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MutT Homolog 1 Inhibitor Karonudib Attenuates Autoimmune Hepatitis by Inhibiting DNA Repair in Activated T Cells

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Autoimmune hepatitis (AIH) is an inflammatory liver disease driven by the hyperactivation of various intrahepatic antigen-specific T cells due to a breach of immune tolerance. Studies in immunometabolism demonstrate that activated T cells harbor increased levels of reactive oxygen species that cause oxidative DNA damage. In this study, we assessed the potential of DNA damage repair enzyme MutT homolog 1 (MTH1) as a therapeutic target in AIH and karonudib as a novel drug for patients with AIH. We report herein that MTH1 expression was significantly increased in liver samples from patients with AIH compared to patients with chronic hepatitis B and nonalcoholic fatty liver disease and from healthy controls. In addition, the expression of MTH1 was positively correlated with AIH disease severity. We further found abundant T cells that expressed MTH1 in AIH. Next, we found that karonudib significantly altered T-cell receptor signaling in human T cells and robustly inhibited proliferation of human T cells *in vitro*. Interestingly, our data reflected a preferential inhibition of DNA damage repair in activated T cells by karonudib. Moreover, MTH1 was required to develop liver inflammation and damage because specific deletion of MTH1 in T cells ameliorated liver injury in the concanavalin A (Con A)-induced hepatitis model by inhibiting T-cell activation and proliferation. Lastly, we validated the protective effect of karonudib on the Con A-induced hepatitis model. *Conclusion:* MTH1 functions as a critical regulator in the development of AIH, and its inhibition in activated T cells reduces liver inflammation and damage. (*Hepatology Communications* 2021;0:1-16).

Autoimmune hepatitis (AIH) is an inflammatory liver disease characterized by elevated levels of serum transaminase and immunoglobulin G, the presence of autoantibodies (such as antinuclear antibodies), the existence of interface hepatitis, and portal plasma cell infiltration in liver histology.[1] Strong evidence suggests that AIH is driven by the expansion of various antigen-specific T cells due to a breach of immune tolerance.[2] The mainstay for AIH treatment is the recommendation

Abbreviations: 8-oxoG, 8-oxo-7,8-dihydroguanine; AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; CD, clusters of differentiation; CDK2, cyclin-dependent kinase 2; CHB, chronic hepatitis B; Con A, concanavalin A; dGTP, deoxyguanosine triphosphate; GGT, gamma-glutamyltransferase; HC, healthy control; IFN-γ, interferon-gamma; IHC, immunohistochemistry; IL, interleukin; KO, knockout; MTH1, MutT homolog 1; NAFLD, nonalcoholic fatty liver disease; ns, no significance; OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly(adenosine diphosphate ribose) polymerase; PBS, phosphate-buffered saline; Th, T helper; TNF-α, tumor necrosis factor alpha; Treg, T regulatory; WT, wild type; γ-H2AX, phosphorylated histone H2AX.

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of predniso(lo)ne to induce remission and the combination of predniso(lo)ne and azathioprine to maintain therapeutic efficacy.[3] Although different medicines have been developed to treat AIH, adverse effects are a limitation, e.g., predniso(lo)ne causes osteoporosis[4] and exacerbates diabetes[5] and azathioprine brings hematologic abnormalities, such as leukopenia[6] and myelodysplastic syndromes.[7] In addition, many patients do not respond to these medicines.[8] Therefore, it is imperative to develop new and more effective immunosuppressive drugs.

T cells are the critical immune cells that fight against pathogens, protecting the immune homeostasis in physiologic conditions. However, the aberrantly activated T cells could secret robust proinflammatory cytokines, causing injury to normal tissues and leading to inflammatory or autoimmune diseases. To facilitate the full-effector status, these immune cells alter their metabolic pattern that accompanies the overwhelming amounts of reactive oxygen species (ROS) production.[9] Existing studies consider DNA damage as one of the major outcomes of ROS overproduction.[10,11] The nucleotides present in the nucleotide pool are more susceptible to oxidative damage than those in the DNA strands.[12] Among them, guanine has the lowest redox potential and the deoxyguanosine triphosphate (dGTP) pool is the most vulnerable target for oxidation, leading to the formation of 8-oxo-7,8-dihydroguanine (8-oxoG).[13]

MutT homolog 1 (MTH1) is an enzyme belonging to the nucleotide diphosphate X phosphohydrolase family. The main role of MTH1 is to hydrolyze 8-oxo-dGTP to 8-oxo-deoxyguanosine monophosphate (dGMP), which prevents the misincorporation of the former into DNA.[14,15] Previous studies showed that MTH1 expression was elevated in tumor cells and was correlated with prognosis and survival.[16,17] Studies have shown that a prerequisite to successful elimination of cancer using MTH1 inhibitor is the misincorporation of oxidized nucleotides into DNA.[18,19] Currently, a clinical trial is underway to verify whether the MTH1 inhibitor karonudib is effective in the treatment of cancer in humans (NCT03036228).

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TABLE 1. CHARACTERISTICS OF PATIENTS WITH AIH, CHB, OR NAFLD AND HCS.

	AIH (n = 40)	CHB (n = 19)	NAFLD (n = 24)	HC (n = 8)
Age (years)	51.80 ± 1.626	39.95 ± 2.087	45.75 ± 2.484	30.63 ± 1.908
Sex (F/M)	37/3	6/13	14/10	4/4
ALT (U/L)	141.2 ± 21.30	57.22 ± 30.46	94.13 ± 17.07	21.63 ± 2.521
AST (U/L)	142.7 ± 24.44	38.46 ± 11.72	52.63 ± 6.980	18.75 ± 1.319
ALP (U/L)	115.1 ± 14.02	72.42 ± 4.841	98.50 ± 8.458	83.25 ± 6.038
GGT (U/L)	112.3 ± 19.90	25.66 ± 4.938	154.0 ± 64.65	18.38 ± 2.322
TBIL (μmol/L)	21.29 ± 2.614	14.25 ± 1.768	16.14 ± 2.276	10.10 ± 1.196
IgG (g/L)	15.70 ± 0.6543	12.48 ± 1.156	13.87 ± 0.9054	NA

Continuous data are shown as mean ± SEM.
Abbreviations: F/M, female/male; IgG, immunoglobulin; NA, not applicable; TBIL, total bilirubin.

Given that hyperactive T cells and cancer cells are both rapid proliferating cells and have a similar metabolic pattern, we explored the potential role of MTH1 in the pathogenesis of AIH. We first investigated expression levels of MTH1 in T cells of liver tissues of patients with AIH. Next, we assessed the effects of karonudib on human T cells *in vitro*. Lastly, we examined the role of MTH1 on concanavalin A (Con A)-induced liver injury using mice with the specific deletion of MTH1 in T cells and the novel MTH1 inhibitor karonudib *in vivo*.

Materials and Methods

STUDY SUBJECTS AND LIVER SAMPLES

Liver samples at diagnosis (n = 91) were collected from 40, 19, and 24 patients with AIH, hepatitis B virus, and nonalcoholic fatty liver disease (NAFLD), respectively. We also included 8 healthy controls (HCs). Notably, the patients who were diagnosed with AIH were confirmed using the simplified scoring system for AIH that was proposed by the International Autoimmune Hepatitis Group in 2008.[20] The patients diagnosed with chronic hepatitis B (CHB) and NAFLD met the standard criteria of CHB and NAFLD.[21,22] For the immunohistochemistry (IHC) study, liver samples from patients with AIH, CHB, and NAFLD were collected from ultrasound-guided needle liver biopsies; the eight HC liver samples were derived from explant donors before liver transplantation. Clinical characteristics of the 91 subjects are noted in Table 1. All participants were enrolled in Shanghai Renji Hospital and provided written informed consent. The study was carried out under the principles of the declaration of Helsinki and approved by the ethics committees of Renji Hospital.

