An inhibitor of leukotriene-A_4 hydrolase from bat salivary glands facilitates virus infection

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Bats are increasingly accepted as potential reservoirs of many viruses that cause zoonotic disease outbreaks through spillover to other animals and humans. However, our understanding of the factors that contribute to virus spillover from bats is very limited. Here, we identified and characterized an immunosuppressant protein (MTX) that is highly concentrated in the submandibular salivary gland of the bat, *Myotis pilosus*. By selectively inhibiting the epoxide hydrolase function of leukotriene-A_4 hydrolase (LTA_4H) to inhibit LTA_4 hydrolysis and the generation of leukotriene B_4 (LTB_4), a potent lipid chemoattractant for host defense against infection, MTX inhibited the antiviral responses of the host and facilitated viral infection. MTX had no effect on the aminopeptidase function of LTA_4H and therefore did not impair the antiinflammatory function of LTA_4H. MTX potently inhibited proinflammatory proteases (i.e., plasmin, trypsin, and elastase) to induce immune tolerance and maintain high stability. In mouse models, influenza A virus (IAV) H1N1 infection and pathogenicity were exacerbated by MTX but were reversed by interfering with the effects of MTX on LTA_4H or exogenous LTB_4 administration. This study provides deeper insight into immunologically privileged sites for microbial community residence in bats and supports the therapeutic potential of targeting MTX-LTA_4H.

Myotis pilosus | salivary gland | toxin | immunosuppressive | virus transmission

Certain high-impact zoonotic disease outbreaks have been linked to bat-borne viruses, including severe acute respiratory syndrome coronavirus (SARS-CoV), rabies virus, influenza virus, Hendra and Nipah viruses, Ebola and Marburg hemorrhagic fever filoviruses, Middle East respiratory syndrome (MERS) coronavirus, and most recently, SARS-CoV-2 (1–3). Bats are increasingly being considered as potential reservoirs harboring a diverse and complex microbial community, including many known and unknown viruses (4–11). More than 15 families of zoonotic viruses have been identified in >200 species of bats worldwide (4–11), many of which can spill over into animal and human populations and cause diseases. Thus, there may be immunologically privileged sites for microbial community residence and/or factors contributing to bat-borne pathogen spillover. However, information on bat biology and immunology remains insufficient to clarify the generation of immunological privilege and immune tolerance. Furthermore, studies on the factors contributing to the risk of bat pathogen spillover and cross-species transmission are limited.

Pathogen infection events in bat tissue also remain poorly understood, although virus–host interactions between the Egyptian fruit bat (*Rousettus aegyptiacus*) and Marburg virus provide strong evidence of viral shedding through oral secretions, indicating a viable route for horizontal infection (12). Thus, we speculate that host factors related to the oral cavities of bats may facilitate virus residence and invasion. As a ubiquitously expressed proinflammatory epoxide hydrolase, leukotriene A_4 hydrolase (LTA_4H) bears two opposing roles, i.e., proinflammation by producing leukotriene B_4 (LTB_4) and antiinflammation (13, 14). LTB_4 is a potent chemoattractant, which acts primarily on neutrophils, eosinophils, T cells, and mast cells (15–17). LTB_4 exerts its biological functions via two types of G protein–coupled receptors (GPCRs): i.e., LTB_4 receptors 1 and 2 (BLT1/2) (18, 19). Produced by its rate-limiting enzyme LTA_4H, LTB_4 activates the extracellular signal–regulated kinase (ERK), protein kinase B (AKT), and nuclear factor-κB (NF-κB) subunit p65 signaling pathway through BLT; licenses

Significance

An immunosuppressant protein (MTX), which facilitates virus infection by inhibiting leukotriene A_4 hydrolase (LTA_4H) to produce the lipid chemoattractant leukotriene B_4 (LTB_4), was identified and characterized from the submandibular salivary glands of the bat *Myotis pilosus*. To the best of our knowledge, this is a report of an endogenous LTA_4H inhibitor in animals. MTX was highly concentrated in the bat salivary glands, suggesting a mechanism for the generation of immunological privilege and immune tolerance and providing evidence of viral shedding through oral secretions. Moreover, given that the immunosuppressant MTX selectively inhibited the proinflammatory activity of LTA_4H, without affecting its antiinflammatory activity, MTX might be a potential candidate for the development of antiinflammatory drugs by targeting the LTA_4-LTA_4H-LTB_4 inflammatory axis.

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The authors declare no competing interest.

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inflammasome activation; increases the expression of the Toll/Interleukin 1 receptor (TIR) adaptor MyD88 and the transcription factors NF-κB, activator protein-1 (AP-1), and purine rich box-1 (PU.1); and promotes the production of proinflammatory factors, leading to an inflammatory response in the host, which intensifies the production of LTA_4H and LTB_4 and activates the antiviral immune system (20–25). In this work, we identified and characterized a LTA_4H inhibitor (MTX) from the submandibular salivary glands (also known as "submaxillary" glands) of the bat *Myotis pilosus* at a high concentration (~1% of total protein) and explored its effects on host immunity and virus invasion. By targeting LTA_4H with high affinity to inhibit the production of the proinflammatory mediator LTB_4, MTX effectively inhibited proinflammatory functions and antiviral immunity of the host to facilitate virus infection.

Results

MTX Is Locally Concentrated in Bat Salivary Glands and Acts as an Inhibitor of LTA_4H. We purified and characterized a protein with a molecular weight of 13 kDa from the salivary glands of *M. pilosus*. This protein (MTX) inhibited the activity of several proinflammatory enzymes, including plasmin (inhibitory constant K_i = 79.99 nM), trypsin (K_i = 67.51 nM), and elastase (K_i = 35.94 nM), and showed a half-life of 16 h in mouse plasma (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S1). The cDNA encoding the MTX precursor (*SI Appendix*, Fig. S1*E*) of 135 amino acid (aa) residues, including an 18-aa predicted signal peptide, and mature MTX was cloned. BLAST searching showed that MTX was a double-knot Kazal-type serine protease inhibitor. Immunoblot analysis and enzyme-linked immunosorbent assay (ELISA) indicated that MTX was distributed in the submaxillary salivary glands at a high concentration (~1% of total protein, 7 to 10 μg/mg), but was not found in other tissues at significant concentrations (Fig. 1*A*). We expressed and purified MTX (*SI Appendix*, Fig. S2) to analyze its enzyme inhibitory activity and function in vitro and in vivo. Based on the coimmunoprecipitation analysis, MTX directly interacted with LTA_4H (*SI Appendix*, Fig. S3), as confirmed by surface plasmon resonance (SPR) (Fig. 1*C*) and native-polyacrylamide gel electrophoresis (PAGE) analyses (Fig. 1*D*). SPR analysis revealed that the equilibrium dissociation constant (*KD*) for the interaction between MTX and LTA_4H was 0.45 nM, thus showing a high affinity. MTX selectively inhibited the epoxide hydrolase function of LTA_4H (K_i: 1.23 μM) to inhibit leukotriene A_4 (LTA_4) hydrolysis and block the generation of leukotriene B_4 (LTB_4) (Fig. 1 *E*–*G*). However, MTX did not affect the aminopeptidase function of LTA_4H (*SI Appendix*, Fig. S4*A*) and therefore did not impair its antiinflammatory function. In addition, MTX had no effect on phospholipase A2 (PLA2), cytochrome p450 (CYP450), and total cyclooxygenase (COX) enzyme activities (*SI Appendix*, Fig. S4 *B, C* and *D*) in vitro.

