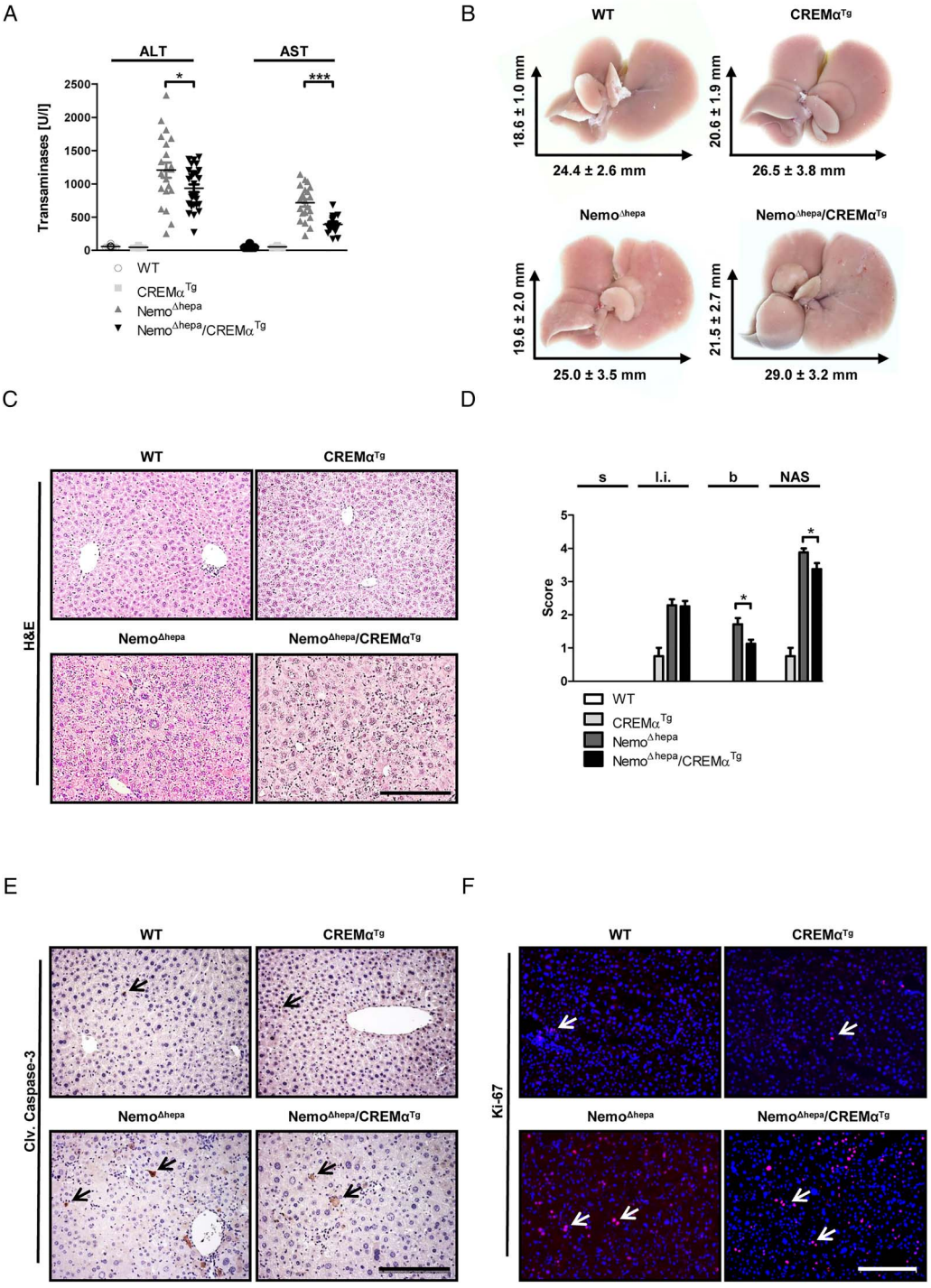

Figure 1 CREMα ameliorates the onset of chronic liver disease in Nemo$^{\Delta hepa}$ mice. (A) Serum ALT and serum AST levels (Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$/CREMα^{Tg}). Data are shown as mean±SEM of n=21–28 mice per group (*p<0.05). (B) Macroscopic view of the livers as indicated. (C) Microscopic picture of H&E staining (scale bar: 200 μm). (D) Histological scoring of H&E-stained paraffin samples concerning steatosis (s), lobular inflammation (l.i.), ballooning (b) and total non-alcoholic fatty liver disease activity score. Data are shown as mean±SEM of n=4–8 mice per group (*p<0.05). (E) Immunohistochemical staining for cleaved caspase-3 (scale bar: 200 μm). (F) Immunofluorescence staining for Ki-67 (red: Ki-67, blue: DAPI, scale bar: 200 μm). ALT, alanine aminotransferase; AST, aspartate aminotransferase; CREMα, cyclic AMP-responsive element modulator α; WT, wild type.

ALT levels were significantly reduced in 52-week-old Nemo$^{\Delta hepa}$/CREMα^{Tg} compared with Nemo$^{\Delta hepa}$ mice (figure 3A). Macroscopic liver examination revealed solid tumours in both Nemo$^{\Delta hepa}$ and Nemo$^{\Delta hepa}$/CREMα^{Tg} mice (figure 3B). However, HCC was found in 100% of Nemo$^{\Delta hepa}$ mice, whereas it was detected in only 80% of Nemo$^{\Delta hepa}$/CREMα^{Tg} mice (see figure 3C; lower panel and online supplementary figure S4A). Furthermore, the liver-to-body weight ratio was significantly reduced in Nemo$^{\Delta hepa}$/CREMα^{Tg} compared with Nemo$^{\Delta hepa}$ mice, respectively (figure 3C; upper panel).

Interestingly, the absolute as well as the relative area of HCC were significantly increased in Nemo$^{\Delta hepa}$ compared with Nemo$^{\Delta hepa}$/CREMα^{Tg} mice (see figure 3C; lower panel and online supplementary figure S4B).

Both CD4$^+$ and CD8$^+$ T cells are mostly considered to be significant players in inhibiting, impeding and killing tumour cells.