MICE

Female C57BL/6J mice were procured from the Shanghai Laboratory Animal Center. MTH1 loxP mice were bred by GemPharmatech Co., Ltd. The clusters of differentiation (CD)4 cre mice were kindly provided by Professor Nan Shen (Renji Hospital). All mice used in this study were 6-8 weeks old and were contained in specific pathogen-free conditions in the animal facility of Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China. All animal experiments were carried out following recommendations from the Guide for the Care and Use of Laboratory Animals, Ethics Committee of Renji Hospital.

IHC STAINING

Formalin-fixed, paraffin-embedded tissue sections from liver biopsies were verified using IHC and confocal laser scanning microscopy experiments, as described.[23] Briefly, after antigen retrieval, liver samples were incubated with goat serum for 30 minutes before they were incubated with primary antibody MTH1 (ab200832; Abcam) at 4°C overnight. After three washes with phosphate-buffered saline (PBS), the slides were incubated with a horseradish peroxidase-conjugated secondary antibody for IHC at room temperature for 30 minutes. Liver sections were blindly evaluated by two pathologists. The expression of MTH1 was scored on a 0-4-point scale per high-power field. Cases were scored

as follows: 1 if expression area <25%, 2 if ≥25%-50%, 3 if ≥50%-75%, and 4 if ≥75%.

CONFOCAL STAINING

Confocal laser scanning microscopy was used for the detection of costaining markers, as described.[24] Briefly, after antigen retrieval, liver samples were incubated with donkey serum for 30 minutes before being incubated with primary antibodies MTH1 (ab200832; Abcam), CD3 (60181-1-Ig; Proteintech), CD4 (ab67001; Abcam), and CD8 (ab17147; Abcam) at 4°C overnight. After three washes with PBS, the slides were incubated with fluorochrome-conjugated secondary antibodies (1:200; Invitrogen) at room temperature for 30 minutes. Consequently, the nucleus was stained using 4′,6-diamidino-2-phenylindole (Southern Biotech, Birmingham, AL). Histologic immunofluorescence was determined using an LSM 710 laser scanning confocal microscope (Carl Zeiss, Jena, Germany).

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism 6 software. All values were expressed as mean ± SEM. Statistical differences for normally distributed data were analyzed by the Student *t* test. Correlations were determined by Spearman's rank correlation coefficient for nonparametric data or Pearson's correlation coefficient for normally distributed data. In all tests, $P < 0.05$ was considered statistically significant. Details on other materials and methods are provided in the Supporting Materials.

Results

INCREASED EXPRESSION OF MTH1 IN LIVERS OF PATIENTS WITH AIH

The hypothesis is that MTH1 could be needed for activated T cells to detoxify the deoxyribonucleoside triphosphate (dNTP) pools to maintain genome integrity during proliferation. Therefore, we used IHC to examine MTH1 protein expression in the liver from patients with AIH, CHB, or NAFLD and from HCs. We found that MTH1 was predominantly located in immune cells whereas the hepatocytes barely expressed MTH1 in the three diseases or HCs. Moreover, there was a significantly increased abundance of MTH1-positive cells in AIH compared to either HCs ($P < 0.01$) or CHB and NAFLD (both $P < 0.0001$), as illustrated in Fig. 1A,B. Notably, MTH1 expression was positively correlated with degrees of inflammation ($r = 0.6989$, $P < 0.0001$) other than the different stages of fibrosis ($r = 0.3119$, $P = 0.0725$) in AIH, as shown in Fig. 1C. The expression of MTH1 was positively correlated with serum levels of alanine aminotransferase (ALT) ($r = 0.6467$, $P < 0.0001$), aspartate aminotransferase (AST) ($r = 0.3534$, $P = 0.0404$), alkaline phosphatase (ALP) ($r = 0.4657$, $P = 0.0063$), and gamma-glutamyltransferase (GGT) ($r = 0.52$, $P = 0.0016$) (Fig. 1D,E). In summary, the above results suggest that MTH1 is highly expressed in AIH and positively correlated with disease severity.

INCREASED EXPRESSION OF MTH1 IN HEPATIC T CELLS OF PATIENTS WITH AIH

To identify the cellular source of MTH1 in patients with AIH, we conducted an immunofluorescence double-staining assay for MTH1 and CD3, CD4, and CD8 and found there were plentiful T cells that were colocalized with MTH1 in AIH (Fig. 2A; Supporting Fig. S1A,B). Similarly, the number of MTH1+CD3+ T cells was positively correlated with the degree of hepatic inflammation ($r = 0.8115$, $P < 0.0001$) but not with the different stages of fibrosis ($r = 0.1160$, $P = 0.5980$) in patients with AIH (Fig. 2B). Additionally, positive correlations were found between numbers of MTH1+CD3+ T cells and the serum levels of ALT ($r = 0.7008$, $P = 0.0002$), AST ($r = 0.6862$, $P = 0.0003$), ALP ($r = 0.7128$, $P = 0.0001$), and GGT ($r = 0.6853$, $P = 0.0004$) (Fig. 2C,D). In summary, the above results suggest that MTH1 is highly expressed in T cells and the numbers of MTH1+CD3+ T cells are related to the disease severity of AIH.

EFFECT OF MTH1 ON ACTIVATION AND FUNCTION OF HUMAN T CELLS *IN VITRO*

To assess the expression of MTH1 on T cells, CD3+ T cells isolated from healthy human volunteers were first treated with or without anti-CD3/

