Tenofovir alafenamide alleviates nonalcoholic steatohepatitis in mice by blocking the phosphorylation of AKT in intrahepatic mononuclear phagocytes

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February 22, 2024

Abstract

Background and Purpose: Although the prevalence of nonalcoholic steatohepatitis (NASH) is rapidly increasing, effective therapy is lacking. Tenofovir alafenamide (TAF) is a widely used antiviral drug for hepatitis B. In this study, we investigated the potential pharmacological effects of TAF on NASH. Experimental Approach: Two different NASH mouse models were established: 1) by subcutaneous injection of streptozotocin (0.2 mg) and feeding the mice a high-fat, high-cholesterol (HFHC) diet, and 2) feeding the mice a choline-deficient, L-amino acid-defined, high-fat (CDAHF) diet. Key Results: Serum alanine aminotransferase and triglyceride levels in TAF-treated NASH mice were significantly lower than those in the mock-treated ones. The livers from the TAF-treated NASH mice showed attenuated mononuclear phagocyte (MP) infiltration compared to those from the mock-treated ones. TAF-treated NASH mice exhibited decreased liver infiltration of activated MPs (IAIE+/PD-L1+/MerTK+). In ex vivo experiments using sorted human CD14+ monocytes treated with lipopolysaccharide (LPS) and/or TAF, we confirmed the decreased level of phosphorylated AKT in TAF-treated LPS-stimulated monocytes compared to that in the mock-treated ones. Mouse liver immunoblotting showed that phosphorylation levels of AKT were significantly lower in the TAF-treated NASH group than in the mock-treated group. Conclusion and Implications: TAF exerts anti-inflammatory effects in NASH livers by attenuating AKT phosphorylation in intrahepatic activated MPs. Therefore, TAF may serve as a new therapeutic option for NASH.

Introduction

Non-alcoholic steatohepatitis (NASH) is characterized by hepatic steatosis, hepatocellular damage, and liver inflammation, progressing to more severe stages, including cirrhosis or hepatocellular carcinoma (Abdelmalek, 2021; Huby & Gautier, 2021). Although NASH is highly prevalent, there are currently no effective treatments for this disease (Raza, Rajak, Upadhyay, Tewari, & Anthony Sinha, 2021). NASH treatments are currently being developed for various targets, and several clinical trials are underway. Only few drugs are in phase III trials, whereas others remain in phase I or II trials (Yoo et al., 2019). The US Food and Drug Administration has not yet approved any specific drug for NASH treatment; therefore, developing new and improved therapeutic drugs is essential (Kang et al., 2021).

Complex crosstalk between hepatocytes, hepatic stellate cells (HSCs), and diverse immune cells is activated during NASH progression (Huby & Gautier, 2021; H. L. Lee et al., 2022). During the process, inflammatory signals increase the mononuclear phagocyte (MP) pool size in the liver. Single-cell RNA sequencing of NASH mice liver showed increased size of MP clusters compared with normal mice liver (Xiong et al., 2019). Recent studies have focused on the diversity of MP in NASH mouse models (Remmerie et al., 2020; Seidman et al., 2020; Tran et al., 2020). Intrahepatic MPs are traditionally classified into two populations, Kupffer

cells (KCs) and monocyte-derived macrophages (MoMFs). KCs are liver-resident phagocytes; MoMFs are recruited from the circulation to the hepatic injury site (Krenkel et al., 2018). Monocytes infiltrate the liver, and recruited monocytes differentiate into intrahepatic MPs, promoting HSC activation in the chronically inflamed liver (Sung, 2021; P. S. Sung et al., 2022). A recent single cell analysis also demonstrated that the recruited and intrahepatic MPs exhibit distinct inflammatory phenotypes during nonalcoholic fatty liver disease (NAFLD) progression (Krenkel et al., 2020).

Two tenofovir prodrugs are currently available for treating human immunodeficiency and hepatitis B virus infections. Tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF) are commonly used antiviral agents with potent antiviral activity, causing liver fibrosis regression (S. W. Lee et al., 2021; Zhao et al., 2020). Another report demonstrated that TDF regulates liver fibrosis by downregulating the PI3K/Akt/mTOR signaling pathway, leading to the activation of HSC apoptosis (S. W. Lee et al., 2021). TAF treatment also resulted in liver fibrosis regression, similar to TDF effects in a fibrosis mouse model (Zhao et al., 2020). However, there are currently no reports showing the effects of TAF treatment on NASH regression. This study investigated the potential therapeutic effects of TAF on NASH in two different *in vivo* NASH models. Moreover, we examined the effect of TAF on the PI3K/Akt/mTOR signaling pathway in intrahepatic MPs.