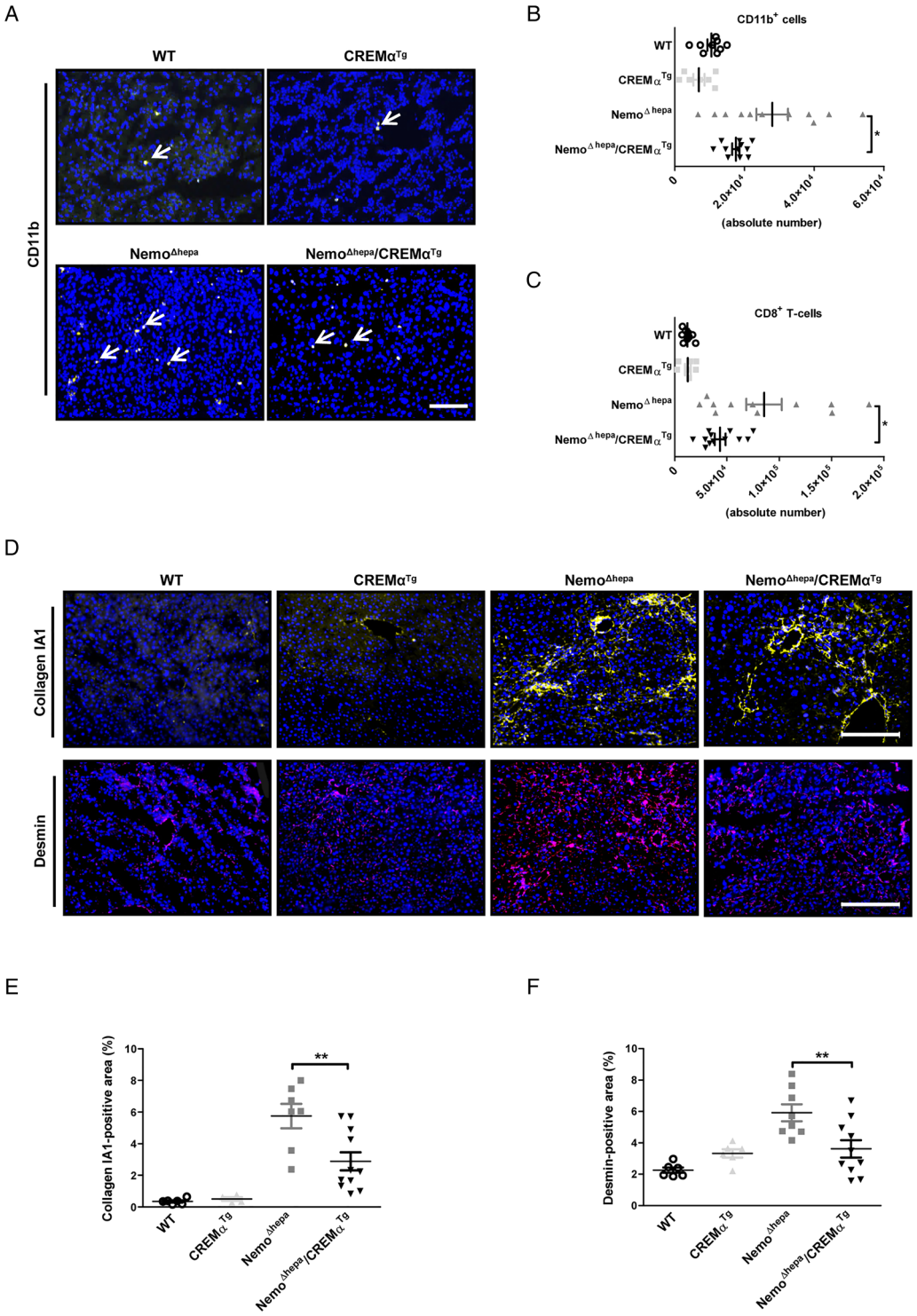

Figure 2 Overexpression of CREMα in T cells reduces hepatic inflammation in NEMO$^{\Delta hepa}$ mice. (A) Immunofluorescence staining for CD11b (yellow: CD11b, blue: DAPI, scale bar: 100 μm). Fluorescence-activated cell sorting analysis of infiltrating cells: Absolute number of (B) CD11b$^+$ DCs; (C) CD8$^+$ T cells; and (D) immunofluorescence staining for Collagen IA1 (yellow: Collagen IA1, blue: DAPI, scale bar: 200 μm). Immunofluorescence staining for desmin (red: Desmin, blue: DAPI, scale bar: 200 μm). (E)+(F) Quantification of collagen and desmin-positive area (Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$/CREMα^{Tg}). Data are shown as mean±SEM of n=6–10 mice per group (*p<0.05, **p<0.01). CREMα, cyclic AMP-responsive element modulator α; WT, wild type.

Moreover, it has been recently shown that loss of CD4$^+$ T cells promotes HCC development.[13] To evaluate the immunosuppressive effect of cyclic AMP-responsive element modulator-α (CREMα)^{Tg}-overexpressing CD4$^+$ T cells, we challenged Hepa 1–6 cells, a HCC cell line, with isolated splenic and hepatic CD4$^+$ T cells from wild type (WT) and Nemo$^{\Delta hepa}$/CREMα^{Tg} mice by testing its ability to form anchorage-independent colonies. As control, we used tumour cell clones that grow in soft agar.

Surprisingly, CD4$^+$ T cells derived from a Nemo$^{\Delta hepa}$/CREMα^{Tg} environment augmented the transformation potential of the HCC cell line. Conversely, splenic CD4$^+$ T cells isolated