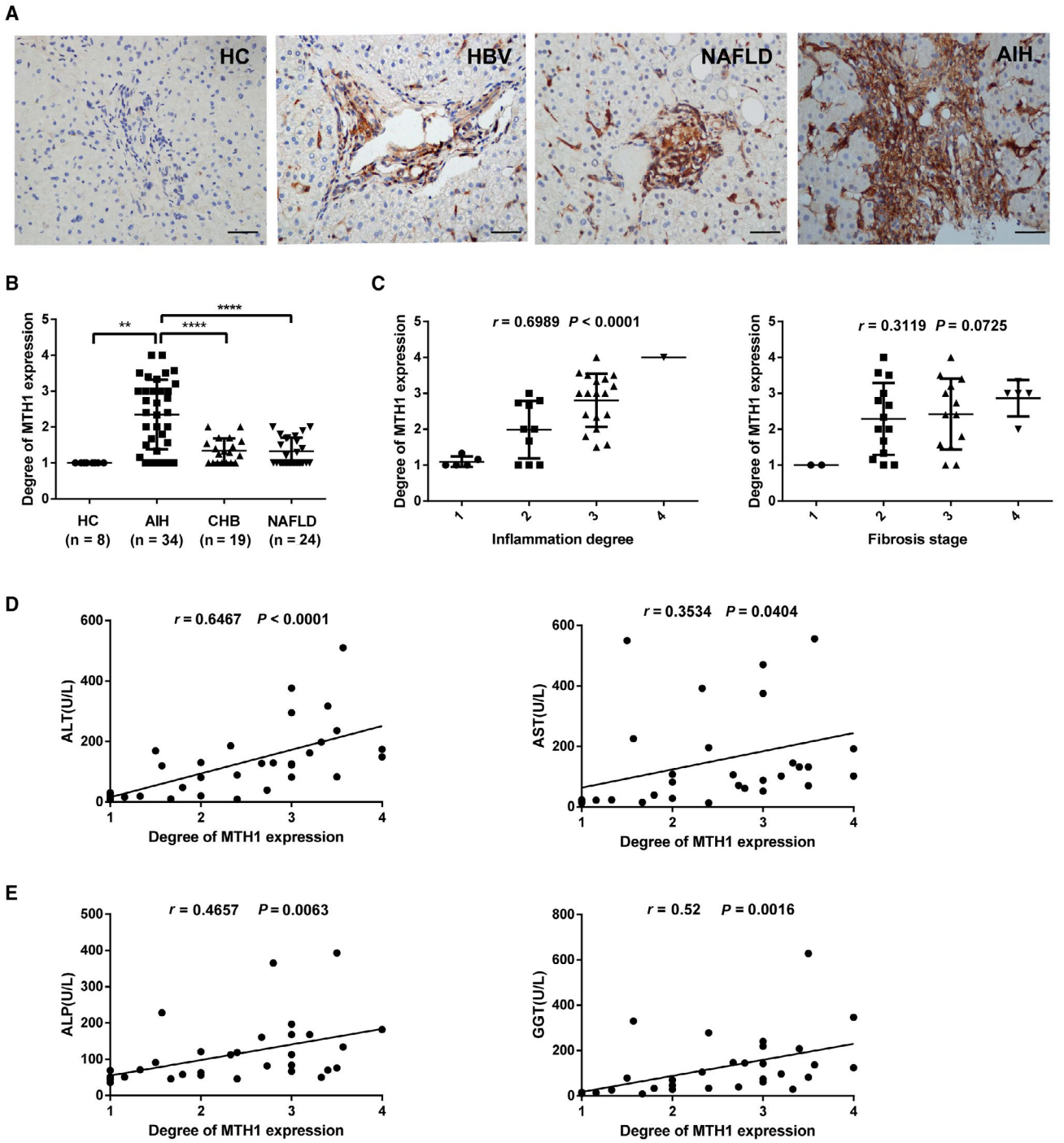

FIG. 1. Increased expression of MTH1 in livers of patients with AIH. (A,B) Representative IHC staining (magnification ×400) and statistical analysis of hepatic MTH1 expression in HCs (n = 8), HBV (n = 19), NAFLD (n = 24), and AIH (n = 34). Scale bar, 50 μm. (C) Degree of hepatic MTH1+ cells was positively correlated to the degree of hepatic inflammation but showed no difference among advanced fibrosis stages. (D) Degree of hepatic MTH1+ cells in portal areas was positively correlated with serum disease activity biomarkers ALT and AST in patients with AIH. (E) There was a positive correlation of the degree of hepatic MTH1+ cells with levels of serum ALP and GGT. Bars reflect the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Abbreviation: HBV, hepatitis B virus.

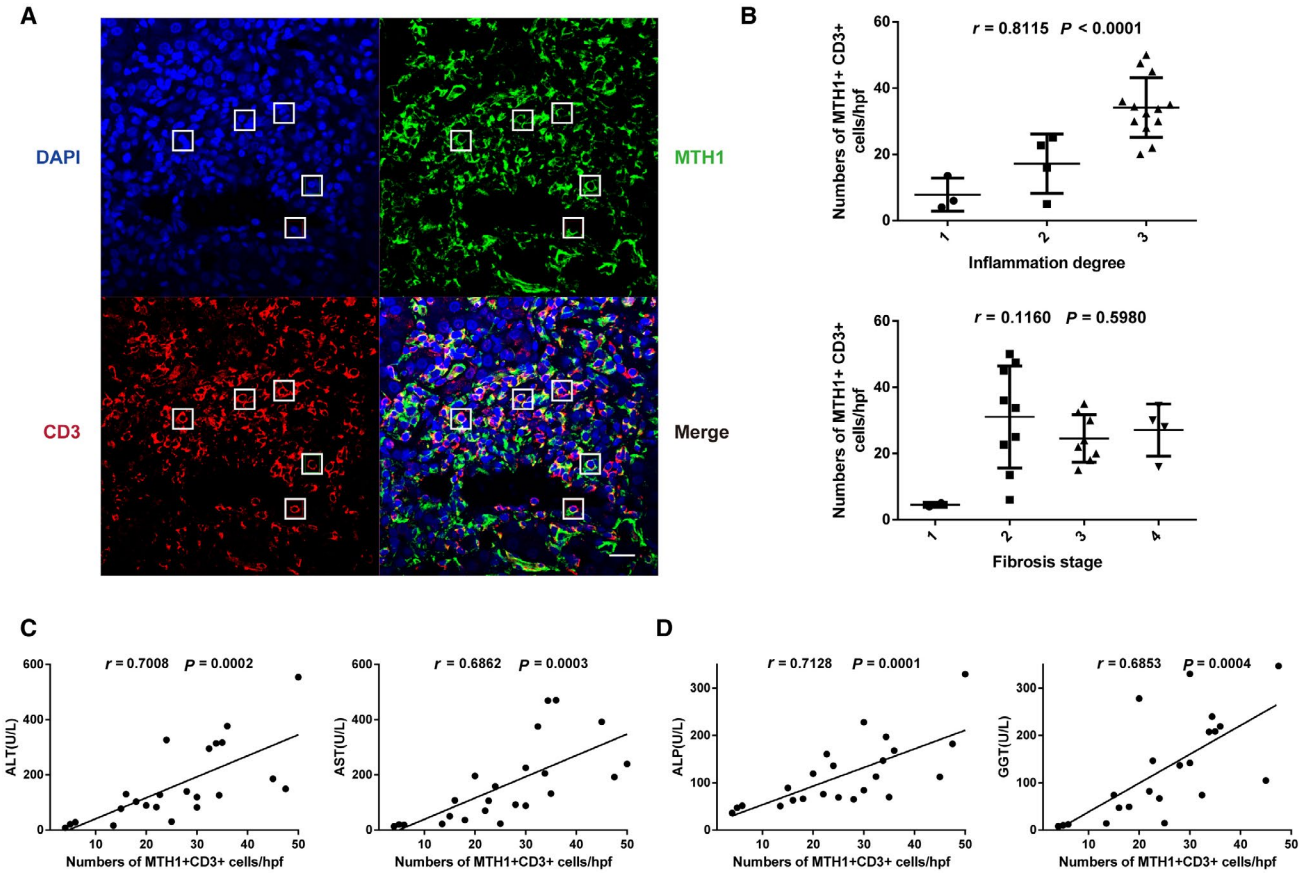

FIG. 2. Hepatic MTH1+CD3+ T cells correlated with disease activity in AIH. (A) Representative confocal staining of CD3 (red), MTH1 (green), and DAPI (for nuclei in blue) (magnification ×400) in the liver of patients with AIH. Scale bar, 20 μm. (B) Number of MTH1+CD3+ T cells in portal areas was positively correlated with degree of hepatic inflammation but showed no clear link with fibrosis stages in AIH. (C) Number of MTH1+CD3+ T cells in portal areas had a significant positive correlation with levels of serum ALT and AST in patients with AIH. (D) Number of MTH1+CD3+ T cells in portal areas was positively correlated with levels of serum ALP and GGT. Bars reflect the mean ± SEM. Abbreviations: DAPI, 4 0, 6-diamidino-2-phenylindole; hpf, high-power field.

CD28 beads for 72 hours. We found that activated T cells had an increased expression of MTH1 compared to resting T cells (Fig. 3A). To fully elucidate the impact of MTH1 on T cells, we stimulated CD3+ T cells from healthy human volunteers with anti-CD3/CD28 beads for 72 hours with or without karonudib. Compared to the vehicle controls, the percentage of activated T cells (characterized by CD25+ or CD69+) was significantly decreased by karonudib treatment (Fig. 3B,C). We further tested the effect of karonudib on T-cell subsets. We found that karonudib had an evident inhibitory effect on proinflammatory cells, such as T helper 1 (Th1) cells, CD4+ T cells expressing tumor necrosis factor alpha (TNF-α), and CD8+ T cells expressing interferon-gamma (IFN-γ) and TNF-α (Supporting Fig. S2A,B). However, karonudib had no inhibitory effect on T regulatory (Treg) cells and Th17 cells (Supporting Fig. S2A). Another interesting finding is that karonudib did not change the number of resting T cells. However, the number of activated T cells decreased significantly when treated with karonudib (Fig. 3D). Therefore, we further conducted cell-trace proliferation assays to examine whether karonudib had an antiproliferative property. Intriguingly, the administration of karonudib robustly inhibited both CD4 and CD8 T-cell proliferation derived from both HCs and patients with AIH who were treatment naive (Fig. 3E). We also observed