LTA_4H Is Elevated in Response to H1N1 Infection. LTA_4H plays a key role in catalyzing the final and rate-limiting step of LTB_4 biosynthesis, which has been implicated in many acute and chronic inflammatory diseases (13, 14, 26–29). Significantly elevated plasma concentrations of LTA_4H and LTB_4 were observed in the H1N1-infected mouse model (Fig. 1 *H* and *I*), suggesting that the increase in LTB_4 production following LTA_4H up-regulation is an antiviral response of the host.

MTX Augments H1N1 Infection by Inhibiting Inflammatory Axis LTA_4-LTA_4H-LTB_4. As a key factor in eicosanoid storms caused by infection, LTA_4H is a potent contributor to inflammation (13). Furthermore, eicosanoid storms are associated with the occurrence of cytokine storms (30, 31). Given the potent ability of MTX to inhibit LTA_4H, we tested its effects on the inflammatory axis LTA_4-LTA_4H-LTB_4 and cytokine production induced by viral infection (Fig. 2 *A* and *B* and *SI Appendix*, Figs. S5 *A* and *D–I* and S6). Following H1N1 infection, LTA_4H, inflammatory mediator LTB_4 (derived from LTA_4 hydrolysis by LTA_4H), proinflammatory cytokines interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β), and inflammatory cell chemokine interleukin 8 (IL-8) were significantly elevated in all tested cells (i.e., nonsmall cell lung cancer [NSCLC] line A549 cells (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S6*E*), human lung fibroblast cell line Medical Research Council 5 (MRC-5) cells (*SI Appendix*, Fig. S6 *B, D, G*), plasmacytoid dendritic cells (pDCs) (*SI Appendix*, Fig. S6 *A, C,* and *H*), and human umbilical vein endothelial cells (HUVECs) (*SI Appendix*, Figs. S5 *A* and *D–I* and S6*F*). In contrast, these elevations were inhibited by administration of MTX or antibodies against BLT1/2 (0.5 μg/mL of anti-BLT1 antibody mixed with 0.5 μg/mL of anti-BLT2 antibody). In addition, MTX also reduced the production of prostaglandin E_2 (PGE_2) and cysteinyl leukotrienes (CysLTs) induced by H1N1 in A549 cells (Fig. 2 *B*, *iii* and *iv*). LTA_4H is up-regulated during inflammation (32–35). Here, as illustrated in Fig. 2 *A, ii,* and *B*, *i*, both MTX and anti-BLT1/2 treatment inhibited the elevation of LTA_4H induced by H1N1 infection, which was likely due to the inhibition of inflammation. The antiinflammatory activity of MTX and anti-BLT1/2 impaired the antiviral ability of the cells and augmented H1N1 infection, as observed by the increase in virus nucleoprotein (NP) (H1N1 PR8 NP) expression and virus titer (Fig. 2*A*).

MTX Inhibits LTB_4 Receptor Signaling Induced by H1N1 Infection. MTX inhibited LTB_4 production by inhibiting LTA_4H, suggesting that it may also inhibit LTB_4 receptor signaling. The activation of various inflammatory signaling pathway proteins, i.e., ERK, AKT, and NF-κB subunit p65, is related to LTB_4 and BLT (20–25). As illustrated in Fig. 2 *C*, *i* and *SI Appendix*, Fig. S5*B*, H1N1 infection induced trimerization and dimerization of the LTB_4 receptor BLT1, and thus activated the receptor in A549 cells (Fig. 2 *C*, *i*) and HUVECs (*SI Appendix*, Fig. S5*B*). In contrast, MTX and anti-BLT1/2 antibodies inhibited trimerization and dimerization. Consistently, MTX and anti-BLT1/2 also blocked the downstream signals of the LTB_4 receptor and inhibited p65 activation and ERK and AKT phosphorylation induced by H1N1 infection in A549 cells (Fig. 2 *C*, *ii–iv* and *SI Appendix*, Fig. S7*A*) and HUVECs (*SI Appendix*, Fig. S5*C*). Exogenous addition of LTB_4 in A549 cells increased the phosphorylation of ERK and AKT and activation of p65 (Fig. 2*D* and *SI Appendix*, Fig. S7*B*), thus suppressing NP expression (Fig. 2*E* and *SI Appendix*, Fig. S7*C*). The addition of BLT1 antagonists U75302 and MTX inhibited the activation of inflammatory signaling pathway proteins and promoted the expression of NP (Fig. 2 *D* and *E* and *SI Appendix*, Fig. S7 *B* and *C*). In addition, MTX and BLT antibodies also inhibited the interferon (IFN) signaling pathway (*SI Appendix*, Fig. S8). Thus, MTX inhibited immune activation following viral infection by inhibiting LTB_4 receptor activation.

MTX Inhibits Neutrophil Chemotaxis to Facilitate H1N1 Infection. Neutrophil chemotaxis can be promoted by LTB_4 and chemokines and plays an important role in antiviral immunity in hosts (36). Given the inhibitory activity of MTX on the LTA_4-LTA_4H-LTB_4 axis inflammation, we tested its effects on neutrophil chemotaxis in cell coculture systems of A549-polymorphonuclear neutrophils (PMNs) (Fig. 3) and HUVECs-PMNs (*SI Appendix*, Fig. S9). H1N1 infection promoted neutrophil chemotaxis and activation, as indicated by the up-regulation of myeloperoxidase (MPO) expression of PMNs on the A549 cells and HUVECs (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S9*A*). However, MTX and anti-BLT1/2 antibodies inhibited neutrophil activation, as indicated by the down-regulation of MPO expression (Fig. 3 *A* and *B*