Experimental Procedures

Isolation of peripheral human CD14$^+$monocytes and in vitro experiments

Peripheral blood mononuclear cells were isolated from a healthy adult donor using Ficoll–Hypaque density gradient centrifugation. CD14$^+$ monocytes (anti-CD14 microbeads, MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) were separated from the peripheral blood mononuclear cells using the OctoMACS separator and starting kits (MACS, Miltenyi Biotec) (Park et al., 2020). Separated human CD14$^+$ monocytes were plated in 90 × 20-mm cell culture dishes (SPL Life Sciences, Pochon, Korea) at a density of 1×10^6 cells/mL in serum-free Roswell Park Memorial Institute 1640 medium. The medium was pre-treated with TAF for 6 h. The control group was treated with an equal volume of dimethyl sulfoxide (DMSO). The medium was refreshed with 1 μg/mL LPS or mock for 24 h. The cells were then incubated at 37 °C with 5% CO_2. We then performed fluorescence staining (AKT, CD45, human leukocyte antigen-DR isotype [HLA-DR], phospho-AKT, programmed death-ligand 1 [PD-L1], and LIVE/DEAD dye). The research protocol conformed to the Declaration of Helsinki; the institutional review board of St. Mary's Hospital in Seoul (KC20TISI0817) approved the study. Informed consent was obtained from all the participants.

Establishment of in vivo streptozotocin (STZ)-injected, high-fat, high-cholesterol (HFHC)-fed NASH mouse model

Sixteen days pregnant female C57BL/6J mice were purchased from the Jackson Laboratory (Japan). STZ (0.2 mg; Sigma-Aldrich, St. Louis, MO, USA) was injected subcutaneously into 3-day-old male C57BL/6J mice. STZ was dissolved in 0.1 M sodium citrate buffer (pH 4.5) before administration. One month later, the male mice were randomly divided into four groups: normal, STZ-injected normal carbohydrate (Teklad Global 18% Protein Rodent Diet; TD 2018C, Envigo, Indianapolis, IN, USA) diet, STZ-injected HFHC (Western Diet; D12079B, 41 kcal% fat, 43 kcal% carbohydrates; Research Diets, New Brunswick, NJ, USA) diet-fed, and STZ-injected TAF-treated HFHC diet-fed groups (TAF, 5 mg/kg; Gilead Sciences, Foster City, CA, USA). TAF was administered daily for 4 weeks. Blood was collected from the orbital venous plexus every 2 weeks, and random glucose levels were analysed. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and triglyceride (TRIG) levels were measured using a chemical analyser according to the manufacturer's protocol (Vettest 8008 Chemistry Analyzer; IDEXX Laboratories, Westbrook, ME, USA). Mice were intraperitoneally anesthetized with Rompun (10 mg/kg) and Zoletil (40 mg/kg), and their livers were removed. Mice livers were dissociated using a dissociation kit (MACS, Miltenyi Biotec) and gentleMACS Dissociator (MACS, Miltenyi Biotec). Fluorescence staining was then performed (CD11b, F4/80, CD45, Ly6G, PD-L1, IAIE, MerTK, and LIVE/DEAD dyes).

The Korea Excellence Animal Laboratory Facility at the Korea Food and Drug Administration was accredited in 2007 by the Institutional Animal Care and Use Committee (IACUC) and the Department of Laboratory

Animals (DOLA) at the Catholic University of Korea, Songeui Campus, and acquired full Assessment and Accreditation by the Laboratory Animal Care International accreditation in 2018. All animal care and experimental procedures were conducted according to the Institutional Animal Care and Use Committee and were approved by the Center for Medical Science of the Catholic University of Korea (2021-0329-02).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7 (GraphPad, Inc., San Diego, CA, USA). Continuous variables were assessed using independent t -tests. The relationships between the two parameters were examined using Pearson's correlation tests. Statistical significance was set at p <0.05.

Additional methods are presented in the supplementary information.

RESULTS

TAF treatment ameliorates liver injury in the NASH mouse model

We established a NASH mouse model using STZ and HFHC diets to evaluate the effects of TAF on NASH (Fig 1A). Liver function tests were performed by measuring the serum ALT, AST, and triglyceride (TRIG) levels to confirm the NASH model and TAF effects. ALT, AST, and TRIG levels were lower in the TAF-treated HFHC diet-fed group than in the mock-treated ones (Fig 1B). Body weight and random glucose levels were measured for 2 weeks. The total liver weight of the TAF-treated HFHC diet-fed mice was lower than that of the mock-treated ones. (Supplementary Fig S1A). Haematoxylin and eosin (H&E) staining of paraffin-embedded liver sections showed that hepatocyte necrosis and sinusoidal congestion were significantly reduced in the livers of the TAF-treated HFHC diet-fed group than in those of the mock-treated ones. Sirius red staining showed that the collagen deposition area was decreased in the livers of the TAF-treated HFHC diet-fed group compared to those of the mock-treated one. Moreover, the IHC results showed that the percentages of LPS- and toll-like receptor 4 (TLR4)-positive cells were higher in the mock-treated HFHC diet group than in the TAF-treated group (Fig 1C). The TLR4- or LPS-positive cell frequency was lower in the TAF-treated HFHC diet group than in the mock-treated one. The area ratio of stained collagen was lower in the TAF-treated HFHC diet group than in the mock-treated one. NASH Activity Score (NAS) was decreased in the livers of the TAF-treated HFHC diet-fed group compared to those of the mock-treated one (Fig 1D).