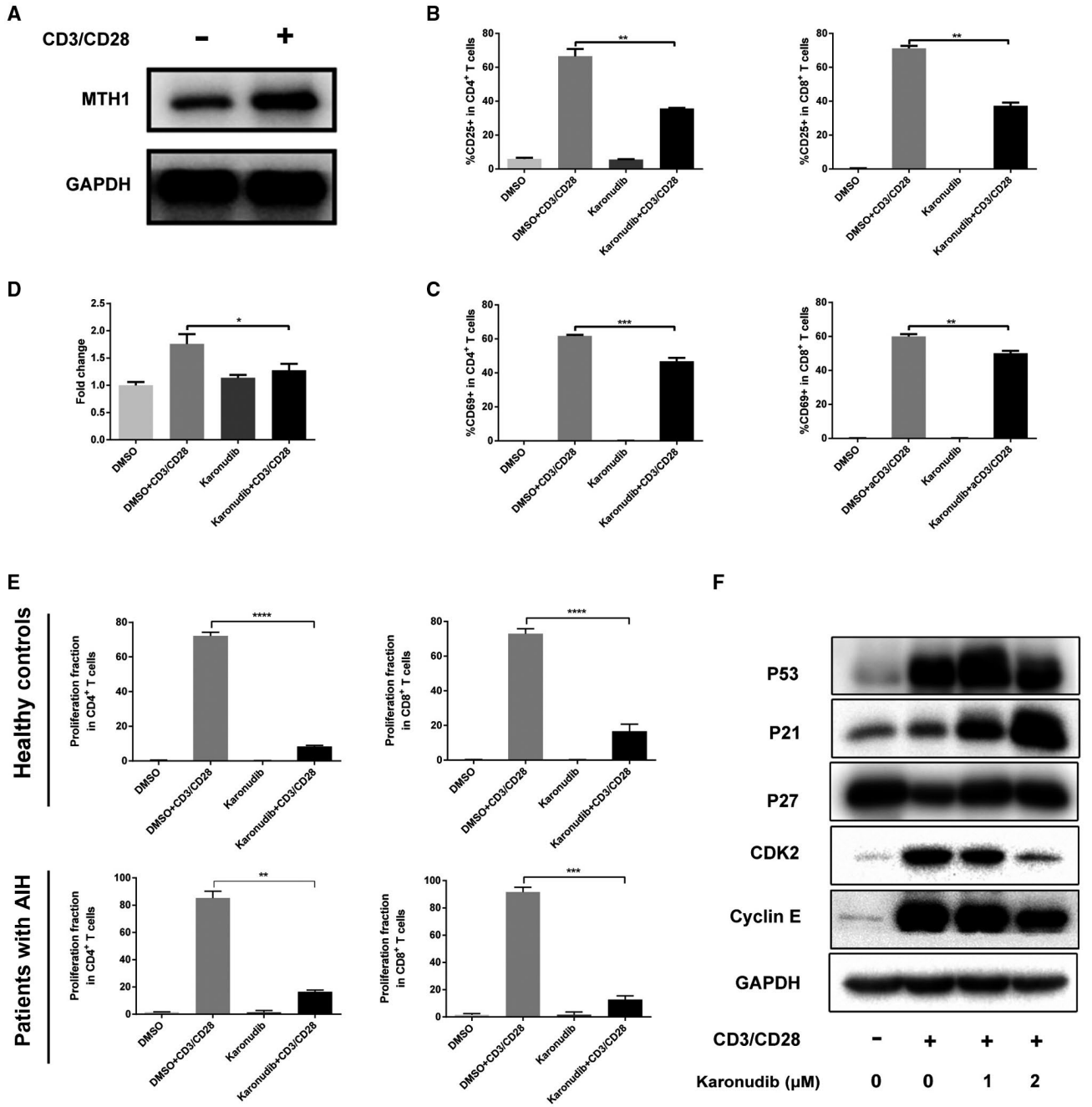

FIG. 3. Karonudib significantly inhibited T-cell proliferation in human T cells *in vitro*. Isolated human CD3+ T cells were cultured with/without anti-CD3/CD28 beads for 72 hours. Representative western blot analyses of MTH1 72 hours after anti-CD3/CD28 beads stimulation. (B,C) Isolated T cells were activated with anti-CD3/CD28 beads with or without 2 μM karonudib for 72 hours. The percentage of CD25+ and CD69+ T cells was determined on day 3 by flow cytometry. (D) Analysis of T-cell number treated with karonudib for 72 hours after anti-CD3/CD28 beads stimulation. (E) Statistical analysis of the T-cell proliferation assay treated with/without 2 μM karonudib for 72 hours from HCs and patients with AIH who were treatment naive. (F) Representative western blot analyses of P53, P21, P27, CDK2, and cyclin E 72 hours after anti-CD3/CD28 beads stimulation. The GAPDH blot was used as a loading control. Data are from one experimental representative of at least three independent experiments and represent triplicate wells. Bars reflect the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Abbreviations: DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

that the expression of proteins that facilitated the cell cycle, such as cyclin E and cyclin-dependent kinase 2 (CDK2), were inhibited, whereas the expression of proteins, such as P53, P21, and P27, that limited the speed of the cell cycle were significantly up-regulated (Fig. 3F).

Studies showed that activation of protein kinase B (AKT), nuclear factor kappa B (NF-κB), and extracellular signal-regulated kinase (ERK) pathways are closely associated with T-cell activation and proliferation.[25-27] Our study showed that the phosphorylated proteins of AKT and P65 were strongly inhibited by karonudib (Supporting Fig. S3A,B), while the expression of the phosphorylated proteins of ERK pathways remained unchanged (Supporting Fig. S3C). Because Th1 cells play an important role in AIH, we next sought to define whether the MTH1 inhibitor can modulate the differentiation of Th1 in an *in vitro* setup. Remarkably, compared to the vehicle controls, the percentage of CD4+ T cells expressing IFN-γ was significantly lower (Supporting Fig. S4A). Collectively, our data suggest that the treatment using karonudib reduced human T-cell activation and function *in vitro*.

KARONUDIB INCREASED DNA DAMAGE IN ACTIVATED HUMAN T CELLS *IN VITRO*

The traditional role of MTH1 is to hydrolyze oxidized dNTPs into deoxyribonucleoside monophosphates to prevent the incorporation of oxidative DNA damage.[15] Previous studies have verified that several MTH1 inhibitors prevent DNA damage repair, inducing DNA damage and cytotoxicity in cancer cells.[18,19] The comet assay detected that activated T cells treated with karonudib for 72 hours showed evident comet tails, which suggested significant DNA damage (Fig. 4A,B). Subsequently, we determined the levels of proteins of two classic markers of DNA damage, phosphorylated histone H2AX (γ-H2AX) and cleaved poly(adenosine diphosphate ribose) polymerase (PARP), using western blot. The expression of these markers was elevated when the activated T cells were treated with karonudib (Fig. 4C). Another interesting finding was that the proapoptotic property of karonudib worked selectively on activated cells and lacked obvious proapoptotic effects on the resting T cells (Fig. 4D,E). Therefore, these results imply that karonudib could increase the extent of DNA damage specifically in activated T cells.

SPECIFIC DELETION OF MTH1 IN T CELLS INHIBITED Con A-INDUCED HEPATITIS BY DECREASING ACTIVATION OF HEPATIC T CELLS

To verify the role of MTH1 in experimental T-cell-mediated hepatitis, our study developed mice with a T-cell-specific deletion of MTH1 by breeding the $MTH1^{loxP/loxP}$ mice and the CD4-cre strain. Because mature $CD4^+$ and $CD8^+$ T cells in the periphery develop from double-positive T cells, the expression of Cre under the control of CD4 deletes LoxP-flanked genes in both $CD4^+$ and $CD8^+$T cells.[28] The specific deficiency of MTH1 in T cells alone could rescue Con A-induced hepatitis to a great extent, and this was indicated by the sharp decline in the levels of serum ALT and AST (Fig. 5A). Histologic assessment of liver tissues reaffirmed the amelioration of liver injury in $CD4^{cre}MTH1^{loxP/loxP}$ (MTH1 knockout [KO]) mice, which was revealed by a narrowed area of hepatic necrosis (Fig. 5B). Moreover, MTH1 KO mice that were tail-vein injected with Con A had a remarkably decreased level of inflammatory mediators compared to that of wild-type (WT) mice that received a similar treatment (Fig. 5C). Therefore, these data imply that specific deletion of MTH1 in T cells alone can protect mice from Con A-induced liver injury.