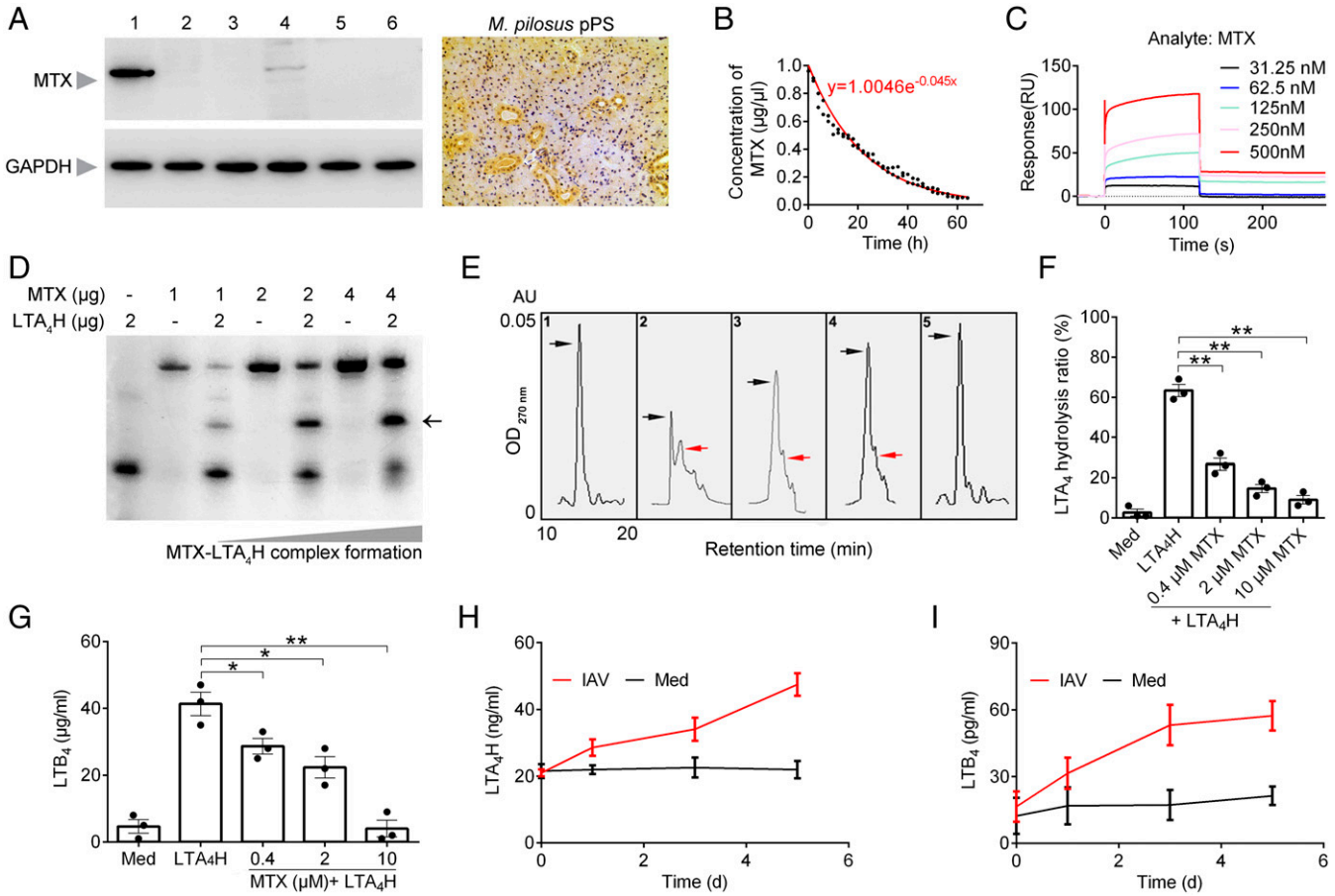

Fig. 1. MTX is concentrated in bat salivary glands and acts as an inhibitor of LTA_4H, which is up-regulated by virus infection. (*A*) Immunoblot (*Left*) and immunohistochemical (*Right*) analyses of MTX distribution in bat tissues. Lane 1, salivary gland; lane 2, heart; lane 3, liver; lane 4, skin; lane 5, muscle; lane 6, blood. pPS, posterior principal salivary gland. (*B*) Stability analysis of MTX in mouse plasma by ELISA. (*C*) Interaction between MTX and LTA_4H by SPR. (*D*) MTX-LTA_4H complex (marked by arrow) formation analyzed by native-PAGE. RP-HPLC, reverse-phase high-performance liquid chromatography (*E*) and LTA4 hydrolysis ratio (*F*) analyses showing that MTX inhibits LTA_4 (50 μM) hydrolysis by LTA_4H (100 nM) to generate LTB_4. (*E*) Box 1, LTA_4 alone; box 2, LTA_4 and LTA_4H; boxes 3 to 5, LTA_4, LTA_4H, and 0.4 to 10 μM MTX; black arrow shows LTA_4 peak; red arrow shows LTB_4 peak. (*F*) LTA_4 hydrolysis ratio = (1 – LTA_4 peak area of treatment group/LTA_4 peak area of LTA_4 alone [Med] group) %. (*G*) ELISA analysis of concentration of LTB_4 in the LTA_4 mixture preincubated with or without MTX and LTA_4H. Concentration of LTA_4H (*H*) and LTB_4 (*I*) in mouse plasma after H1N1 virus (IAV) infection was determined by ELISA. Each symbol (*B*, *F*, and *G*) represents an individual technical replicate in one experiment. Small horizontal lines (*F–I*) (*F* and *I* [$n = 6$]) indicate mean ± SEM (SEM). Data are representative (*A* and *C–E*) of three independent experiments and are from (*B* and *F–I*) two independent experiments. $*P < 0.05$, $**P < 0.01$ by one-way ANOVA with Fisher's protected *t* tests.

and *SI Appendix*, Fig. S9*A*) and elevation of viral load (NP expression, Fig. 3 *A* and *G* and *SI Appendix*, Fig. S9 *A* and *E*) and viral titers in the cells (Fig. 3*F*). Notably, 2 μM MTX and anti-BLT1/2 antibody treatment nearly completely inhibited neutrophil activation. As expected, MTX and anti-BLT1/2 antibodies in the cell coculture systems inhibited LTA_4H, LTB_4, and IL-8 production (Fig. 3 *C–E* and *SI Appendix*, Fig. S9 *B–D*).