We established a NASH mouse model using a choline-deficient, L-amino acid-defined, high-fat (CDAHF) diet to evaluate TAF's role in NASH. (Fig 1E). ALT, AST, and TRIG levels were lower in the TAF-treated CDAHF diet-fed group than in the mock-treated one (Fig 1F). Body weight and random glucose levels were measured for 2 weeks. The total liver weight of the TAF-treated CDAHF diet-fed group was lower than that of the mock-treated one (Supplementary Fig S1B). H&E staining of paraffin-embedded liver sections showed that hepatocyte necrosis and sinusoidal congestion were significantly reduced in the TAF-treated CDAHF diet-fed group's livers compared to those of the mock-treated one (Supplementary Fig S1C).

The number of PD-L1^{+}/IAIE^{+}/MerTK^{+}intrahepatic MPs is lower in TAF-treated STZ, HFHC diet-fed NASH mice.

We performed flow cytometry analysis after mouse liver digestion to confirm the role of intrahepatic MPs in the NASH mouse model using the livers of STZ and HFHC diet-fed mouse treated with mock or TAF. A gating strategy for quantifying intrahepatic MPs using flow cytometry is shown in Supplementary Figure S2A.

A representative gating strategy for intrahepatic recruited (CD11b^{high} F4/80^{low}) and resident (CD11b^{low} F4/80^{high}) MPs is shown in Fig 2A. The TAF-treated HFHC diet-fed group showed significantly decreased intrahepatic recruited (CD11b^{high}F4/80^{low}) MPs than the mock-treated one. However, there were no significant differences in resident (CD11b^{low} F4/80^{high}) MPs (Fig 2B) between the TAF-treated HFHC diet-fed group and the mock-treated one. Programmed death ligand 1 (PD-L1), IAIE, MER proto-oncogene, and tyrosine kinase (MerTK) were used as CD11b^{+}F4/80^{+} MP activation surface markers. The intrahepatic IAIE^{+} and

MerTK$^+$ MP mean fluorescence intensity (MFI) values were significantly lower in the TAF-treated HFHC diet group than in the mock-treated one (Fig 2C). The intrahepatic PD-L1$^+$/IAIE$^+$/MerTK$^+$-positive MP number was significantly decreased in the TAF-treated HFHC diet group than in the mock-treated one (Fig 2D). Furthermore, the number of CD11b$^+$ F4/80$^+$ MPs per g liver weight was significantly reduced in the TAF-treated HFHC diet group than in the mock-treated one (Supplementary Fig S2B). Figure 2E shows that tumour necrosis factor-α, chemokine (C-C motif) ligand 2, and interleukin-1 beta mRNA expression levels were significantly lower in the TAF-treated HFHC diet group than in the mock-treated one, indicating reduced inflammation.

The number of PD-L1$^+$/IAIE$^+$/MerTK$^+$intrahepatic MPs is lower in TAF-treated CDAHF diet-fed NASH mice

We performed flow cytometry analysis after mouse liver digestion to confirm the role of intrahepatic MPs role in the NASH mouse model using the liver of CDAHF diet-fed mouse treated with mock or TAF. A representative gating strategy for intrahepatic recruited (CD11b^{high} F4/80^{low}) and resident (CD11b^{low} F4/80^{high}) MPs is shown in Fig 3A. The TAF-treated CDAHF diet-fed group showed considerably decreased intrahepatic recruited (CD11b^{high}F4/80^{low}) MPs than the mock-treated one. However, there were no significant differences in resident (CD11b^{low} F4/80^{high}) MPs between the TAF-treated CDAHF group and mock-treated one (Fig 2B). The intrahepatic IAIE$^+$ and MerTK$^+$ MP MFI values were significantly lower in the TAF-treated CDAHF diet group than in the mock-treated one (Fig 3C). The intrahepatic PD-L1$^+$/IAIE$^+$/MerTK$^+$-positive MP number was remarkably decreased in the TAF-treated HFHC diet group (Fig 3D). Furthermore, the number of CD11b$^+$F4/80$^+$ MPs per g liver weight was significantly reduced in the TAF-treated CDAHF diet group than in the mock-treated one (Supplementary Fig S2C).