Because T cells play a critical role in the pathogenesis of Con A-induced hepatitis, we further explored the effects of MTH1 on T cells in mice. We tested T-cell activation markers (CD25 and CD69) and naive T-cell markers (CD44 and CD62L) in lymphocytes of both liver and spleen by using MTH1 KO mice. We found that the proportion of CD25+ T cells markedly decreased in both the liver and spleen of these mice when compared to model mice (Fig. 6A,B). We also observed a similar trend of CD69+ T cells in both liver and spleen of MTH1 KO mice (Fig. 6C,D). Additionally, the proportion of naive T cells was increased in both liver and spleen of MTH1 KO mice when compared to model mice (Fig. 6E,F). We further tested whether specific deletion of MTH1 in T cells had any effect on T-cell subsets in the Con

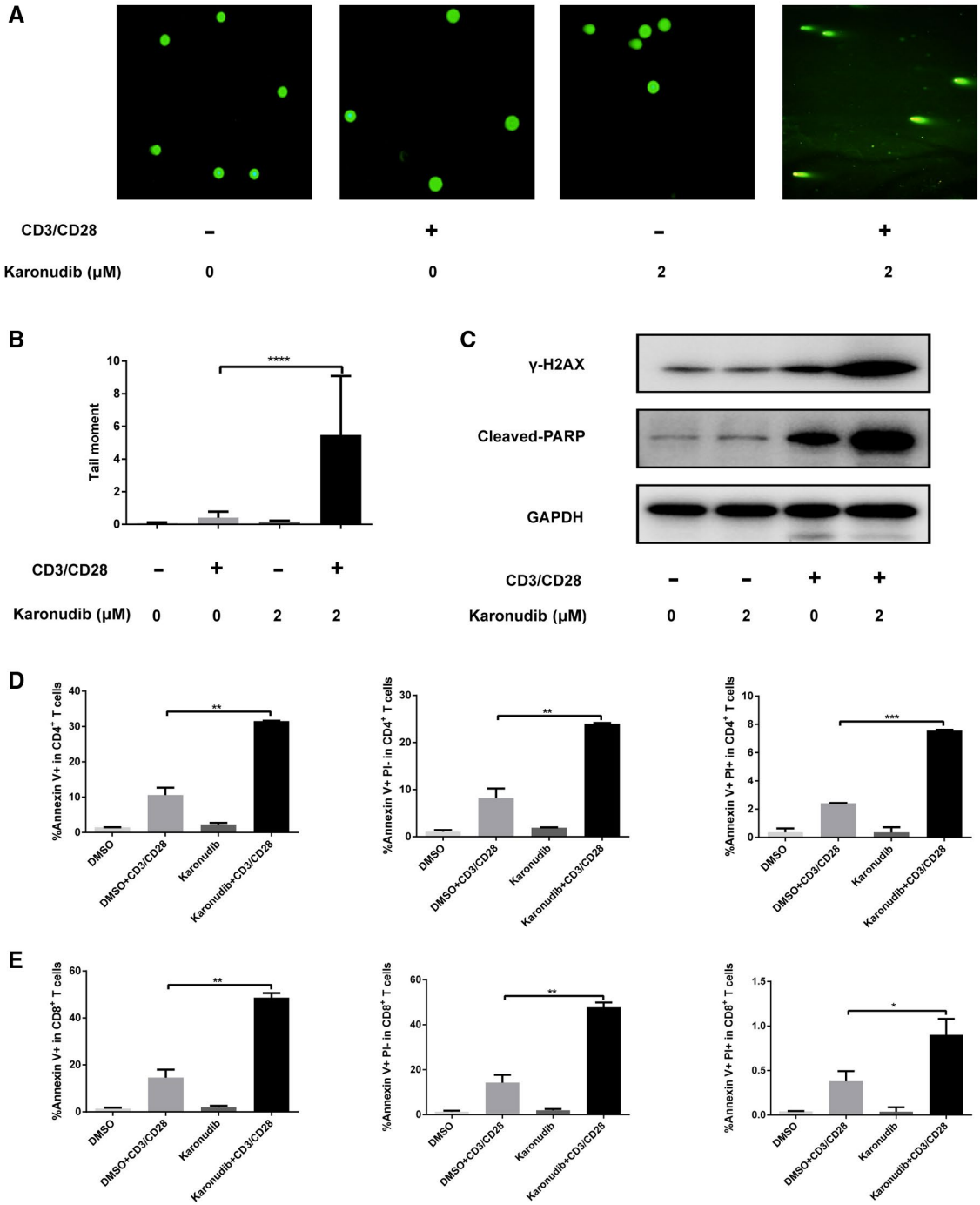

FIG. 4. Karonudib rendered activated T cells more susceptible to DNA damage *in vitro*. (A) Representative fields corresponding to each treatment were photographed. Isolated human T cells were activated with/without anti-CD3/CD28 beads in the presence of DMSO or 2 μM karonudib for 72 hours. The alkaline comet assay was conducted and nucleoids were visualized by epifluorescence microscopy using a fluorescein isothiocyanate filter. (B) Quantification of comet tail moment. Values represent mean ± SEM from three independent experiments (100 comets per experiment). (C) Levels of cleaved-PARP and phosphorylated histone H2AX (γ-H2AX) were determined by immunoblot analysis. The GAPDH blot was used as a loading control. (D,E) Intracellular flow cytometry assessment of annexin V and propidium iodide expression in anti-CD3/CD28 beads-stimulated CD4+ and CD8+ T lymphocytes treated with 2 μM karonudib or DMSO after 72 hours. Data are from one experimental representative of at least three independent experiments. Bars reflect the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Abbreviations: DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