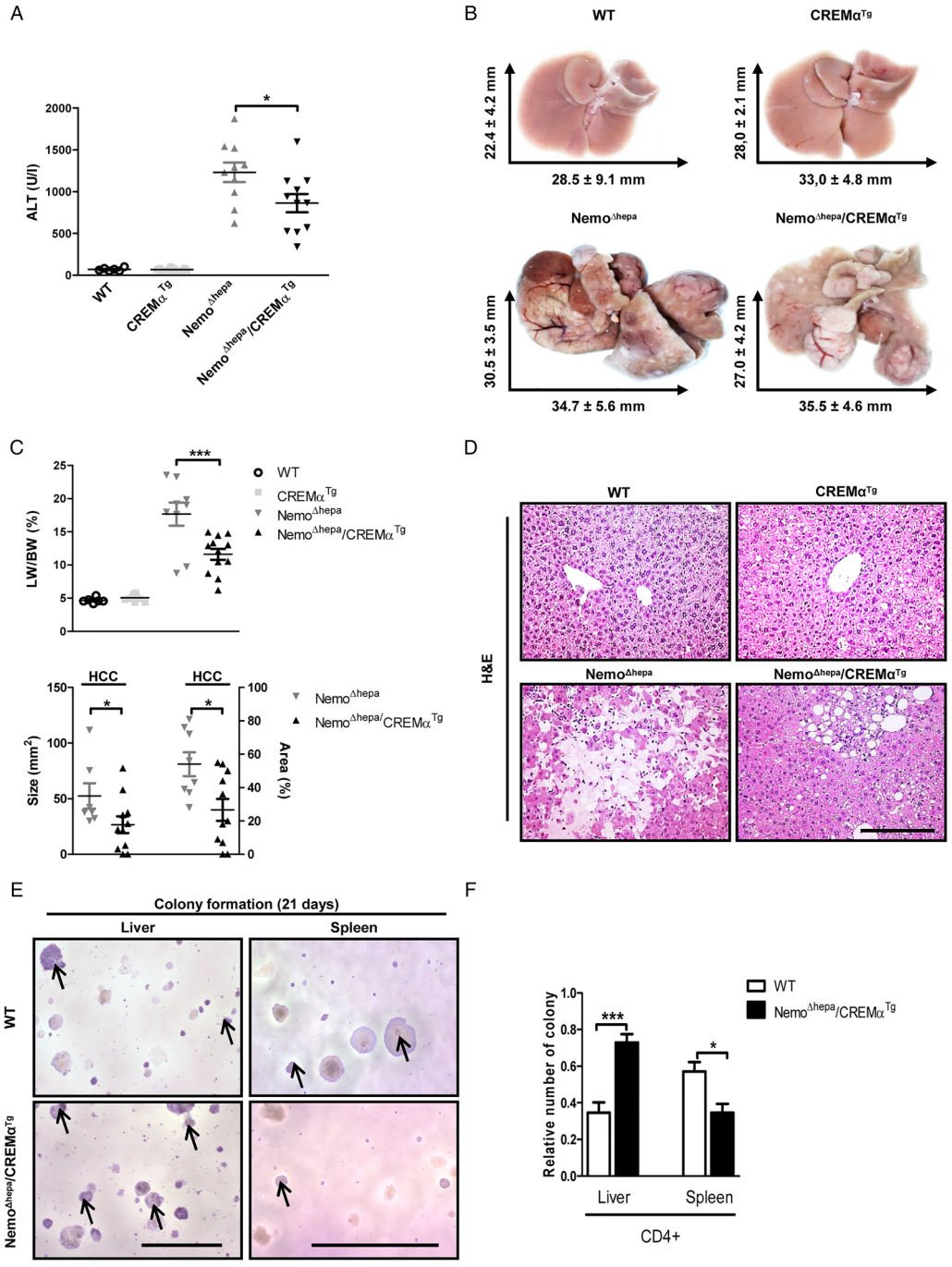

Figure 3 Nemo$^{\Delta hepa}$/CREMα^{Tg} mice display reduced carcinogenesis. (A) Serum aspartate aminotransferase levels (Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$/CREMα^{Tg}). Data are shown as mean±SEM of n=6–12 mice per group (*p<0.05). (B) Liver pictures and average liver size. (C, upper panel) Liver versus body weight (LW/BW) ratio (Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$/CREMα^{Tg}). Data are shown as mean±SEM of n=6–12 mice per group (***p<0.001). (C, lower panel) Quantification of absolute and relative area of HCC. Data are shown as mean±SEM of n=8–11 mice per group (*p<0.05). (D) Macroscopic picture of H&E staining (scale bar: 200 μm). (E) Soft agar colony formation assays were performed in Hepa 1–6 challenged with CD4^{+} T cells isolated from spleen and liver of WT and Nemo$^{\Delta hepa}$/CREMα^{Tg} mice. After 21 days of incubation, colonies were stained with 0.01% crystal violet (scale bar: 1 and 5 mm). (F) The number of colonies was counted and shown in a graphic presentation of the soft agar colony formation assays of mean±SEM of triplicated experiments (***p<0.001). ALT, alanine aminotransferase; CREMα, cyclic AMP-responsive element modulator α; HCC, hepatocellular carcinoma; WT, wild type.

from Nemo$^{\Delta hepa}$/CREMα^{Tg} mice, but with a CREMα^{Tg} phenotype, significantly reduced the tumourigenic capacity of Hepa 1–6 cells (see figure 3E, F and online supplementary figure S4C). These results question whether CD4^{+} T cells exert a crucial role in tumour development in Nemo$^{\Delta hepa}$/CREMα^{Tg} or decreased carcinogenesis is a secondary effect of the reduced inflammation observed in these livers.

Overexpression of CREMα in T cells reduces tumour malignancy in NEMO$^{\Delta hepa}$ *mice*

Since 1-year Nemo$^{\Delta hepa}$/CREMα^{Tg} livers showed less HCC, and the impact of CREMα overexpression in T cells for tumour growth or malignant transformation and proliferation was further investigated in 52-week-old livers. Immunoblotting for PCNA, cyclin A and pRb revealed decreased protein expression

of these proliferation markers in Nemo$^{\Delta hepa}$/CREMα^{Tg} compared with Nemo$^{\Delta hepa}$ livers (figure 4A). These data were confirmed by reduced Ki-67 immunostaining in liver tissue of Nemo$^{\Delta hepa}$/CREMα^{Tg} mice (figure 4B).

To characterise changes in the molecular signature of HCC derived from Nemo$^{\Delta hepa}$/CREMα^{Tg} and Nemo$^{\Delta hepa}$ animals, we next investigated *c-myc* expression, a transcription factor associated with the conversion of preneoplastic liver lesions into HCC.[14 15] mRNA expression of *c-myc* was significantly reduced in Nemo$^{\Delta hepa}$/CREMα^{Tg} mice compared with Nemo$^{\Delta hepa}$ mice (figure 4C). Additionally, western blot as well as immunostaining against glutamine synthetase, an enzyme shown to be upregulated in human and murine HCC tissue,[16] were enhanced in Nemo$^{\Delta hepa}$ compared with Nemo$^{\Delta hepa}$/CREMα^{Tg} mice (see figure 4D and online supplementary figure S4D). Altogether, these results suggest that Tg overexpression of CREMα in T cells exerts a beneficial effect on tumour progression.

The simultaneous transfer of CREMα Tg T cells and haematopoietic cells ameliorates liver injury in Nemo$^{\Delta hepa}$ mice

First, we excluded a direct effect of CREMα^{Tg} T cells on hepatocyte physiology. Therefore, we stimulated primary hepatocytes of WT, CREMα^{Tg}, Nemo$^{\Delta hepa}$ and Nemo$^{\Delta hepa}$/CREMα^{Tg} mice for 6 h with TNF-α (10 ng/mL). In line with previous observations, Nemo-deficient hepatocytes were hypersensitive to TNF-α,[10] whereas WT hepatocytes were resistant (see online supplementary figure S5A, C). However, there was no difference in sensitivity towards TNF-α between Nemo$^{\Delta hepa}$ and Nemo$^{\Delta hepa}$/CREMα^{Tg} hepatocytes.

To further validate the effect of CREMα^{Tg} T cells on Nemo$^{\Delta hepa}$ hepatocytes, we stimulated primary isolated hepatocytes from WT and Nemo$^{\Delta hepa}$ mice with supernatant (SN) of unprimed (Th0) or Treg primed conditions (transforming growth factor (TGF)-β, TGF-β+retinoic acid (RA) or SN of activated HSC (7d) from WT or CREMα^{Tg} T cells). Interestingly, we found that stimulation of WT hepatocytes with these conditions did not influence their survival (see online supplementary figure S5B, D). However, challenge of Nemo$^{\Delta hepa}$ hepatocytes with Th0 CREMα^{Tg} T cells SN increased ALT levels, whereas priming with T-cell SN—causing their differentiation towards a regulatory T-cell (Treg) phenotype—significantly ameliorated the levels of transaminases in Nemo$^{\Delta hepa}$ hepatocytes (see online supplementary figure S5B,D). Altogether these results indicate that CREMα^{Tg} Treg attenuate Nemo$^{\Delta hepa}$-derived hepatocyte damage.

Consequently, we investigated whether amelioration of CLD progression was caused by haematopoietic cells, and, especially, T cells. We, thus, performed adoptive transfer experiments. Six-week-old recipient mice were irradiated and transplanted with bone marrow-derived cells (BMDCs). In addition, these animals received a boost injection of T cells. As control, Nemo$^{\Delta hepa}$ mice received either WT or CREMα^{Tg} BMDCs in order to verify the importance of the adoptive T-cell transfer. Mice were sacrificed 8 weeks later, and parameters of liver injury, hepatic inflammation and liver fibrosis were assessed, to analyse the impact of the transplanted cells (figure 5A).

Liver histology of transplanted mice revealed reduced liver damage in Nemo$^{\Delta hepa}$ mice receiving CREMα^{Tg} cells, accompanied by significantly lower serum ALT values (figure 5B, C). This effect was more pronounced in animals that received BMDCs and T cells, indicating the crucial effect of pre-existing T cells. Furthermore, Nemo$^{\Delta hepa}$ mice receiving CREMα Tg cells displayed significantly reduced collagen deposition as evidenced by Sirius red staining (figure 5D, E). Thus, haematopoietic-derived CREMα Tg T cells are essential to confer protection against CLD progression in NEMO$^{\Delta hepa}$ mice.