Cell surface PD-L1 expression of activated intrahepatic MPs decreased by ex vivo TAF treatment

We established an animal TAA-induced liver injury model to identify the*ex vivo* TAF effects on inflamed intrahepatic MPs. TAA was injected intraperitoneally for 8 weeks (Fig 4A). H&E staining of paraffin-embedded liver sections showed that hepatocyte necrosis and sinusoidal congestion were significantly higher in the TAA-treated group's livers than in those of the no TAA-treated ones. Sirius red staining showed that the collagen deposition area was increased in the TAA-treated group's livers compared to that in the no TAA-treated group (Fig 4B). The TAA-treated injured livers were perfused with collagenase, and MPs were isolated using gradient centrifugation. The isolated MPs were treated with different TAF concentrations (Fig 4C). TAF- or mock-treated MPs were analysed using flow cytometry. The number of PD-L1$^+$ cells among the total analysed MPs was decreased by TAF in a concentration-dependent manner (Fig 4D). TAF treatment decreased the MFI of PD-L1 in CD11b$^+$F4/80$^+$ intrahepatic MPs (Fig 4E).

TAF blocks phosphorylation of AKT in MPs

Immunoblotting was performed to determine the signalling pathway blocked by TAF in *in vivo* STZ, HFHC diet-fed NASH mouse model treated with mock or TAF. The p-AKT, AKT, p-mTOR, mTOR, and GAPDH protein levels were detected in the NASH mice livers. p-AKT and p-mTOR levels were decreased in the livers of the STZ-injected TAF-treated HFHC diet-fed mice compared to those of the STZ-injected mock-treated HFHC diet-fed mice (Fig 5A).

We investigated TAF's effects on human monocytes, as monocytes are recruited to damaged livers and differentiate into MPs. We sorted CD14$^+$ monocytes in the peripheral blood mononuclear cells. Sorted human CD14$^+$ monocytes were pre-treated with TAF for 6 h and then stimulated with or without LPS for 24 h. The CD14$^+$ monocyte population was determined using flow cytometry. PD-L1$^+$ and human leukocyte antigen-DR isotype (HLA-DR)$^+$ populations decreased with increasing TAF concentrations compared to those in the mock group (Fig 5B). The HLA-DR$^+$/PD-L1$^+$ cell number increased after LPS treatment but decreased in a concentration-dependent manner in the TAF-stimulated group (Fig 5C). Moreover, TAF treatment decreased HLA-DR and PD-L1 MFI in activated CD14$^+$monocytes compared to that in the mock group (Fig 5D). Representative AKT and p-AKT level plots in activated human CD14$^+$monocytes treated with mock or TAF were analysed using flow cytometry. The phosphorylated AKT population was

significantly reduced following TAF addition to activated human $CD14^+$ monocytes based on p-AKT MFI values; AKT phosphorylation was blocked by TAF treatment (Fig 5E). A schematic representation of the effect of TAF on NASH liver is presented in Fig 5F.

Discussion

In this study, we confirmed that TAF alleviated NASH in two different NASH mouse models by decreasing the activity of recruited and intrahepatic MPs by blocking the AKT pathway. Our results demonstrated that liver damage and $IAIE^+$/PD-$L1^+$/$MerTK^+$intrahepatic MP number were lower in TAF-treated NASH mice. Moreover, LPS-stimulated TAF-treated $CD14^+$ human monocytes expressed lower PD-$L1^+HLA\text{-}DR^+$ levels than LPS-stimulated mock-treated ones. These results suggest that TAF decreased the numbers of recruited and intrahepatic MPs in the NASH liver. Moreover, pAKT levels were decreased in TAF-treated NASH mice livers and isolated peripheral human $CD14^+$ monocytes, suggesting that TAF blocks AKT phosphorylation in MPs.

During NASH, recruited and intrahepatic MPs differentiate into pro-inflammatory MPs (Sung, 2021). The single-cell transcriptome analysis of NASH mouse livers showed that genes most specific to MPs were primarily NASH-induced genes (Xiong et al., 2019). Another recent single cell analysis also demonstrated that the recruited and intrahepatic MPs exhibit distinct inflammatory phenotypes during NAFLD progression (Krenkel et al., 2020). Other studies also demonstrated that in NASH/ALD livers, $CD68^+$ MP numbers increased with the progression of fibrosis (Pil Soo Sung et al., 2022), and $Trem2^+CD9^+$ MPs derived from the recruited MPs display a profibrogenic phenotype (Ramachandran et al., 2019). Therefore, it is critical to investigate the mechanisms that target the activated MPs to treat NASH.