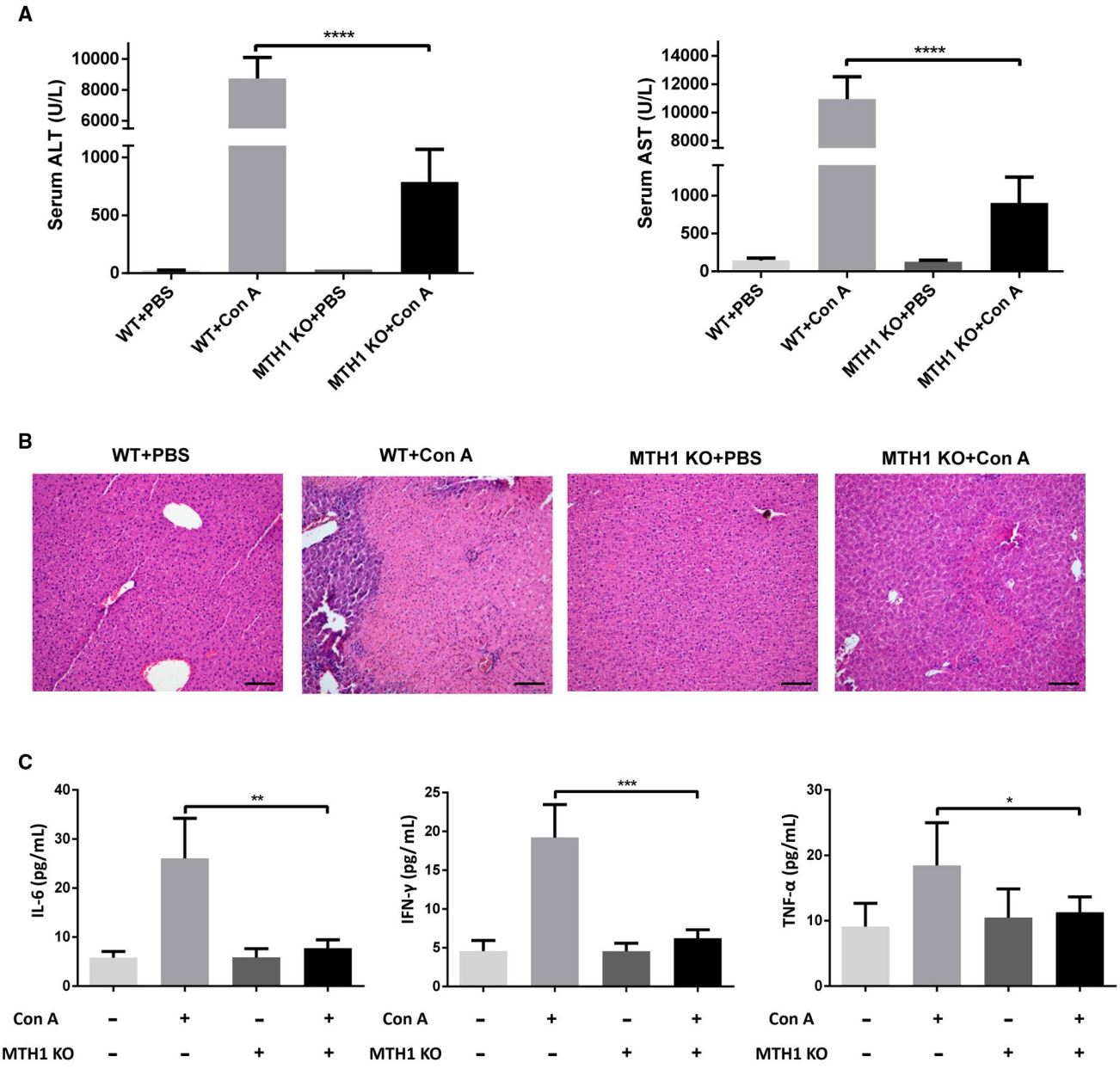

FIG. 5. Target deletion of MTH1 in T cells protected from Con A-induced liver injury. (A) Serum levels of ALT and AST were assessed at 24 hours following Con A (8 mg/kg) injection. (B) Histologic analysis of mouse livers was performed using hematoxylin and eosin staining (magnification ×400; scale bar, 50 μm) 24 hours after Con A injection (8 mg/kg). (C) Con A-induced elevation of serum inflammatory cytokines was ameliorated in MTH1 KO mice. Results represent the mean ± SEM (n = 4-6 mice per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

A model. We found that MTH1 KO mice had a decreased level of IFN-γ in both CD4+ and CD8+ T cells (Fig. 6G,J). However, no differences were found for Treg cells and Th17 cells between MTH1 KO mice and WT mice (Fig. 6H,I). In addition, evident inhibition of T-cell proliferation was observed in MTH1 KO mice, as shown in Fig. 6K. Taken together, our findings showed that the specific deficiency of MTH1 in T cells inhibited the activation and proliferation of T cells in mice.

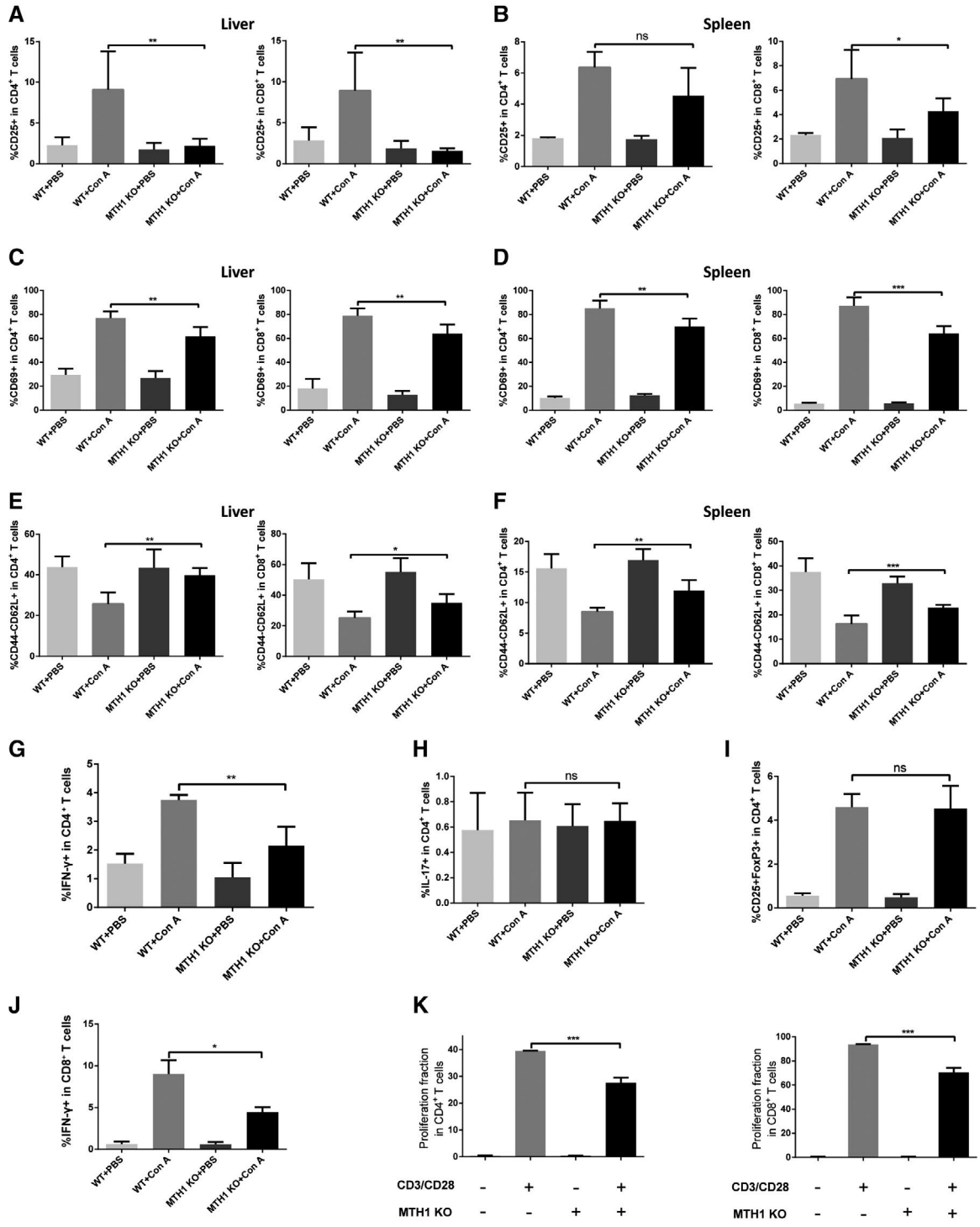

FIG. 6. Specific deletion of MTH1 in T cells inhibited Con A-induced hepatitis by decreasing the activation of hepatic T cells. (A,B) Frequency of CD25+ cells in hepatic and splenic CD4+ T and CD8+ T cells are shown. (C,D) Frequency of CD69+ cells in hepatic and splenic CD4+ T and CD8+ T cells are shown. (E,F) Frequency of naive cells (CD44-CD62L+) in hepatic and splenic CD4+ T and CD8+ T cells are shown. (G-I) Summary graphs of (G) IFN-γ+, (H) IL-17+, and (I) CD25+FoxP3+ in hepatic CD4+ T cells. (J) Frequency of IFN-γ+ cells in hepatic CD8+ T cells. (K) Statistical analysis of the T-cell proliferation assay from T cells of WT mice and MTH1 KO mice for 72 hours. Data are from one experimental representative of at least three independent experiments and represent triplicate wells. Graphs reflect mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

MTH1 INHIBITOR KARONUDIB ATTENUATED CON A-INDUCED HEPATITIS BY INHIBITING T-CELL ACTIVATION AND PROLIFERATION

To further confirm the role of MTH1 in experimental T-cell-mediated hepatitis, we administrated the MTH1 inhibitor karonudib to a Con A-induced liver injury murine model. Mice administered with karonudib were completely protected from Con A-induced liver injury, which was demonstrated by the sharp decrease in serum ALT and AST levels when compared to Con A-treated mice without any karonudib administration (placebo group) (Fig. 7A). Through histologic studies, karonudib significantly alleviated the degree of necrosis compared to the placebo group (Fig. 7B). Additionally, the administration of karonudib significantly decreased serum levels of proinflammatory cytokines, such as interleukin (IL)-6, IFN-γ, and TNF-α, in the Con A-induced hepatitis model (Fig. 7C). Taken together, these results suggest that karonudib treatment could be efficient in treating Con A-induced hepatitis.