MTX Facilitates H1N1 Infection in Mice. The in vivo effects of MTX on H1N1 infection were investigated using wild-type (WT) and *LTA4H* gene knockout ($LTA_4H^{-/-}$) mice. As illustrated by immunofluorescence analysis in Fig. 4*A*, after 1 d of H1N1 infection (intranasal inoculation with 10^3 tissue culture infection dose 50% [$TCID_{50}$] of H1N1 PR8), both low viral load and high neutrophil activation (indicated by MPO expression) were found in the lungs of male WT mice. Conversely, very high viral loads and low neutrophil activation were observed in the lungs of male $LTA_4H^{-/-}$ mice. The treatment of MTX (50 μg per mouse) and anti-BLT1/2 antibody (5 μg of BLT1 antibody mixed with 5 μg of BLT2 antibody per mouse) increased viral susceptibility in WT mice with similar viral loads and neutrophil activation as observed in $LTA_4H^{-/-}$ mice. Inflammation can promote LTA_4H up-regulation (32–35), and thus MTX and anti-BLT1/2 antibody treatment likely down-regulated LTA_4H expression (Fig. 4 *A–D* and *SI Appendix*, Fig. S10 *A* and *C*) by inhibiting inflammation and consequently reducing the production of LTB_4 (Fig. 4*E*). The formation of neutrophil extracellular traps (NETs) was also inhibited (*SI Appendix*, Fig. S10 *A* and *B*). The concentrations of LTB_4, IL-6, IL-1β, TNF-α, and CXCL1 and gene expression levels of *IL-6*, *IL-1β*, *TNF-α*, and *CXCL1* were much lower in the lung tissues of $LTA_4H^{-/-}$ mice infected with the H1N1 virus than in that of WT mice (Fig. 4 *E–I*). Lipidomic analysis also showed the down-regulation of LTB_4 and prostaglandins (PGs) in the MTX-treated group after H1N1 infection (*SI Appendix*, Fig. S10*D*). A large number of cytokines (i.e., IL-1β, IL-6, and TNF-α) are known to enhance PGE_2 production by affecting PLA2 and COX (37–40). Although MTX had no effect on PLA2 and COX enzyme activities in vitro, MTX may have reduced the production of PGs (*SI Appendix*, Fig. S10*D*) through reduced inflammation in vivo. Moreover, in mice, exogenous administration of LTB_4 may also increased the content of PGE_2 by increasing lung cytokines (*SI Appendix*, Fig. S11 *D–F*). Upon H1N1 infection, the chemotaxis and activation