The NASH mouse models show pathological features similar to that of human NASH (Saito et al., 2015). Therefore, we established a NASH mouse model using a combination of chemical (STZ) and dietary interventions with HFHC diets. A recent study on human NAFLD livers suggested that gut-derived LPS induces liver damage by activating MPs via the TLR-4 pathway (Carpino et al., 2020). Our IHC results showed that LPS and TLR-4 expression was significantly reduced in the TAF-treated HFHC diet-fed mice compared to the mock-treated ones. A previous study revealed that TAF mitigates ALT levels in chronic hepatitis B virus infection maybe due to metabolic factors associated with NAFLD (Sripongpun et al., 2022). The NASH mouse model showed elevated serum ALT, AST, and TRIG levels, indicating severe liver damage. After TAF administration in the NASH mouse model, serum ALT, AST, and TRIG levels were remarkably restored to normal ranges. $CD11b^{high}F4/80^{low\ +}$, used as a recruited MP marker, exhibited a higher percentage in the mock-treated HFHC diet group and was markedly decreased in the TAF-treated group. Previous studies have revealed that TDF, an antiviral agent used to treat chronic hepatitis B, has additional immune-modulatory effects (S. W. Lee et al., 2021; Zhao et al., 2020). To investigate anti-inflammatory effects of TAF on inflamed intrahepatic MPs, MPs were isolated from a TAA-induced liver injury mouse model and treated with TAF *ex vivo* . The activation status of the isolated MPs was significantly reduced after TAF treatment, suggesting that TAF has anti-inflammatory effects.

Akt is known as a key player in signal transduction and regulation of cellular processes. The PI3K/AKT pathway regulates MP survival, migration, proliferation, and inflammatory signals and promotes the development of inflammation (Linton, Moslehi, & Babaev, 2019). The PI3K/Akt pathway is activated by TLR4 or other cytokines, chemokines, and Fc receptors. TLR activation ultimately affects the activation state of macrophages (Lopez-Pelaez, Soria-Castro, Bosca, Fernandez, & Alemany, 2011). Moreover, LPS increases the phosphorylation of Akt (S. J. Lee, Seo, & Kim, 2015). Our data demonstrated that AKT phosphorylation was inhibited by TAF administration in a NASH mouse model. Moreover, pAKT levels were decreased in TAF-treated activated peripheral human $CD14^+$ monocytes, suggesting that TAF blocks AKT phosphorylation in MPs. Further human studies are necessary to discover the safe and effective anti-inflammatory dose of TAF in patients with NASH.

Conclusion

We demonstrated that TAF alleviates NASH in the liver by downregulating the AKT signalling pathway,

resulting in MP inactivation. Therefore, TAF may be an effective therapeutic agent for treating NASH.

References

1. Huby, T., & Gautier, E. L. (2021). Immune cell-mediated features of non-alcoholic steatohepatitis. *Nature Reviews. Immunology,* 1-15. Advance online publication.

2. Abdelmalek, M. F. (2021). Non-alcoholic fatty liver disease: another leap forward. *Nature reviews. Gastroenterology & hepatology, 18* (2),85-86.

3. Raza, S., Rajak, S., Upadhyay, A., Tewari, A., & Anthony Sinha, R. (2021). Current treatment paradigms and emerging therapies for NAFLD/NASH. *Frontiers in bioscience (Landmark edition), 26* (2), 206-237.

4. Yoo, J. J., Kim, W., Kim, M. Y., Jun, D. W., Kim, S. G., Yeon, J. E., Lee, J. W., Cho, Y. K., Park, S., Sohn, J. H., & the Korean Association for the Study of the Liver (KASL)-Korea Non-alcoholic fatty liver Study Group (KNSG) (2019). Recent research trends and updates on non-alcoholic fatty liver disease. *Clinical and molecular hepatology, 25* (1), 1-11.

5. Kang, S. H., Lee, H. W., Yoo, J. J., Cho, Y., Kim, S. U., Lee, T. H., Jang, B. K., Kim, S. G., Ahn, S. B., Kim, H., Jun, D. W., Choi, J. I., Song, D. S., Kim, W., Jeong, S. W., Kim, M. Y., Koh, H., Jeong, S., Lee, J. W., Cho, Y. K.,... Korean Association for the Study of the Liver (KASL) (2021). KASL clinical practice guidelines: Management of non-alcoholic fatty liver disease. *Clinical and molecular hepatology, 27* (3), 363-401.

6. Lee, H. L., Lee, J., Cha, J. H., Cho, S., Sung, P. S., Hur, W., Yoon, S. K., & Bae, S. H. (2022). Anti-fibrotic effects of branched-chain amino acids on hepatic stellate cells. *The Korean journal of internal medicine, 37* (1), 53-62.

7. Xiong, X., Kuang, H., Ansari, S., Liu, T., Gong, J., Wang, S., Zhao, X. Y., Ji, Y., Li, C., Guo, L., Zhou, L., Chen, Z., Leon-Mimila, P., Chung, M. T., Kurabayashi, K., Opp, J., Campos-Perez, F., Villamil-Ramirez, H., Canizales-Quinteros, S., Lyons, R.,... Lin, J. D. (2019). Landscape of Intercellular Crosstalk in Healthy and NASH Liver Revealed by Single-Cell Secretome Gene Analysis. *Molecular cell, 75* (3), 644-660.e5.