Similar to the results of MTH1 KO mice, the proportion of CD25+ cells among CD4+ or CD8+ T cells from karonudib-treated mice was significantly decreased compared to the placebo group (Fig. 8A,B). Karonudib also inhibited the expression of CD69 in T cells of the liver and spleen from Con A-treated mice (Fig. 8C,D). In addition, the proportion of naive T cells in karonudib-treated mice was significantly increased in both the liver and spleen, as shown in Fig. 8E,F. We further tested the effect of karonudib on T-cell subsets in the Con A model. We found that karonudib had an evident inhibitory effect on proinflammatory cytokines, such as IFN-γ, in both CD4+ and CD8+ T cells and a slightly decreased level of IL-17 in CD4+ T cells (Fig. 8G,H,J). However, karonudib had no inhibitory effect on Treg cells (Fig. 8I). Interestingly, there was an obvious suppression of mouse activated T-cell proliferation using the karonudib treatment *in vitro* (Fig. 8K). All these results show that karonudib attenuated Con A-induced hepatitis by inhibiting T-cell activation and proliferation.

Discussion

The most striking translational finding of this study is that MTH1 is extensively expressed and clinically relevant in AIH. Karonudib and specific knockout of MTH1 in T cells protected the mice from Con A-induced liver injury. Following MTH1 inhibition, intrahepatic T-cell activation was suppressed and the proportion of naive T cells was increased. In addition, karonudib robustly decreased the levels of these proinflammatory mediators in activated T cells and inhibited Th1 differentiation. These effects are based on DNA damage susceptibility of the hyperactive T cells.

A previous study showed that DNA damage is detectable once the T cells have been activated.[29] In our study, we observed that the activated T cells expressed higher levels of MTH1 than the resting T cells to counteract the deleterious effects of DNA damage. Clinically, the adoption of karonudib in the treatment of cancer is to induce cell-cycle arrest, which suppresses the rapid proliferation of cancer cells. Hyperactive T cells and cancer cells are both rapid proliferating cells and have a similar metabolic pattern.[30,31] Interestingly, karonudib rendered activated T cells more susceptible to DNA damage, evidenced by the comet assay and increased protein levels of γ-H2AX and cleaved PARP, consistent with a study that demonstrated MutT contributed to the fidelity of DNA.[32] The carboxyfluorescein succinimidyl ester assay also showed that karonudib inhibited T-cell proliferation. Additionally, the expression of cyclin E and CDK2, which facilitated the cell cycle, were inhibited, whereas the expression of P21, P27, and P53, which inhibited the cell cycle, were enhanced. We therefore showed that the karonudib inhibition property in T cells is consistent with the role of karonudib in cancer cells, which is to induce cell-cycle arrest.

The DNA damage response/repair (DDR) pathway is well elucidated in studies of cancer biology. Many antitumor therapeutics exploit DNA damage by overwhelming repair mechanisms to trigger cancer-cell death.[33,34] Different cells have been armed with proper mechanisms to defend themselves against oxidative DNA damage caused by misincorporation of 8-oxoG into DNA. Among them, MTH1, 8-oxoguanine DNA glycosylase 1 (OGG1), and MutY DNA glycosylase (MUTYH) are the main enzymes that eliminate the devastating effects caused by 8-oxoG.[35-37] Mainly, MTH1 works in the nucleotide pool and depletes 8-oxoG by hydrolyzing 8-oxo-dGTP to 8-oxo-dGMP at the source.[38]

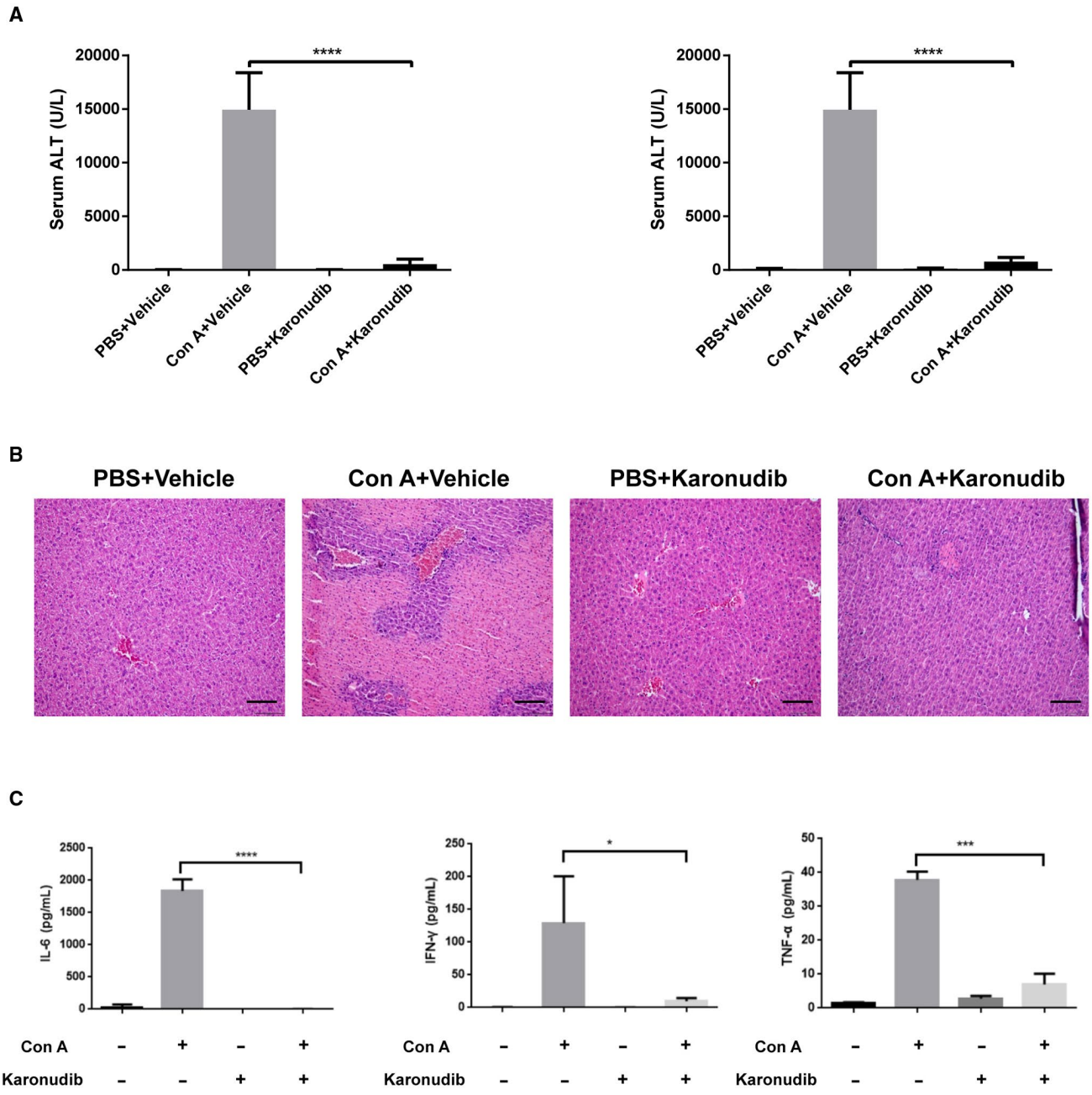

FIG. 7. Karonudib significantly attenuated Con A-induced liver injury. (A) Serum of ALT and AST levels at 24 hours in Con A (10 mg/kg)-treated mice that were administered simultaneously with/without karonudib by gavage. (B) Representative hematoxylin and eosin staining (magnification ×400) of liver tissues from mice treated with Con A for 24 hours; scale bar, 50 μm. Extensive necrosis in hepatocytes was observed in Con A-treated mice administered simultaneously without karonudib by gavage, while a significant reduction of necrosis was observed in mice administered simultaneously with karonudib by gavage. (C) Con A-induced elevation of serum inflammatory cytokines was ameliorated in mice treated with karonudib by gavage. Data are from one experimental representative of at least three independent experiments. Results represent the mean ± SEM (n = 4-6 mice per group). $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