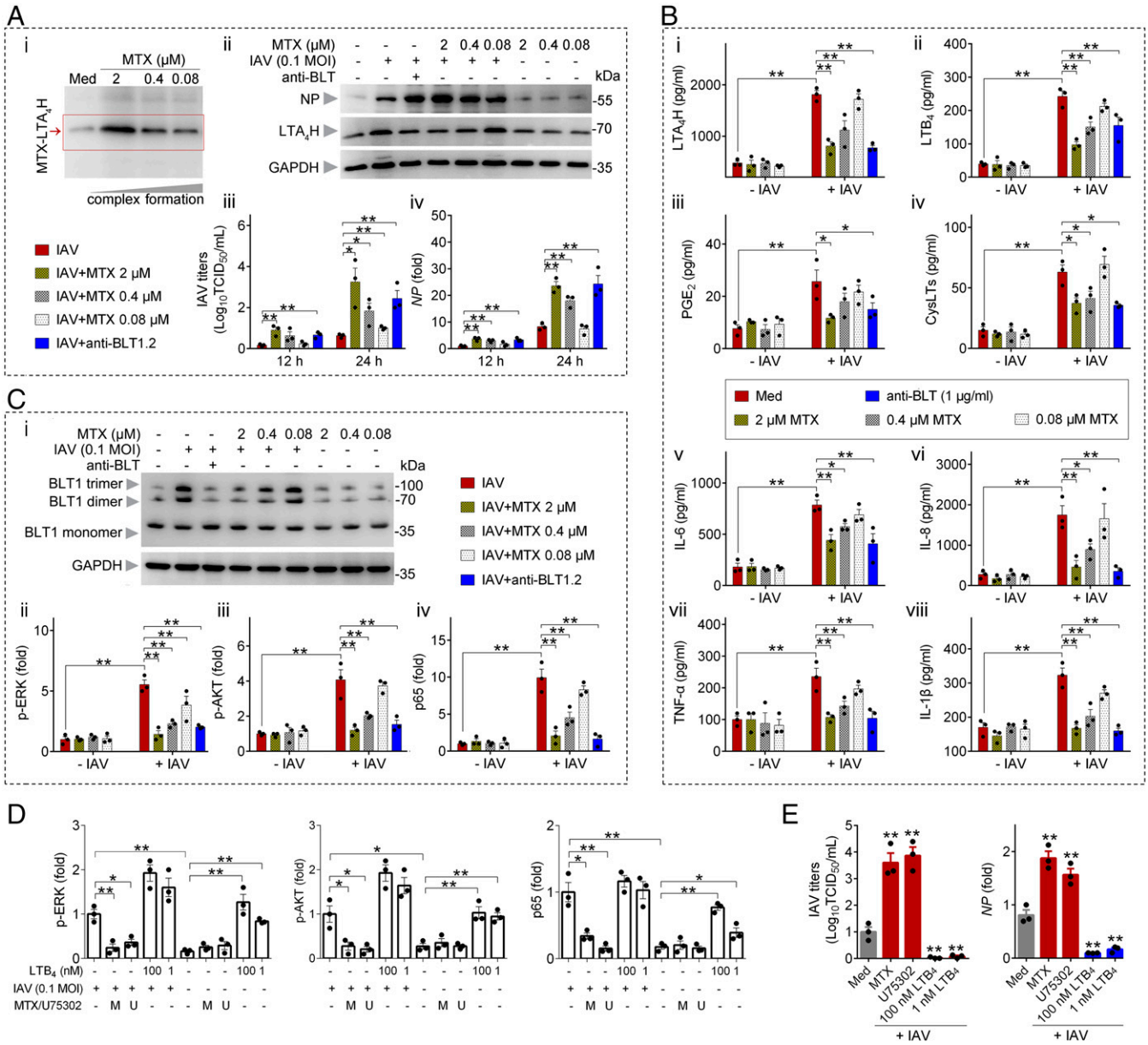

Fig. 2. MTX augments H1N1 infection in A549 cells by inhibiting LTB_4 receptor signaling and LTA_4-LTA_4H-LTB_4 inflammatory axis. (*A*) A549 cells were infected with H1N1 virus (IAV) for 12 or 24 h in the presence (+) or absence (–) of anti-BLT1/2 antibodies (anti-BLT1 antibody [0.5 μg/mL] mixed with anti-BLT2 antibody [0.5 μg/mL]) or different concentrations of MTX. *A*, *i*, Immunoblot analysis of MTX-LTA_4H complex in cell supernatant after viral infection for 24 h. *A*, *ii*, Immunoblot analysis of NP (H1N1 nucleoprotein) and LTA_4H (24 h). IAV titers (*A*, *iii*) and *NP* gene expression (*A*, *iv*) were determined by $TCID_{50}$ and qRT-PCR (relative to 12 h IAV group), respectively. (*B*) Concentrations of LTA_4H (*i*), LTB_4 (*ii*), PGE_2 (*iii*), CysLTs (*iv*), IL-6 (*v*), IL-8 (*vi*), TNF-α (*vii*), and IL-1β (*viii*) in cell supernatants from *A* measured by ELISA (24 h). *C*, *i*, Effects of MTX on trimerization and dimerization of LTB_4 receptor 1 (BLT1) in A549 cells induced by H1N1 virus (IAV) infection for 1 h in the presence or absence of anti-BLT1/2 antibodies (anti-BLT1 antibody [0.5 μg/mL] mixed with anti-BLT2 antibody [0.5 μg/mL] or different concentrations of MTX by immunoblot analysis. Cell lysates were cross-linked for 15 min with disuccinimidyl suberate. (*C*, *ii–iv*) Effects of MTX on downstream signals of LTB_4 receptor activation promoted by H1N1 infection for 1 h determined by immunoblot analysis. Quantification of signaling intensity of p-ERK (*ii*) and p-AKT (*iii*) using GAPDH as the loading control, and quantification of p65 (*iv*) signaling intensity using histone as the loading control (blots in *SI Appendix*, Fig. S2*A*). (*D*) LTB_4 activated the downstream inflammatory signaling pathway of BLT1 to inhibit viral infection. A549 cells treated with U75302 (BLT1 antagonist, 100 nM), MTX (2 μM), or LTB_4 in the presence or absence of H1N1 virus. After 1 h, we detected activation of signaling pathway proteins p65, ERK, and AKT and their phosphorylation by immunoblot analysis (blots in *SI Appendix*, Fig. S2*B*). After 24 h of virus infection in each treatment group, IAV titer and *NP* gene expression were detected by $TCID_{50}$ and qRT-PCR, respectively (*E*). Each symbol (*A*, *iii* and *iv*; *B*; *C*, *ii–iv*; *D*; and *E*) indicates an individual technical replicate in one experiment, and small horizontal (*A*, *iii* and *iv*; *B*; *C*, *ii–iv*; *D*; and *E*) lines indicate mean ± SEM. Data are representative of (*A*, *i* and *ii*; and *C*, *i*) and are from (*A*, *iii* and *iv*; *B*; *C*, *ii–iv*; *D*; and *E*) three independent experiments. $*P < 0.05$, $**P < 0.01$ by one-way ANOVA with Fisher's protected *t* tests.

of neutrophils, eosinophils, DCs, $CD16^+CD3^-$ natural killer (NK) cells, and $CD8^+$ T cells in the lungs of WT mice were significantly stimulated. However, MTX, anti-BLT1/2, and *LTA_4H* knockout suppressed chemotaxis and activation of these cells (Fig. 4 *J–N* and *SI Appendix*, Fig. S12). These results suggest that MTX plays an immunosuppressive role by inhibiting LTA_4H.

MTX Exacerbates Pathological Injuries Caused by H1N1 Infection. After 9 d of H1N1 infection, compared with low virus NP expression in the lungs and livers of WT mice, immunofluorescence indicated high NP expression in *$LTA_4H^{-/-}$* mice and in mice administered either MTX or anti-BLT1/2 antibodies (Fig. 5*A*). In addition, TdT-mediated dUTP nick-end labeling

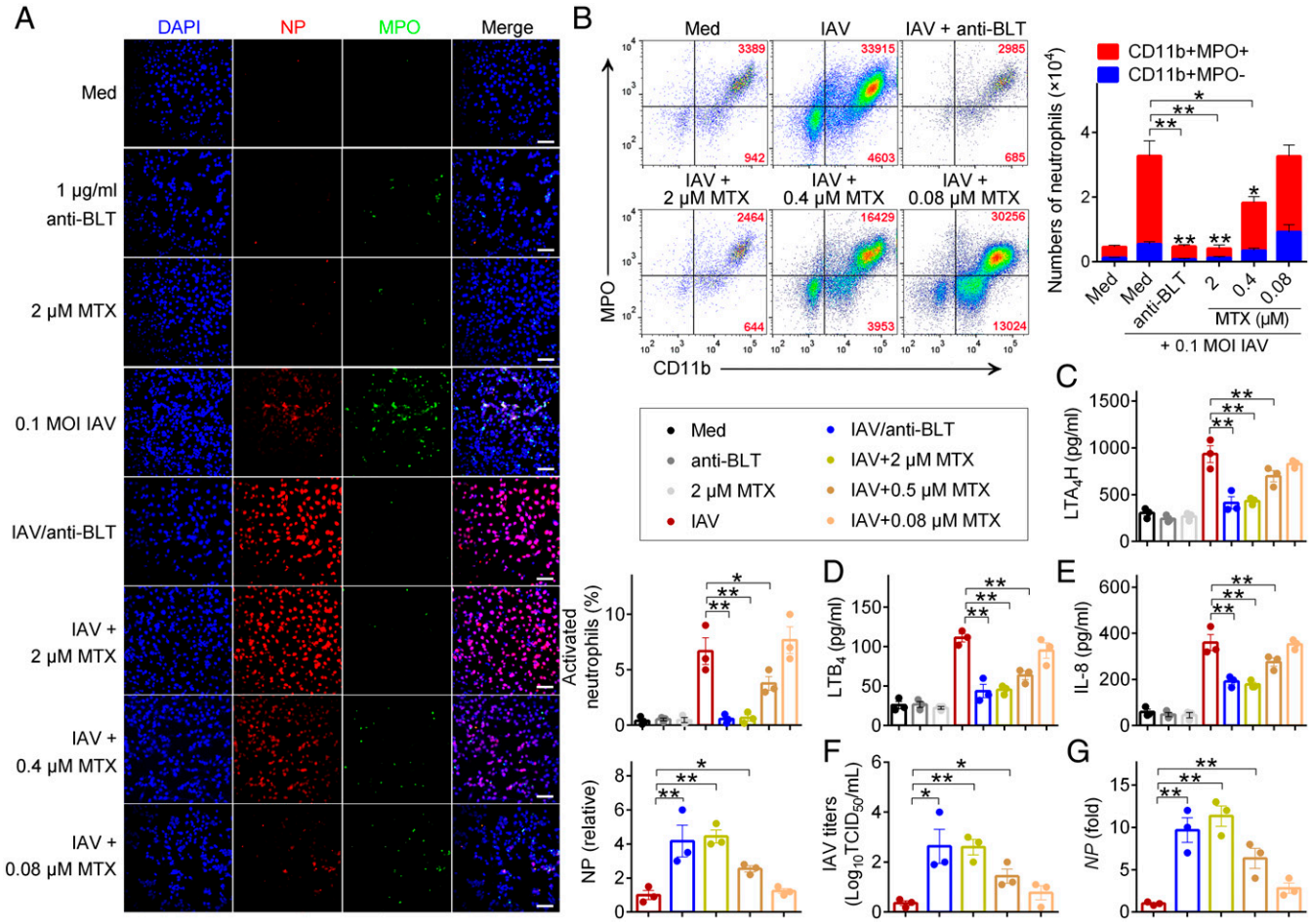

Fig. 3. MTX inhibits neutrophil chemotaxis to facilitate H1N1 infection. (*A*) Effects of MTX on neutrophil chemotaxis in the A549-PMN cell coculture system. Neutrophil activation was indicated by MPO expression on A549 cells. MPO and NP were analyzed by immunofluorescence 24 h after H1N1 virus (IAV) infection. (Scale bars, 50 μm.) (*B*) Flow cytometry analysis of neutrophil chemotaxis, with the number of neutrophils in the lower layer of the Transwell coculture system from *A* was calculated. Concentrations of LTA_4H (*C*), LTB_4 (*D*), and IL-8 (*E*) in cell supernatant from *A* were analyzed by ELISA; and IAV titer (*F*) and *NP* gene expression level (*G*) were detected by $TCID_{50}$ and qRT-PCR, respectively. Each symbol (*A*, *Right*, *C–G*) indicates an individual technical replicate in one experiment, and small horizontal lines (*A*, *Right*, *B*, *Right* [$n = 3$], and *C–G*) indicate mean ± SEM. Data are representative of (*A* and *B, Left*) and are from (*A, Right*; and *C–G*) three independent experiments. $*P < 0.05$, $**P < 0.01$ by one-way ANOVA with Fisher's protected *t* tests.