Overexpression of CREMα does not induce a predominant Th17 response in hepatic T cells

CREMα-overexpressing mice display a Th17 phenotype, characterised by enhanced production of IL-17, a proinflammatory as well as a profibrotic cytokine,[5] and manifest accelerated inflammatory response in in vivo models of contact dermatitis and acute lung injury. Surprisingly, Nemo$^{\Delta hepa}$/CREMα^{Tg} mice showed reduced CLD progression. We, thus, examined the mRNA transcripts of the lineage transcription factor of Th17 cells, *Rorγt*, and their signature cytokine, *IL17A*, in 8-week-old CREMα^{Tg} and Nemo$^{\Delta hepa}$/CREMα^{Tg} livers. Interestingly, T cells isolated from spleens of Nemo$^{\Delta hepa}$/CREMα^{Tg} mice showed a significant increase in *Rorγt* and *IL17A* mRNA expression (figure 6A).

Since we observed major differences in the effect of CREMα Tg T cells on the Nemo$^{\Delta hepa}$ phenotype, associated with their differentiation pattern,[17] we further investigated the Th17 phenotype in hepatic T cells (figure 6B). Indeed, our FACS analysis revealed that CREMα^{Tg} control mice showed no increase in Rorγt^{+} T cells in the liver. In contrast, enhancement of Rorγt^{+} T cells was observed in Nemo$^{\Delta hepa}$ livers compared with Nemo$^{\Delta hepa}$/CREMα^{Tg} livers (figure 6B), suggesting that CREMα overexpression does not induce a predominant Th17 response in hepatic T cells.

CREMα overexpression changes the inhibitory profile of hepatic T cells

As Th17 cells and Tregs develop through reciprocal pathways, we next investigated whether reduced *Rorγt* expression might be linked with an enhanced differentiation of hepatic T cells into the Treg lineage.

NanoString Technology, a direct multiplexed measurement of gene expression in 8-week-old WT, CREMα^{Tg}, Nemo$^{\Delta hepa}$ and Nemo$^{\Delta hepa}$/CREMα^{Tg} hepatic T cells, was performed. Thereby, we observed increased mRNA transcripts for forkhead-box-protein p3 (*foxp3*)—the lineage transcription factor of Tregs in hepatic T cells isolated from Nemo$^{\Delta hepa}$/CREMα^{Tg} mice (figure 6C, D). Additionally, several markers of Treg function such as cytotoxic T-lymphocyte-associated protein 4 *(ctla4)*, an inhibitory receptor, as well as Epstein-Barr virus induced-3 (*ebi3)*, which encodes the IL-27β subunit of IL-35—a cytokine that suppresses inflammatory responses of immune cells, were strongly induced in hepatic T cells of Nemo$^{\Delta hepa}$/CREMα^{Tg} mice (figure 6C, D). In line with these findings, the T-cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif (ITIM) domains (*tigit*), which is able to induce IL-10 production by DCs[18] and thereby mediate immune suppression, was strongly upregulated in hepatic T cells of Nemo$^{\Delta hepa}$/CREMα^{Tg} mice (figure 6D). Consistently, programmed cell death protein-1 (*pdcd1*), a surface receptor that prevents the activation of T cells by inhibiting Treg apoptosis, was also upregulated in hepatic T cells of Nemo$^{\Delta hepa}$/CREMα^{Tg} and Nemo$^{\Delta hepa}$ mice (figure 6D).

To confirm our findings on protein level and to address the question whether the observed changes were specific for hepatic T cells, we performed FACS analysis using antibodies against FOXP3, CTLA4 and T-cell immunoglobulin domain and mucin domain 3 (TIM3) and further included blood-derived T cells in our analysis. TIM3 is widely regarded as a negative regulator of effector T-cell function and exhaustion and additionally has

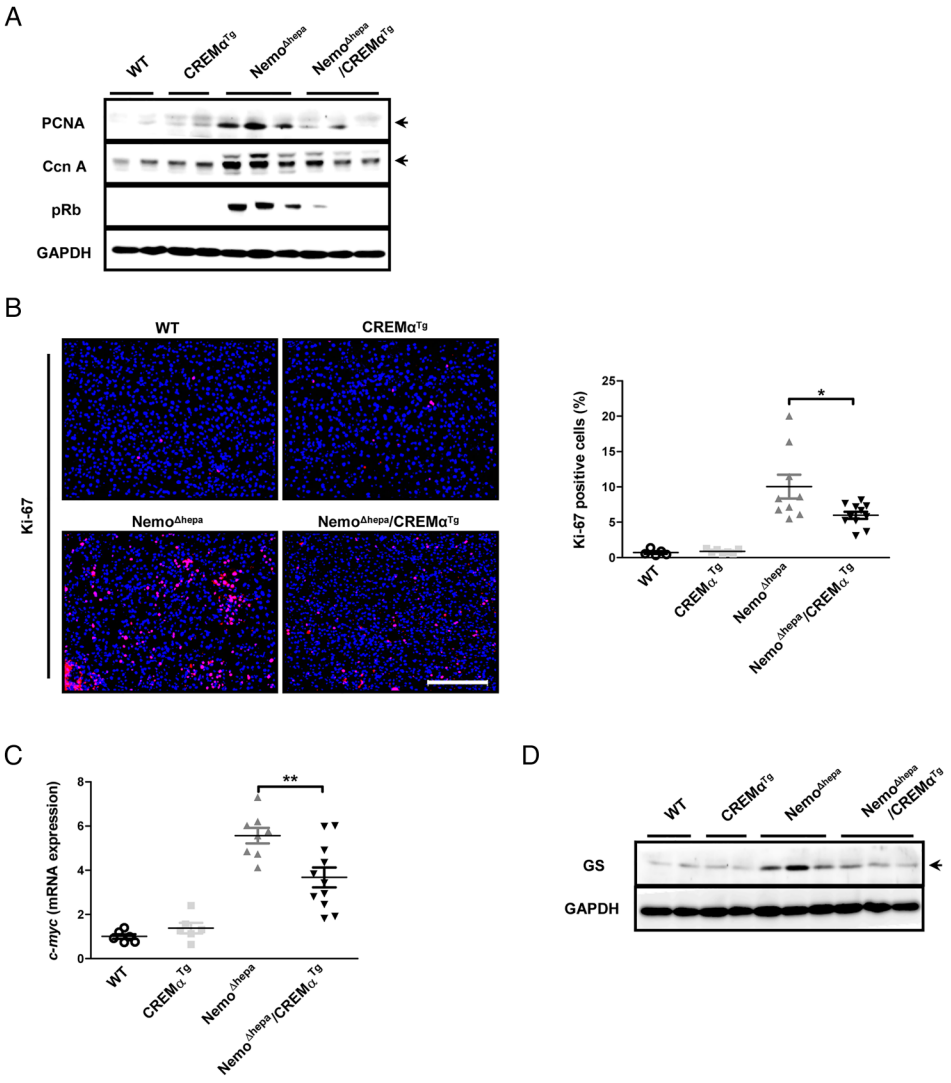

Figure 4 Overexpression of CREMα in T cells reduces tumour malignancy in NEMO$^{\Delta hepa}$ mice. (A) Western blot using liver protein for PCNA, Cyclin A, pRb and GAPDH. (B) Immunofluorescence staining for Ki-67 (red: Ki-67, blue: DAPI, scale bar: 200 μm) and quantification of Ki-67-positive cells (Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$/CREMα^{Tg}). Data are shown as mean±SEM of n=5–11 mice per group (*p<0.05). (C) mRNA expression for *c-myc* (Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$/CREMα^{Tg}). Data are shown as mean ±SEM of n=6–11 mice per group (**p<0.01). (D) Western blot from liver protein for glutamine synthetase and GAPDH. CREMα, cyclic AMP-responsive element modulator α; DAPI, 4′, 6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; WT, wild type.