8. Seidman, J. S., Troutman, T. D., Sakai, M., Gola, A., Spann, N. J., Bennett, H., Bruni, C. M., Ouyang, Z., Li, R. Z., Sun, X., Vu, B. T., Pasillas, M. P., Ego, K. M., Gosselin, D., Link, V. M., Chong, L. W., Evans, R. M., Thompson, B. M., McDonald, J. G., Hosseini, M.,... Glass, C. K. (2020). Niche-Specific Reprogramming of Epigenetic Landscapes Drives Myeloid Cell Diversity in Nonalcoholic Steatohepatitis. *Immunity, 52* (6), 1057-1074.e7.

9. Remmerie, A., Martens, L., Thone, T., Castoldi, A., Seurinck, R., Pavie, B., Roels, J., Vanneste, B., De Prijck, S., Vanhockerhout, M., Binte Abdul Latib, M., Devisscher, L., Hoorens, A., Bonnardel, J., Vandemme, N., Kremer, A., Borghgraef, P., Van Vlierberghe, H., Lippens, S., Pearce, E.,... Scott, C. L. (2020). Osteopontin Expression Identifies a Subset of Recruited Macrophages Distinct from Kupffer Cells in the Fatty Liver. *Immunity, 53* (3), 641-657e14.

10. Tran, S., Baba, I., Poupel, L., Dussaud, S., Moreau, M., Gelineau, A., Marcelin, G., Magreau-Davy, E., Ouhachi, M., Lesnik, P., Boissonnas, A., Le Goff, W., Clausen, B. E., Yvan-Charvet, L., Sennlaub, F., Huby, T., & Gautier, E. L. (2020). Impaired Kupffer Cell Self-Renewal Alters the Liver Response to Lipid Overload during Non-alcoholic Steatohepatitis. *Immunity, 53* (3), 627-640.e5.

11. Krenkel, O., Puengel, T., Govaere, O., Abdallah, A. T., Mossanen, J. C., Kohlhepp, M., Liepelt, A., Lefebvre, E., Luedde, T., Hellerbrand, C., Weiskirchen, R., Longerich, T., Costa, I. G., Anstee, Q. M., Trautwein, C., & Tacke, F. (2018). Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis. *Hepatology (Baltimore, Md.), 67* (2), 1270-1283.

12. Sung, P. S. Crosstalk between tumour-associated macrophages and neighboring cells in hepatocellular carcinoma. *Clinical and molecular hepatology.* Advance online publication.

13. Sung, P. S., Kim, C. M., Cha, J. H., Park, J. Y., Yu, Y. S., Wang, H. J., Kim, J. K., & Bae, S. H. (2022). A Unique Immune-Related Gene Signature Represents Advanced Liver Fibrosis and Reveals Potential Therapeutic Targets. *Biomedicines, 10* (1), 180.

14. Krenkel, O., Hundertmark, J., Abdallah, A. T., Kohlhepp, M., Puengel, T., Roth, T., Branco, D., Mossanen, J. C., Luedde, T., Trautwein, C., Costa, I. G., & Tacke, F. (2020). Myeloid cells in liver and bone marrow acquire a functionally distinct inflammatory phenotype during obesity-related steatohepatitis. *Gut, 69* (3), 551-563.

15. Lee, S. W., Kim, S. M., Hur, W., Kang, B. Y., Lee, H. L., Nam, H., Yoo, S. H., Sung, P. S., Kwon, J. H., Jang, J. W., Kim, S. J., & Yoon, S. K. (2021). Tenofovir disoproxil fumarate directly ameliorates liver fibrosis by inducing hepatic stellate cell apoptosis via downregulation of PI3K/Akt/mTOR signalling pathway. *PLoS One, 16* (12), e0261067.

16. Zhao, J., Han, M., Zhou, L., Liang, P., Wang, Y., Feng, S., Lu, H., Yuan, X., Han, K., Chen, X., Liu, S., & Cheng, J. (2020). TAF and TDF attenuate liver fibrosis through NS5ATP9, TGFbeta1/Smad3, and NF-kappaB/NLRP3 inflammasome signalling pathways. *Hepatology international, 14* (1), 145-160.

17. Park, D. J., Sung, P. S., Kim, J. H., Lee, G. W., Jang, J. W., Jung, E. S., Bae, S. H., Choi, J. Y., & Yoon, S. K. (2020). EpCAM-high liver cancer stem cells resist natural killer cell-mediated cytotoxicity by upregulating CEACAM1. *Journal for immunotherapy of cancer, 8* (1), e000301.