(TUNEL) staining showed a much lower number of apoptotic cells in the lungs and livers of WT mice infected with H1N1 than in the $LTA_4H^{-/-}$, MTX, and anti-BLT1/2 mice (Fig. 5*A*). Hematoxylin and eosin (H&E) staining indicated that tissue injuries, including cell deformation (thickened alveoli septum in lungs) and inflammatory infiltration, were more severe in the $LTA_4H^{-/-}$, MTX, and anti-BLT1/2 mice than in the WT mice (Fig. 5*A*). In addition, at 9 d after infection, the serum H1N1 virus titer in the $LTA_4H^{-/-}$, MTX, and anti-BLT1/2 mice was much higher than that in the WT mice (Fig. 5 *B*, *i*). qRT-PCR also revealed that viral replication in the brain, kidney, lung, and liver was much higher in the $LTA_4H^{-/-}$, MTX, and anti-BLT1/2 mice than in the WT mice at day 9 after infection (Fig. 5 *B*, *ii–v*). Viral load in the lungs of the $LTA_4H^{-/-}$, MTX, and anti-BLT1/2 mice was much higher than that in the WT mice on days 1, 3, and 9 after infection (Fig. 5*C*). After 6 to 14 d of virus infection, body weight decreased significantly in the $LTA_4H^{-/-}$, MTX, and anti-BLT1/2 mice compared with that observed in the WT mice (Fig. 5*D*).

Interfering with the Inhibition of MTX on LTA_4H and Exogenous LTB_4 Administration Inhibits Viral Infection. Significant reductions in LTB_4 (Fig. 4*E*) were observed in the $LTA_4H^{-/-}$, MTX, and anti-BLT1/2 mice, which were associated with severe virus infection (Fig. 4 *A–C*) and low inflammatory response (Fig. 4 *F–N*), suggesting that inhibition of the LTA_4-LTA_4H-LTB_4 inflammatory axis promoted viral infection, and using exogenous LTB_4 administration to block LTA_4-LTA_4H-LTB_4 inhibition elicited by $LTA_4H^{-/-}$ or MTX likely inhibited viral infection. As shown in Fig. 5 *E–G*, the exogenous addition of LTB_4 (inoculated intranasally with 50 ng LTB_4 per mouse) reversed the increase in H1N1 infection in MTX-treated mice and $LTA_4^{-/-}$ mice. In addition, MTX inhibition of the LTA_4-LTA_4H-LTB_4 inflammatory axis was blocked by MTX antibody, exogenous LTB_4, and peptide KLVVDLTDIDPDVA (IM14, for MTX-LTA_4H interaction interference) administration (a designed peptide, *SI Appendix*, Fig. S13) (intravenous injection of LTB_4 0.1 mg/kg, MTX antibody 0.25 mg/kg, or IM14 2 mg/kg), which interfered with the effects of MTX on LTA_4H. Their effects on H1N1 infection were evaluated. As expected, the viral load in the lung was inhibited by exogenous LTB_4, MTX antibody, and IM14 administration (*SI Appendix*, Fig. S11 *A* and *B*). The interferences also inhibited the decrease in body weight caused by viral infection from days 6 to 14 (*SI Appendix*, Fig. S11*C*). Administration of MTX alone exacerbated H1N1 infection and pathological injury, while LTB_4, MTX antibody, and IM14 treatment significantly alleviated infection and injury (*SI Appendix*, Fig. S11*A*). Moreover, exogenous administration of