been shown to enhance the suppressor function of Tregs.[19] Our data showed a general increase in Treg-related markers (FOXP3, CTLA4 and TIM3) in hepatic T cells of 8-week-old Nemo$^{\Delta hepa}$/CREMα^{Tg} mice compared with blood-derived T cells (see figure 6E, F and online supplementary figure S7). Moreover, the bioinformatics analysis of gene arrays revealed that T cells isolated from CREMα^{Tg} livers display upregulation of the IL-4 and IL-2 signalling pathways, involved in T-cell activation and differentiation into Th1, Th2 and Treg. CREMα-overexpressing T cells express high mRNA levels of LIGHT and CD40L associated with T-cell activation (see online supplementary figure S6A).

Interestingly, liver Nemo$^{\Delta hepa}$/CREMα^{Tg} T cells exhibit upregulation of the CX3CR/CX3CL1 signalling pathway, a protective pathway in liver injury,[20] TREM2, which attenuates TLR4-dependent inflammation, and macrophage receptor with collagenous structure (MARCO), important in DCs (see online supplementary figure S6A). Furthermore, CREMα^{Tg} and Nemo$^{\Delta hepa}$/CREMα^{Tg} T cells show differential regulation of cytokine production by IL-17A and IL-17F (see online supplementary figure S6B).

Altogether, our data suggest that the differentiation of T cells together with the activation of protective pathways such as the CX3CR/CX3CL1 confers protection against inflammation and tumourigenesis to Nemo$^{\Delta hepa}$/CREMα^{Tg} livers.

The inflammatory milieu in liver of Nemo$^{\Delta hepa}$mice induces FOXP3 expression in CREMα-expressing T cells

After we observed reduced Rorγt^{+} T cells in the liver, we tested the hypothesis that the inflammatory milieu in Nemo$^{\Delta hepa}$ livers might exert a direct effect on CREMα-expressing T cells. Based on previous publications[5] and to further corroborate the specificity of our results, we included splenic T cells in our experiment. Several studies indicate that activated HSC, especially during inflammation, function to enhance Treg differentiation.[21] In this scenario, an important soluble factor is RA; therefore, we analysed the mRNA expression of RA-related genes in WT and Nemo$^{\Delta hepa}$ liver tissue. Indeed, we found upregulation of genes involved in RA metabolism including aldehyde dehydrogenase 1 family, member A1 (*ALDH1A1*), lecithin retinol acetyltransferase 1 (*LRAT1*), cellular RA binding protein 1 (*CRABP1*) and retinoc acid receptor (*RAR*)-α and RAR-β (figure 7A). To investigate whether HSC-derived RA contributes to the increase in FOXP3 in CREMα^{Tg} mice, we isolated splenic T cells and cultured them with HSC SN and compared the differentiation pattern with T cells cultured under Th0 (without differentiation-inducing cytokines), Treg (TGF-β) or pronounced Treg (TGF-β+RA) conditions.

While CREMα^{Tg} T cells displayed a higher mean fluorescence intensity for FOXP3 under Th0 conditions—reflecting the

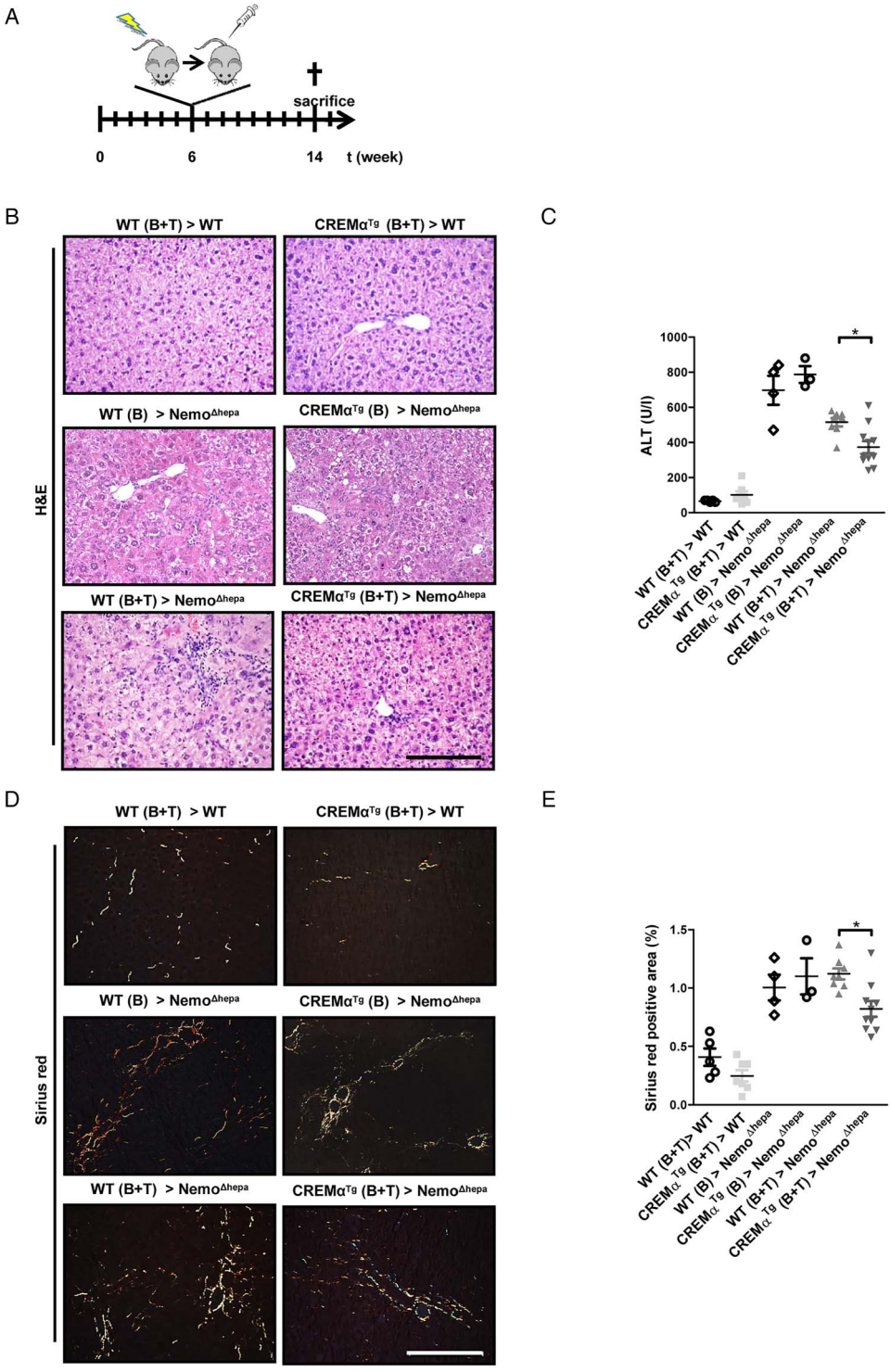

Figure 5 The simultaneous transfer of CREMαTg T cells and haematopoietic cells ameliorates liver injury in Nemo$^{\Delta hepa}$ mice. (A) Outline of the transplantation experiment. WT or Nemo$^{\Delta hepa}$ mice were either transplanted with WT or CREMα^{Tg} bone marrow cells (B) alone or in combination with T cells (B+T). (B) Microscopic picture of H&E staining (scale bar: 200 μm). (C) Serum alanine aminotransferase (ALT) levels (Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$/CREMα^{Tg}). Data are shown as mean±SEM of n=5–11 mice per group (*p<0.05). (D) Immunohistochemical staining for Sirius red (scale bar: 200 μm). (E) Quantification of Sirius red-positive area (WT>Nemo$^{\Delta hepa}$ vs CREMα>Nemo$^{\Delta hepa}$). Data are shown as mean±SEM of n=5–11 mice per group (*p<0.05). CREMα, cyclic AMP-responsive element modulator α; WT, wild type.