OGG1 minimizes any potential genotoxicity by excising the opposite cytosine of 8-oxoG from DNA strands.[39] The MUTYH enzyme removes the adenine that pairs with 8-oxoG.[37] In short, the overall role of the three enzymes is to minimize the serious consequences caused by an increased cellular accumulation

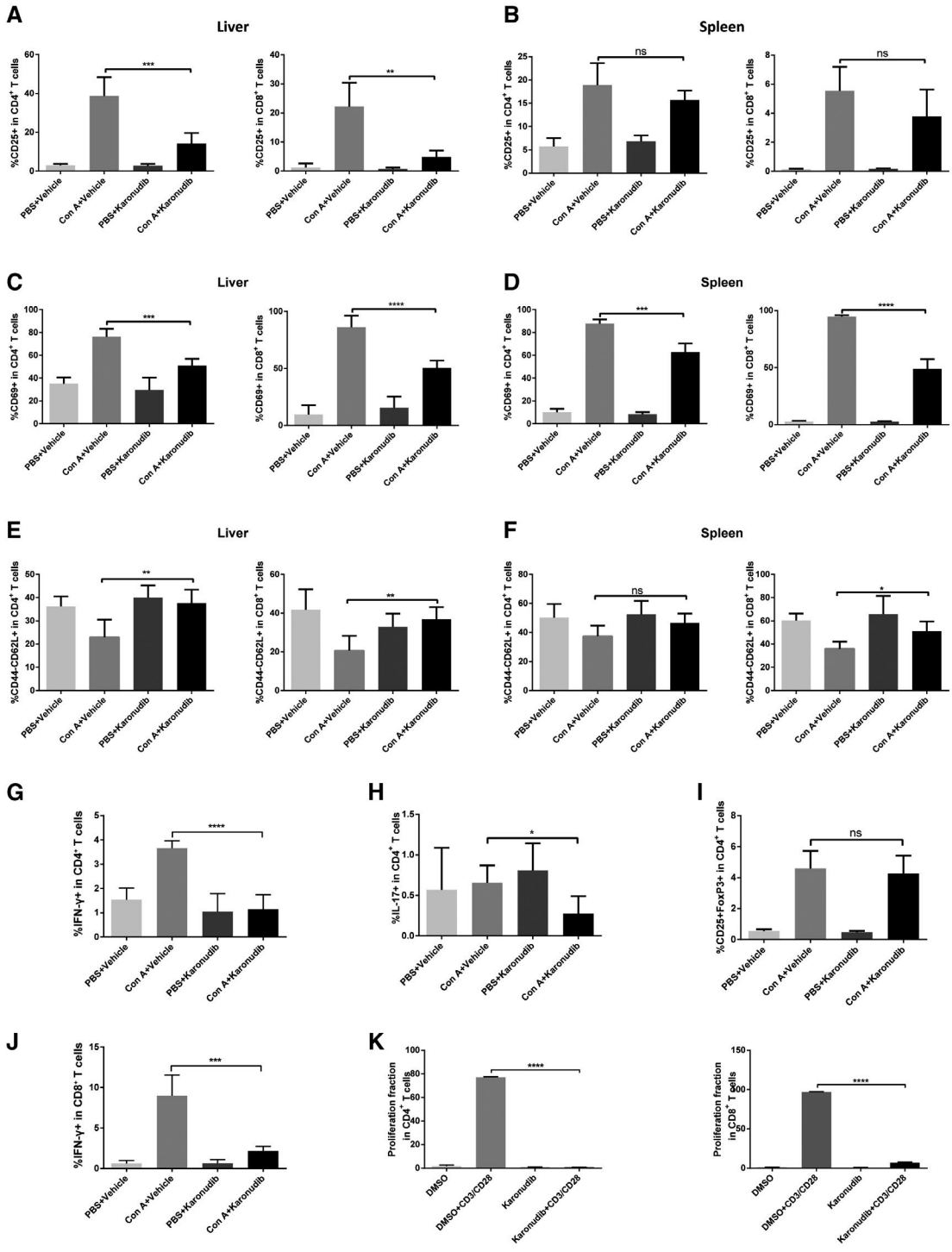

FIG. 8. Karonudib inhibited T-cell activation and increased naive T cells in mice. (A,B) Frequency of CD25+ cells in hepatic and splenic CD4+ T and CD8+ T cells are shown. (C,D) Frequency of CD69+ cells in hepatic and splenic CD4+ T and CD8+ T cells are shown. (E,F) Frequency of naive cells (CD44-CD62L+) in hepatic and splenic CD4+ T and CD8+ T cells are shown. (G-I) Summary graphs of (G) IFN-γ+, (H) IL-17+, and (I) CD25+FoxP3+ in hepatic CD4+ T cells. (J) Frequency of IFN-γ+ cells in hepatic CD8+ T cells. (K) Statistical analysis of the T-cell proliferation assay treated with/without 2 μM karonudib from splenic T cells of mice for 72 hours. Data are from one experimental representative of at least three independent experiments and represent triplicate wells. Graphs reflect mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

of 8-oxoG in cells, thus preventing mutagenesis and cell death.[35]

Several studies have also emphasized the important role of DDR in autoimmune diseases and other chronic inflammation.[40,41] Our research collaborators recently identified the small-molecule inhibitor of OGG1, called TH5487, and found it hampered OGG1 binding to the G-rich regions adjacent to NF-κB binding sites in promoters of proinflammatory genes. TH5487 robustly inhibits the inflammatory response in cultured lung epithelial cells *in vitro* and inhibits TNF-α-induced neutrophilic inflammation *in vivo*.[42]

Currently, the mainstay of treatment for AIH is the use of prednisone, which induces inflammation remission, and the combination of prednisone and azathioprine to maintain therapeutic efficacy. Different drugs have been developed to treat AIH; however, they have not been satisfactory in clinical practice as they had different side effects, such as myelosuppression and infection.[8] Karonudib, as the latest generation of MTH1 inhibitors, has much better oral availability and displays good pharmacokinetic properties.[18] Similar to the use of a small-molecule inhibitor of OGG1 on suppressing inflammation, the adoption of karonudib as the potential treatment of AIH is novel because this therapy makes full use of distinct aspects of T-cell biology in the persistent inflammatory environment. Notably, the high degree of susceptibility that hyperactive T cells displayed relative to resting T cells after they were treated with karonudib could cause minimal damage to reservoir T cells that are vital in other physiological processes, such as prevention of infection and cancer, hopefully bringing more potential therapeutic gains with less potential toxicity.

In conclusion, our study suggests that the nucleotide pool enzyme MTH1 plays an essential role in Con A-induced liver injury as well as in the disease context of AIH by preventing DNA damage in activated T cells. Although MTH1 is initially rooted in cancer treatment, it can be applied in the treatment of T-cell-mediated liver injury. Therefore, the findings from our study have emphasized the high potential of the MTH1 enzyme as a novel therapeutic target in AIH treatment and karonudib as a novel and promising drug for patients with AIH.

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