18. Sung, P. S., Park, D. J., Roh, P. R., Do Mun, K., Cho, S. W., Lee, G. W., Jung, E. S., Lee, S. H., Jang, J. W., Bae, S. H., Choi, J. Y., Choi, J., Ahn, J., & Yoon, S. K. (2022). Intrahepatic inflammatory IgA+ PD-L1-high monocytes in hepatocellular carcinoma development and immunotherapy. *Journal for immunotherapy of cancer, 10* (5), e003618.

19. Ramachandran, P., Dobie, R., Wilson-Kanamori, J. R., Dora, E. F., Henderson, B. E. P., Luu, N. T. Portman, J. R., Matchett, K. P., Brice, M., Marwick, J. A., Taylor, R. S., Efremova, M., Vento-Tormo, R., Carragher, N. O., Kendall, T. J., Fallowfield, J. A., Harrison, E. M., Mole, D. J., Wigmore, S. J., Newsome, P. N., ... Henderson, N. C. (2019). Resolving the fibrotic niche of human liver cirrhosis at single-cell level. *Nature, 575* (7783), 512-518.

20. Saito, K., Uebanso, T., Maekawa, K., Ishikawa, M., Taguchi, R., Nammo, T., Nishimaki-Mogami, T., Udagawa, H., Fujii, M., Shibazaki, Y., Yoneyama, H., Yasuda, K., & Saito, Y. (2015). Characterization of hepatic lipid profiles in a mouse model with nonalcoholic steatohepatitis and subsequent fibrosis. *Scientific reports, 5,*12466.

21. Carpino, G., Del Ben, M., Pastori, D., Carnevale, R., Baratta, F., Overi, D., Francis, H., Cardinale, V., Onori, P., Safarikia, S., Cammisotto, V., Alvaro, D., Svegliati-Baroni, G., Angelico, F., Gaudio, E., & Violi, F. (2020). Increased Liver Localization of Lipopolysaccharides in Human and Experimental NAFLD. *Hepatology (Baltimore, Md.), 72* (2), 470-485.

22. Sripongpun, P., Kim, W. R., Mannalithara, A., Kwong, A., Daugherty, T., Goel, A., & Kwo, P. Y. (2022). Tenofovir Alafenamide Attenuates Effects of Diabetes and Body Mass on Serum Alanine Aminotransferase Activities in Patients With Chronic Hepatitis B. *Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association, 20* (1), 230-2.

23. Linton, M. F., Moslehi, J. J., & Babaev, V. R. (2019). Akt Signalling in Macrophage Polarization, Survival, and Atherosclerosis.*International journal of molecular sciences, 20* (11), 2703.

24. Lopez-Pelaez, M., Soria-Castro, I., Bosca, L., Fernandez, M., & Alemany, S. (2011). Cot/tpl2 activity is required for TLR-induced activation of the Akt p70 S6k pathway in macrophages: Implications for NO synthase 2 expression. *European journal of immunology, 41* (6), 1733-1741.

25. Lee, S. J., Seo, K. W., & Kim, C. D. (2015). LPS Increases 5-LO Expression on Monocytes via an Activation of Akt-Sp1/NF-kappaB Pathways.*The Korean journal of physiology & pharmacology: official*

journal of the Korean Physiological Society and the Korean Society of Pharmacology, 19 (3), 263-268.

FIGURE LEGENDS

Figure 1. Tenofovir alafenamide (TAF) treatment ameliorates liver injury in the nonalcoholic steatohepatitis (NASH) mouse model.(A) Experimental schedule for the NASH mouse model using streptozotocin (STZ) injection and high-fat, high-cholesterol (HFHC) diet. C57BL/6J mice were injected with 0.2 mg STZ and fed HFHC diet or carbohydrate (CHO) diet and treated with mock or TAF. NASH resulted from the HFHC diet after STZ subcutaneous injection for 4 weeks (n = 6-10). (B) Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and triglyceride (TRIG) levels in STZ-injected, HFHC-fed NASH experimental animals treated with mock or TAF. (C) Representative histological analyses of liver sections stained with haematoxylin and eosin, Sirius red, anti-lipopolysaccharide (LPS) and anti-toll-like receptor 4 (TLR4) expression in STZ-injected mock-treated HFHC diet-fed and STZ-injected TAF-treated HFHC diet-fed mice livers. Original magnification: 50×. (D) Comparison of the frequency of LPS- or TLR4-positive cells between STZ-injected, HFHC diet-fed NASH experimental animals treated with mock or TAF. Morphometric analysis of Sirius Red-stained liver section was performed from 5 fields of 5 liver sections per group. Comparison of NASH Activity Score (NAS) from mouse liver specimens among each treatment group. (E) Experimental schedule for the NASH mouse model using choline-deficient, L-amino acid-defined, high-fat (CDAHF) diet-fed mock or TAF. C57BL/6J mice were fed a CDAHF or CHO diet for 6 weeks (n = 7-10). (F) Serum ALT, AST, and TRIG levels in CDAHF diet-fed NASH experimental animals treated with mock or TAF. *p<0.05, **p <0.001, ***p<0.001.