amount of the bound antibody—stimulation with TGF-β or with TGF-β+RA strongly induced FOXP3 expression in WT and, dramatically, in CREMα^{Tg} T cells. Interestingly, challenge with HSC (7d) SN led to an increase of FOXP3 in both WT and CREMα^{Tg} T cells in contrast to quiescent HSC (4d) SN (see figure 7B, C and online supplementary figure S8A, B).

To further evaluate the important role of RA in the TGF-β-dependent induction of FOXP3^{+} Treg and taking into account that the retinoic receptor (RAR)-α is involved in Treg induction,[22 23] we used RO415253, a specific inhibitor of RAR-α. RAR-α inhibition reduced the expression of FOXP3 almost to the level of T cells under Th0 conditions. We observed this effect in both WT and CREMα^{Tg} T cells, suggesting a crucial role of RA in Treg induction (see figure 7B, C and online supplementary figure S8A, B).

To determine whether increased amounts of Treg led to a reduction of other T-cell populations, we additionally assessed the production of signature cytokines IL-17 (Th17) and interferon γ (IFNγ) (Th1). While IL-17 levels showed no major differences under Th0 conditions, CREMα^{Tg} T cells exhibited increased levels of IL-17 upon treatment with TGF-β+IL-6. SN from quiescent and activated HSC reduced the expression of IL-17, in both WT and CREMα^{Tg} T cells (see online supplementary figure S9A, B). Regarding Th1 differentiation, CREMα^{Tg}

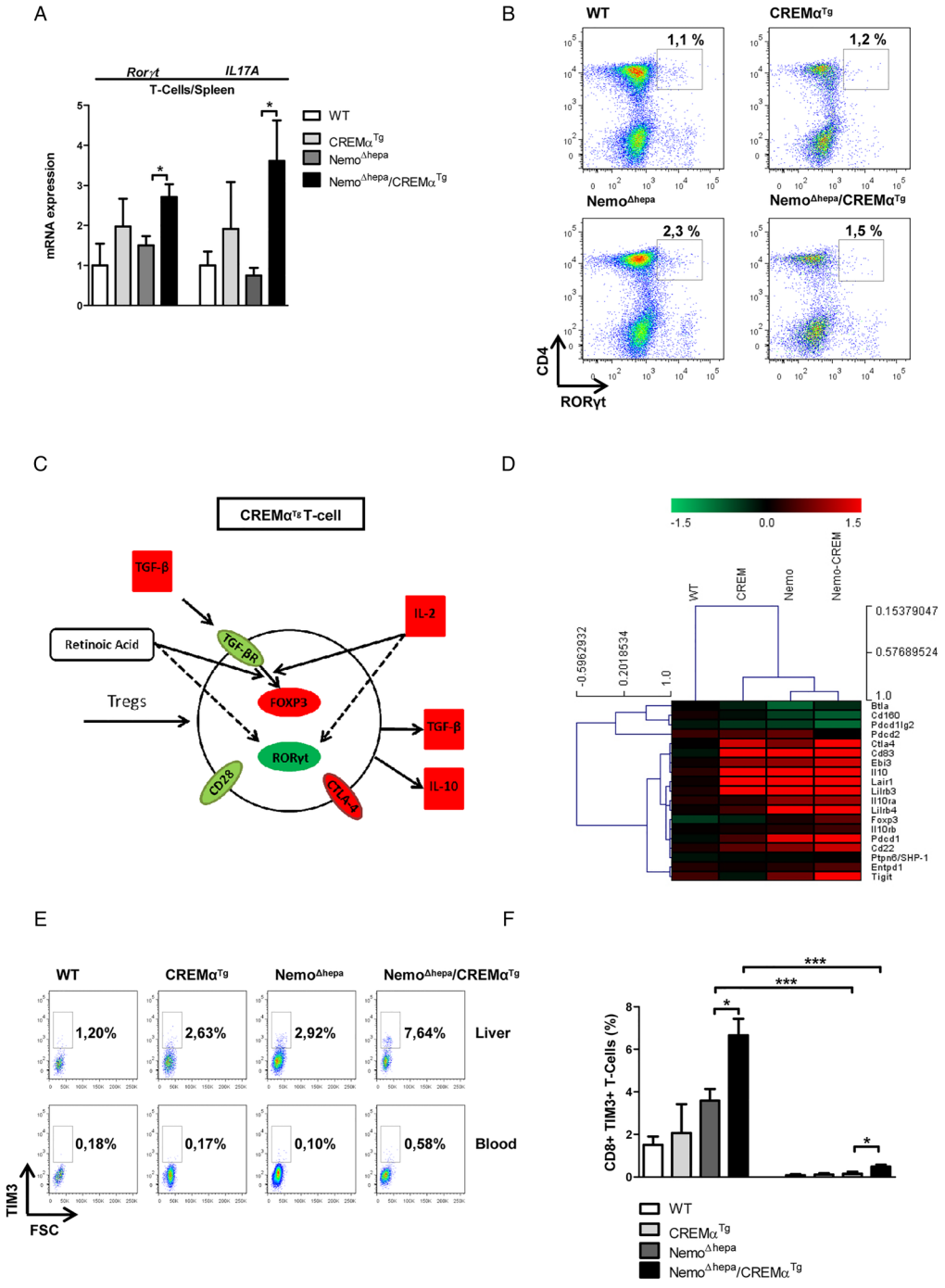

Figure 6 Overexpression of CREMα does not induce a predominant Th17 response in hepatic T cells. (A) qPCR for *Rorγt* and *IL-17A* in splenic T cells (WT vs CREMα^{Tg}, CREMα^{Tg} vs Nemo$^{\Delta hepa}$/CREMα^{Tg}, Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$/CREMα^{Tg}). Data are shown as mean±SEM of n=2–5 mice per group (*p<0.05, **p<0.01). (B) Representative FACS dot plots for RORγt in hepatic leukocytes. Percentages shown are for only this representative FACS plot. (C) Results from N-counter analysis for the differentiation of hepatic T cells into regulatory T cell. (D) Heat map for coinhibitory T-cell epitopes (red: upregulated; green: downregulated). (E) Representative FACS plots for TIM3 in hepatic leukocytes. Percentages shown are for only this representative FACS plot. (F) Percentage of CD8^{+} TIM3^{+} T cells (Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$/CREMα^{Tg}, Nemo$^{\Delta hepa}$ vs Nemo$^{\Delta hepa}$, Nemo$^{\Delta hepa}$/CREMα^{Tg} vs Nemo$^{\Delta hepa}$/CREMα^{Tg}). Data are shown as mean±SEM of n=8–15 mice per group (*p<0.05, ***p<0.001). CREMα, cyclic AMP-responsive element modulator α; FACS, fluorescence-activated cell sorting; RORγt, retinoid receptor-related orphan receptor gamma-t; TIM3, T-cell immunoglobulin domain and mucin domain 3; Treg, regulatory T cell; WT, wild type.