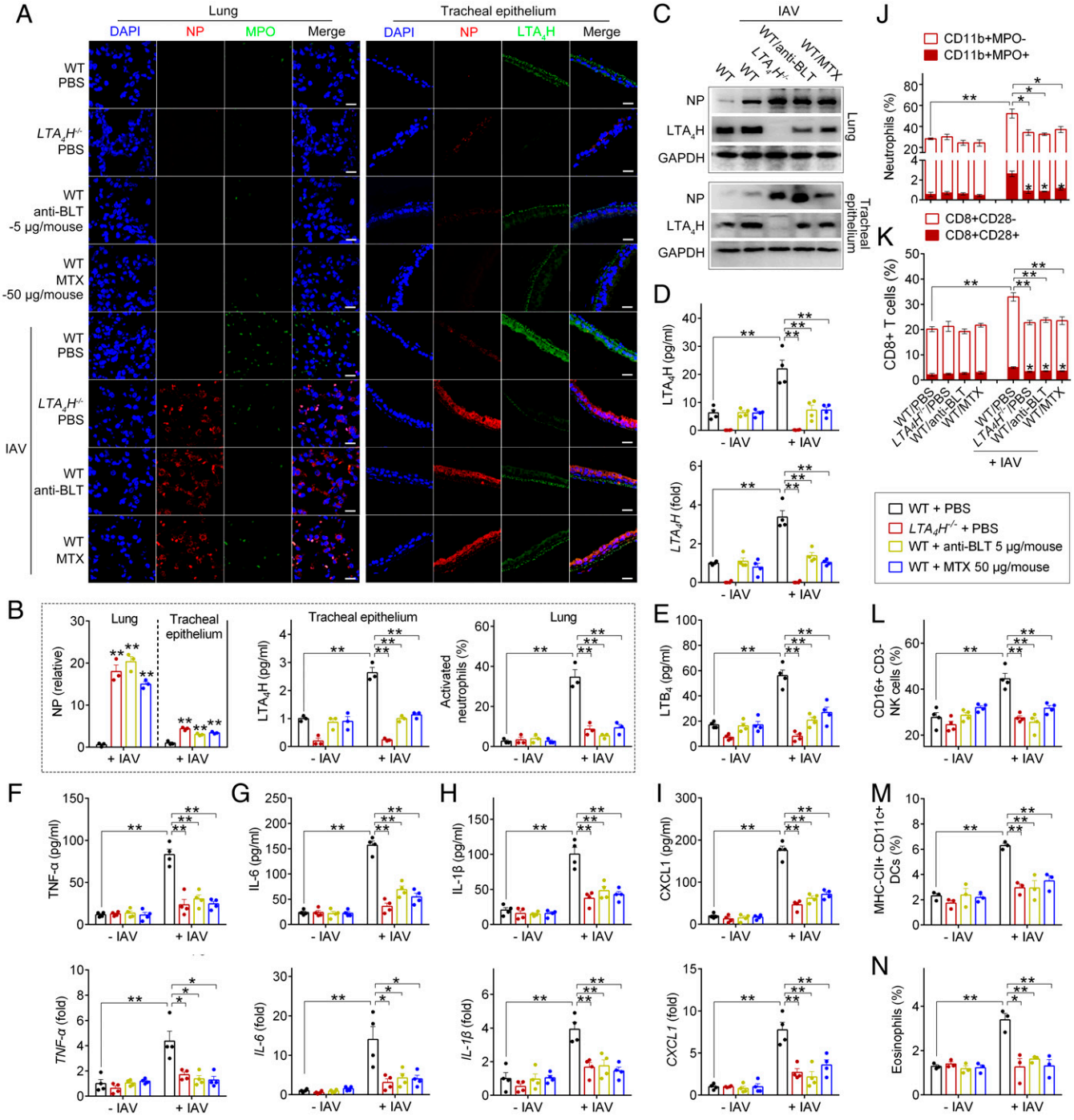

Fig. 4. MTX facilitates H1N1 infection in mice. (*A* and *B*) Effects of MTX (50 μg per mouse) and BLT1/2 antibodies (5 μg per mouse) on H1N1 virus load in lung and trachea of 5-wk-old male WT or *LTA$_4$H*$^{-/-}$ mice using immunofluorescence analysis after 1 d of H1N1 infection (intranasally inoculated with 10^3 $TCID_{50}$ of H1N1 PR8). (Scale bars, 20 μm.) (*C*) Immunoblot analysis of LTA_4H and NP in mouse lung and trachea from *A* and *B*. Concentrations of LTA_4H (*D*, *Up*), LTB_4 (*E*), TNF-α (*F*, *Up*), IL-6 (*G*, *Up*), IL-1β (*H*, *Up*), and CXCL1 (*I*, *Up*) in mouse lung from *A* and *B* were detected by ELISA. Gene expression levels of *LTA$_4$H* (*D*, *Down*), *TNF-α* (*F*, *Down*), *IL-6* (*G*, *Down*), *IL-1β* (*H*, *Down*), and *CXCL1* (*I*, *Down*) in mouse lung tissue from *A* and *B* were detected by qRT-PCR. Flow cytometry analysis of ratio of neutrophils (*J*), $CD8^+$ T cells (*K*), $CD16^+$ $CD3^-$ NK cells (*L*), MHC-CII^+ $CD11c^+$ dendritic cells (DCs) (*M*), and eosinophils (*N*) in cells isolated from mouse lungs from *A* and *B*. Each symbol (*B*, *D–I*, and *L–N*) indicates an individual mouse in one experiment, and small horizontal lines (*B* and *D–N*) (*J* and *K* [$n = 3$]) indicate mean ± SEM. Data are representative of (*A* and *C*) and are from (*B* and *D–N*) three independent experiments. $*P < 0.05$, $**P < 0.01$ by one-way ANOVA with Fisher's protected *t* tests.

LTB_4 increased the levels of IL-6, TNF-α, and PGE_2 in mice lungs (*SI Appendix*, Fig. S11 *D–F*), further demonstrates the immune defense effect of LTB_4 and its induction of cytokine production, as reported previously (20–25). The COX inhibitor piroxicam was administered to eliminate the influence of PGE_2 and PGD_2 on the MTX-LTB_4 pathway during viral infection. As illustrated in *SI Appendix*, Fig. S11 *G–I*, piroxicam had no effect on the increase in viral infection elicited by MTX, further indicating the immunosuppressive effect of MTX.