Figure 2. TAF decreases the number of activated mononuclear phagocytes (MPs) in the STZ-injected, HFHC diet-fed NASH mouse liver.(A) Representative dot plot describing the proportions of recruited/resident MPs. Data are representative of five independent experiments. (B) Recruited ($CD11b^{high}F4/80^{low}$) and resident ($CD11b^{low}F4/80^{high}$) MP cell number per g liver weight and cell percentage in the mock or TAF-treated STZ-injected, HFHC diet-fed NASH mouse model was counted using flow cytometry. (C) Mean fluorescence intensity (MFI) of programmed death ligand 1 (PD-L1), IAIE and MER proto-oncogene, and tyrosine kinase (MerTK) in $CD11b^{+}F4/80^{+}$ cells. (D) Frequency of PD-$L1^{+}$/$IAIE^{+}$/$MerTK^{+}$intrahepatic MP cells in the STZ-injected, HFHC diet-fed NASH mouse model treated with mock or TAF. (E) Relative mRNA expression of inflammatory genes in mock or TAF-treated NASH mouse liver. *p<0.05, **p <0.001, ***p<0.001.

Figure 3. TAF decreases the number of activated MPs in the choline-deficient, L-amino acid-defined, high-fat (CDAHF) diet-fed NASH mouse liver. (A) Representative dot plot describing the proportions of recruited/resident MPs. Data are representative of five independent experiments. (B) Recruited ($CD11b^{high}F4/80^{low}$) and resident ($CD11b^{low}F4/80^{high}$) MP cell number per g liver weight and cell percentage in the mock or TAF-treated CDAHF diet-fed NASH mouse model was counted using flow cytometry. (C) MFI of PD-L1, IAIE and MerTK in $CD11b^{+}$ $F4/80^{+}$ cells. (D) Frequency of PD-$L1^{+}$/$IAIE^{+}$/$MerTK^{+}$intrahepatic MP cells in the CDAHF diet-fed NASH mouse model treated with mock or TAF. *p <0.05, **p<0.001, ***p <0.001.

Figure 4. *Ex vivo* effect of TAF on MPs isolated from the livers of the thioacetamide (TAA)-induced liver injury mouse model. (A) The liver injury mouse model experimental schedule using TAA injection (n = 5). Mice were intraperitoneally injected with 100 or 150 mg/kg TAA three times a week. (B) Representative histological analyses of liver sections stained with H&E, Sirius red in TAA-treated mouse liver. Original magnification: 50×. Morphometric analysis of Sirius Red-stained liver section was done from 5 fields of 5 liver sections per group. (C)*Ex vivo* experimental scheme of MPs isolated from the liver injury mouse model treated with mock or TAF. The mice livers were perfused, and MPs were isolated using gradient centrifugation. (D) Frequency of PD-$L1^{+}$ cells among isolated $CD11b^{+}F4/80^{+}$ cells after treatment with mock or TAF. (E) MFI of PD-L1 in isolated $CD11b^{+}F4/80^{+}$ cells after mock or TAF treatment. *p <0.05, **p <0.01, ***p <0.001.

Figure 5. TAF blocks the phosphorylation of AKT in monocytes/MPs.

(A) The expression of AKT and mTOR in mock or TAF-treated STZ-injected, HFHC diet-fed NASH mice liver tissues were analysed using western blotting. The relative expression of phosphorylated proteins was normalised to total protein expression. Data are representative of three independent experiments. The graph in the right panel shows the quantitative densitometry immunoblotting analysis results. (B) Experimental scheme of isolated peripheral $CD14^{+}$monocytes. Cells were pre-treated with TAF (1, 5 μM) for 6 h and stimulated with 1 μg/mL LPS for 24 h. Dot plots of surface human leukocyte antigen-DR isotype (HLA-DR) and PD-L1 levels in stimulated monocytes treated with mock or TAF. Data are representative of five independent experiments. (C) Frequency of $PD\text{-}L1^{+}/HLA\text{-}DR^{+}$ cells in activated $CD14^{+}$ monocyte cells treated with mock or TAF. (D) MFIs of PD-L1 and HLA-DR in activated $CD14^{+}$ monocyte cells after mock or TAF treatment. (E) Representative dot plots of AKT and pAKT expression in mock or TAF-treated activated peripheral $CD14^{+}$ monocytes analysed using flow cytometry. MFI of pAKT in mock- or TAF-treated activated peripheral $CD14^{+}$ monocytes, analysed using flow cytometry. (F) Schematic representation of the TAF role in NASH liver. $*p<0.05$, $**p<0.001$, $***p<0.001$.