T cells displayed slightly higher basal IFNγ levels, which were strongly diminished in both WT and CREMα^{Tg} T cells upon treatment with TGF-β+IL-6. Alike to IL-17, treatment with SN from quiescent and activated HSC strongly reduced the levels of IFNγ (see online supplementary figure S9A, C). Therefore, our data suggest a specific increase of FOXP3 expression and thus Treg expansion in CREMα^{Tg} T cells stimulated with SN of activated HSC highly dependent on RA.

DISCUSSION

Nemo$^{\Delta hepa}$ mice are characterised by the development of CLD, encompassing spontaneous hepatocyte apoptosis, compensatory

proliferation, and finally leading to HCC development, a process where the strong inflammatory response plays a crucial role. In the current study, we aimed to investigate the impact of a stronger Th17 response, caused by overexpression of CREMα in T cells, on disease progression of Nemo$^{\Delta hepa}$-dependent CLD. In contrast to our working hypothesis, we found reduced liver injury, hepatic fibrogenesis and tumourigenesis. Our data suggest that alterations in the expression pattern of molecules related to inhibitory T-cell function are essential to provide protection against CLD in this model.

CREMα-expressing T cells are characterised by the presence of a Th17 phenotype. In vitro, these cells show enhanced expression of the lineage transcription factor RORγt and the marker cytokines IL-17, IL-21 and IL-22. The present work provides evidence that the Th17 phenotype is altered upon hepatic injury. In accordance with previous publications,[5] T cells isolated from spleens of CREMα-expressing mice showed increased mRNA expression of RORγt and IL-17. Unexpectedly, the expression of RORγt in hepatic T cells was significantly downregulated in Nemo$^{\Delta hepa}$/CREMα^{Tg} mice.

Th17 cells contribute to tissue damage by secreting cytokines such as IL-17 and IL-22 during liver fibrosis.[24 25] Th17 cells are not the only producers of IL-17, even though they represent the major source. Neutrophils, CD8$^+$ T cells and natural killer (NK) cells can also secrete IL-17.[26 27] Indeed, blockade of the IL-17 signalling has been shown to limit inflammation and subsequent hepatocyte damage in mice.[28] Inversely, increased IL-17 signalling exacerbates CCl_4 and bile duct ligation-induced liver fibrosis.[29] In contrast, regulatory T cells have been shown to suppress Th17-mediated inflammation through IL-10 signalling and STAT3.[30 31] Consequently, several studies have demonstrated that Tregs limit fibrosis development by suppressing excessive immune responses in the liver.[32 33] Thus, the increased frequency of Tregs in Nemo$^{\Delta hepa}$/CREMα^{Tg} mice might be responsible for the reduced number of Th17 cells.

The expression of CREMα under control of the CD2 promoter in mice has been shown to reduce the number and alter the composition of immune cells in the liver.[17] Previous work in our laboratory[34] has shown the beneficial effects of depleting NK and NKT cells in Nemo$^{\Delta hepa}$ mice. In the same line of results, Nemo$^{\Delta hepa}$/CREMα^{Tg} animals display reduced amounts of NKT cells, and of proinflammatory macrophages, granulocytes, CD11b$^+$ DCs and CD8$^+$ T cells, suggesting an overall reduced inflammatory response. These results are reflected by the experiments found in 8-week-old Nemo$^{\Delta hepa}$/CREMα^{Tg} mice in comparison to Nemo$^{\Delta hepa}$ animals, showing a less severe phenotype with decreased transaminase levels.

In fact, BMDCs are responsible for the ameliorated liver injury and fibrogenesis in Nemo$^{\Delta hepa}$/CREMα^{Tg} mice. To functionally investigate our phenotype, we performed bone marrow transplantation in combination with an adoptive transfer of splenic T cells to boost the T-cell response. Nemo$^{\Delta hepa}$ mice reconstituted with CREMα BMDCs displayed a significant reduction in markers of liver injury and hepatic fibrogenesis, indicating that haematopoietic cells provide the CREMα-protective effect. It is tempting to speculate that this effect might be due to the induction of Tregs, potent inhibitors of inflammation.[35] However, more experiments need to be performed in order to address the definite role of CREMα in BMDCs, since in the current study, we focused on the role of the overexpression of CREMα in T cells.

Regulatory T cells represent a CD4$^+$ population, which regulates immune responses. Treg can be either generated in the thymus (nTregs) or alternatively in the periphery (iTregs) (eg, the liver).[35] Importantly, Th17 cell and Treg development is a reciprocal process. Thus, the differentiation process of Th17 cells inhibits Treg differentiation and vice versa.[27 30 31 36 37] Indeed, we found increased mRNA and protein levels of lineage transcription factors such as FOXP3 in Nemo$^{\Delta hepa}$/CREMα^{Tg}. Moreover, our data show increased CTLA4 mRNA and protein expression, a molecule that has been linked with Treg function.[38] However, the induction of Treg-associated markers was characteristic for hepatic T cells but not blood-derived T cells. Interestingly, we were able to reproduce our findings in splenic T cells that were challenged with liver-derived soluble mediators. These intriguing results raised the question whether the inflammatory milieu in Nemo$^{\Delta hepa}$ livers exerted a regulatory effect on T cells expressing CREMα.

Several studies have indicated that inflammation-activated HSC enhances Treg differentiation through transdifferentiation into myofibroblasts. Concomitantly, our further functional analysis demonstrates that CREMα transgene expression increases the susceptibility of T cells for differentiation into Treg—during hepatic inflammation and HSC activation as shown by increased levels of FOXP3 *in vivo* and *in vitro*. Hence, the liver-specific inflammatory milieu has an impact in the status of T-cell differentiation.

As the disease progresses from hepatitis to HCC in NEMO$^{\Delta hepa}$ mice, T cells, both CD4$^+$ and CD8$^+$, decrease in numbers with attenuated function and increased expression of inhibitory receptors during HCC. Increased frequencies of Treg, associated with tumour growth, poor prognosis and decreased survival rates, have been observed in patients with gastric and liver cancer.[39–41] Several findings in our model suggested that the lack of a predominant Th17 phenotype of hepatic T cells in Nemo$^{\Delta hepa}$/CREMα^{Tg} mice attenuates tumour development in these mice. First, 1-year-old Nemo$^{\Delta hepa}$/CREMα^{Tg} animals display reduced tumourigenesis. Second, we show *in culture* that CREMα^{Tg} T cells primed with Treg conditions attenuate Nemo$^{\Delta hepa}$-derived hepatocyte damage. Interestingly, with the colony formation assay in vitro we showed that T cells alone do not exert an inhibitory effect on tumour development in Nemo$^{\Delta hepa}$/CREMα^{Tg} livers. Overall, our results suggest that reduced HCC development in vivo is a multifactorial process that requires the interaction between several cell types, thus resulting in a reduced inflammatory environment. Hence, the direct effect of T cells in vivo in this setting might be less crucial than the influence of a variety of factors including growth factors, cytokines and the interplay between T cells and other cell types that contribute to reduced carcinogenesis in Nemo$^{\Delta hepa}$/CREMα^{Tg} livers.

To further dissect the crosstalk between CREMα-overexpressing T cells and Nemo$^{\Delta hepa}$ mice, we evaluated the mechanisms by which soluble mediators are inducing the protective phenotype observed in Nemo$^{\Delta hepa}$/CREMα^{Tg} mice. Through our *in vitro* studies we demonstrate that RA-derived activated HSC induces Tregs in CREMαTg T cells, thereby contributing to reduced inflammatory milieu and, consequently, decreased HCC in Nemo$^{\Delta hepa}$/CREMα^{Tg} livers.

In summary, the effect of the CREMα transgene alters the ratio between Th17 and regulatory T cells, exerting an anti-inflammatory and antitumourigenic effect in Nemo$^{\Delta hepa}$ livers. Thus, our present study shows that molecular defined immunosuppression is able to modulate inflammation-induced cancers and thus opens the gate to develop regulatory T-cell-based therapies for CLD patients.

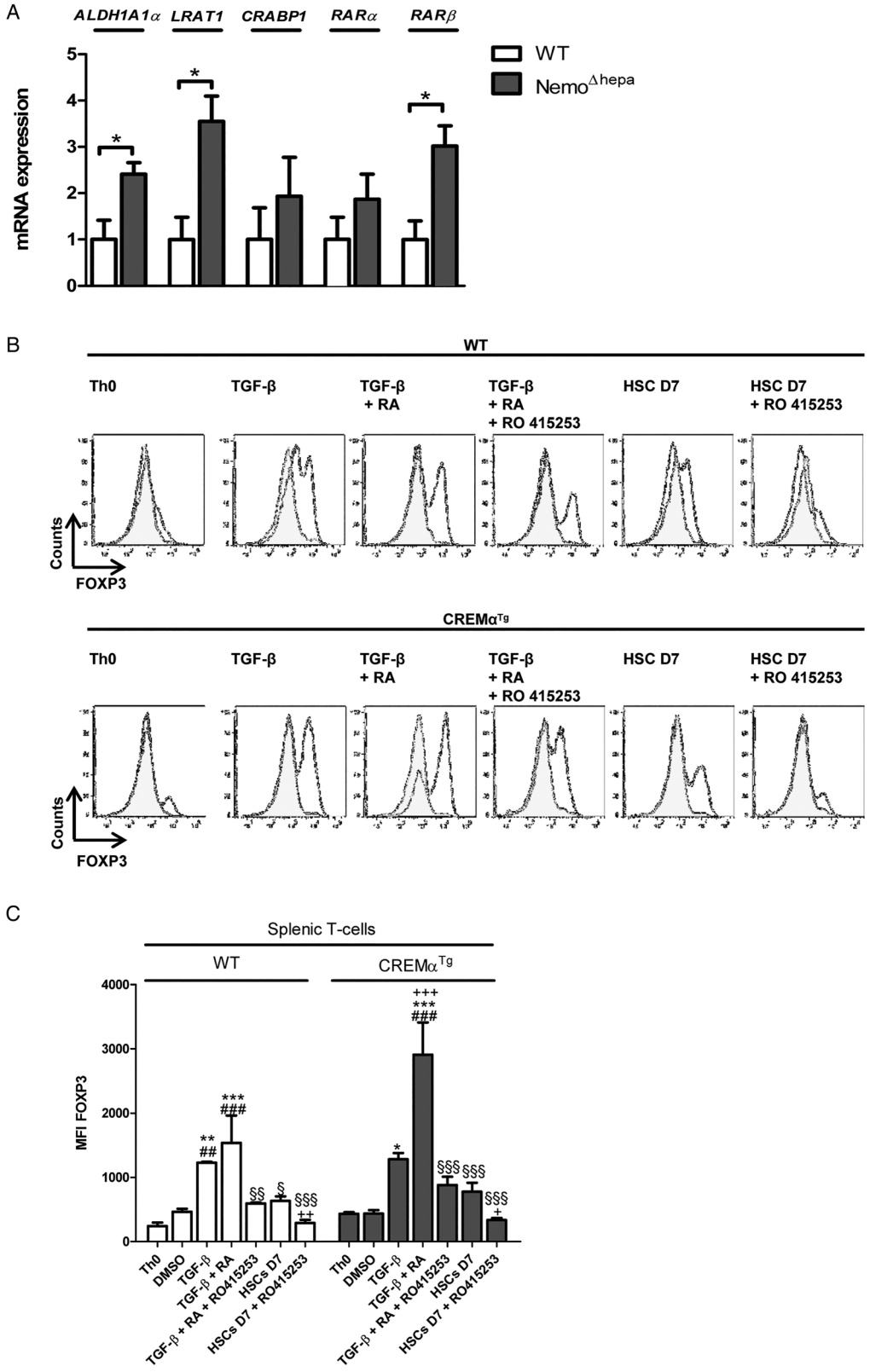

Figure 7 The inflammatory milieu in Nemo$^{\Delta hepa}$ livers induces FOXP3 expression in CREMα-overexpressing T cells. (A) mRNA expression for *ALDH1A1, RARα, RARβ, LRAT1 and CRABP1* (WT vs Nemo$^{\Delta hepa)}$. Data are shown as mean±SEM of n=4–10 mice per group (*p<0.05). Splenic CD4$^+$ T cells were isolated and clonally expanded for 4 days using antibodies against CD3 and CD28 before staining. Additionally, cells were treated with medium (Th0), TGF-β (10 ng/mL), TGF-β (10 ng/mL)+RA (10 nM RA), TGF-β (10 ng/mL)+RA (10 nM)+RO 415253 (1 μM), supernatant of HCS and/or RO 415253 (1 μM). (B) Representative fluorescence-activated cell sorting histogram plots for FOXP3 expression. Filled histograms represent isotype control, and open histograms represent FoxP3 staining. (C) Histograms representing the mean fluorescence intensity of FOXP3. Data shown are the means from one representative experiment, which was repeated twice (##p<0.01, ###p<0.001 compared with Th0; *p<0.05, **p<0.01, ***p<0.001 compared with dimethyl sulfoxide (DMSO); +p<0.05, ++p<0.01 compared with TGF-β; §§ p<0.01, §§§p<0.001 compared with TGF-β +RA). CREMα, cyclic AMP-responsive element modulator α; HSC, hepatic stellate cell; RA, retinoic acid; WT, wild type.

Acknowledgements The authors thank Jörg Bangen for providing SN of activated HSC as well as Christian Liedtke for critically reading the manuscript. Furthermore, we would like to extend our gratitude to Linda Hammerich and Daniela Kroy for helpful advice concerning FACS experiments. FJC is a Ramón y Cajal Researcher (RYC-2014-15242).

Contributors NK and AM: Experimental design, data acquisition, analysis and interpretation, drafting of the manuscript; KO, GH and TL: Data acquisition; KT: Experimental design; FJC: Data acquisition, analysis, study supervision, critical revision of the manuscript; drafting of the manuscript; CT: Experimental design, data interpretation, study supervision, drafting and critical revision of the manuscript.

Funding This work was supported by the IZKF (UKA, RWTH Aachen), the SFB \TRR57 and the START-Program of the Faculty of Medicine (#691405, RWTH Aachen) DFG grant (TR 285/10-1).

Competing interests None declared.

Provenance and peer review Not commissioned; externally peer reviewed.

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