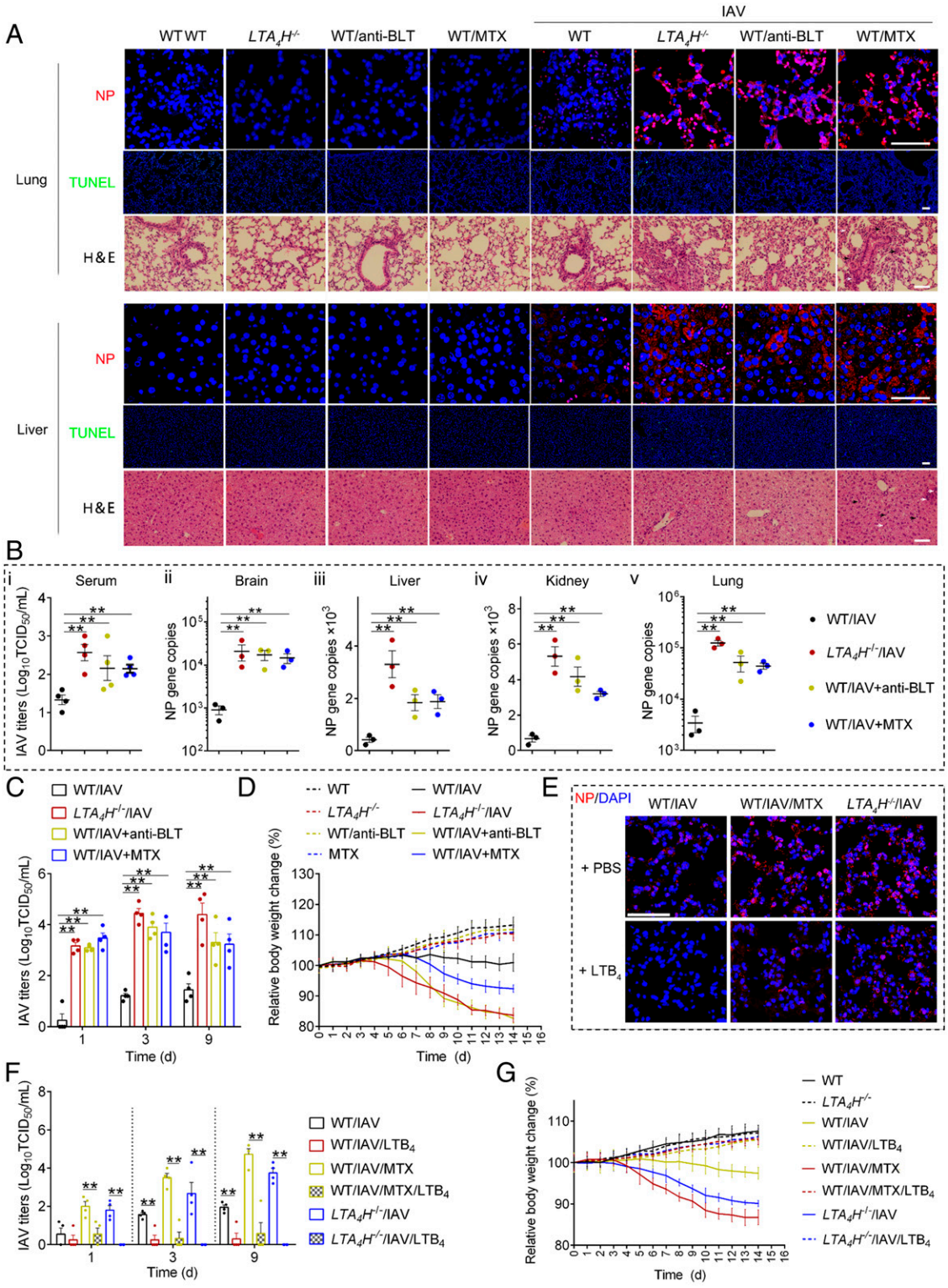

Fig. 5. MTX exacerbates pathological injuries caused by H1N1 infection. (*A–D*) Five-week-old male WT or $LTA_4H^{-/-}$ mice were inoculated intranasally with 10^3 $TCID_{50}$ of H1N1 PR8 alone (IAV) or in combination with BLT1/2 antibodies (5 μg per mouse) or MTX (50 μg per mouse). (*A*) Immunofluorescence analysis of NP, TUNEL, and H&E staining of lung and liver tissues on day 9. (Scale bars, 50 μm.) In the H&E-stained image, white arrow represents inflammatory cell infiltration, and black arrow represents cell deformation. (*B*) H1N1 titer (*i*) in serum of mice and qRT-PCR (assessing viral load) analysis of viral replication in brain (*ii*), liver (*iii*), kidney (*iv*), and lung (*v*) of mice on day 9 after infection. (*C*) H1N1 titers in lungs of mice on days 1, 3, and 9 after infection. (*D*) Daily body weight (relative to initial body weight) of mice within 14 d. (*E–G*) Five-week-old male C57 BL/6J mice (WT) or $LTA_4H^{-/-}$ mice inoculated intranasally with 10^3 $TCID_{50}$ of H1N1 PR8 (IAV) alone or in combination with MTX (50 μg per mouse, IAV/MTX), LTB_4 (50 ng per mouse), or MTX-LTB_4 mixture. (*E*) Immunofluorescence analysis of NP on day 9 after infection. (*F*) H1N1 titers in lungs of mice on days 1, 3, and 9 after infection. (*G*) Daily body weight (relative to initial body weight) of mice within 14 d. (*B*, *C*, and *F*) Each symbol indicates an individual mouse in one experiment. Small horizontal lines indicate mean ± SEM (*B*, *C*, and *F*) or mean ± SD (SD) (*D* [$n = 10$] and *G* [$n = 10$]). Data are representative of (*A* and *E*) and are from (*B–D; F*; and *G*) three independent experiments. $^{**}P < 0.01$ by one-way ANOVA with Fisher's protected *t* tests.

Discussion

Salivary glands have been described as a test bed for new and adaptive roles of secretory proteins (41–43). Although there are ∼1,100 species of bats worldwide, constituting 23% of all mammalian species, only salivary gland proteins associated with hematophagous behavior in vampire bats (three species) have been intensively studied (44–52). Although several viruses have been identified from bats, the crucial factors that profoundly contribute to the modification of host immune response and pathogen spillover are not yet known. In this study, from the bat salivary gland of *M. pilosus*, we identified and characterized a inhibitor (MTX) of LTA_4H, a potent inflammatory driver. Given its high concentration in the bat salivary glands (7 to 10 μg/mg tissue) and high inhibitory activity against host immunity, MTX possibly creates an immunologically privileged environment and induces host immune tolerance to resident microorganisms in bat oral cavities by counteracting the LTB_4-mediated host immune response. Based on its physiological concentration in the bat, MTX administration facilitated H1N1 infection and enhanced H1N1 invasion into a mammalian host by antagonizing the host immune response.

Leukotrienes contribute to antiinfective inflammatory responses (53–55). LTB_4 is an inflammatory lipid mediator and is implicated in many acute and chronic inflammatory diseases (14, 26, 56). LTA_4H is a ubiquitously expressed proinflammatory epoxide hydrolase bearing two opposing roles in immune regulation, including inflammation promotion by catalyzing the conversion of LTA_4 to the inflammatory mediator LTB_4 and antiinflammation by inactivating and degrading the chemotactic tripeptide Pro-Gly-Pro (13, 14). LTA_4H represents an attractive target for the design of superior antiinflammatory drugs as it triggers the final critical and rate-limiting step for LTB_4 biosynthesis (13). Previously, no LTA_4H endogenous inhibitor has been identified in animals. In the current study, we showed that MTX from the bat salivary gland selectively inhibited the epoxide hydrolase function of LTA_4H and suppressed LTA_4 hydrolysis to block LTB_4 generation. However, MTX did not affect the aminopeptidase activity of LTA_4H, and thus did not impair its antiinflammatory function.

As shown in Fig. 4, $LTA_4H^{-/-}$ mice exhibited decreased cytokine expression and escalated viral infection processes following H1N1 infection, which may be the result of decreased LTB_4 production (57, 58). LTB_4 is one of the most potent known chemoattractant, acting on neutrophils, eosinophils, T cells, and mast cells (15–17). LTB_4 causes neutrophil chemotaxis, and NETs formed by neutrophils show strong antibacterial properties and play a key role in antiviral immunity by oxidative bursts and phagocytosis (36). In the current study, the immunosuppressive protein MTX inhibited the chemotaxis of neutrophils and the occurrence of cytokine storm by inhibiting the production of the chemokine LTB_4, and thus promoted viral invasion. Cytokine storms induced by influenza A virus (IAV) can promote the up-regulation of LTA_4H (32–35), which further increases LTB_4 production, BLT inflammatory signaling pathway activation, inflammatory factor release, and immune defense function. Due to its selective inhibition of the hydrolysis of LTA_4 by LTA_4H into LTB_4 without affecting the aminopeptidase activity and antiinflammatory function of LTA_4H in hosts, MTX likely facilitates virus infection. As illustrated in Fig. 5 and *SI Appendix*, Fig. S11, the administration of exogenous LTB_4, MTX antibodies, or IM14 significantly inhibited viral infection and thus alleviated pathological injuries and inflammatory infiltration, while the sympotms were more severe in $LTA_4H^{-/-}$ animals that lack LTB_4. In addition to its direct inflammatory effects on viral infection inhibition, LTB_4 may also contribute to the alleviation of pathological injuries by activating the LTB_4-BLT1/2 pathway to decrease cAMP generation and regulating the cross-talk between PGE_2, IL-1β, and LTB_4 to control inflammation and tissue injury, as reported recently (39, 40).

Trypsin-like proteases participate in inflammatory responses by activating protease-activated receptor 2 (59, 60). Neutrophil elastases are associated with neutrophil-mediated inflammation by regulating the functions of neutrophils as immune response triggers (61, 62). As a key protease related to the fibrinolytic system, plasmin also participates in proinflammatory processes (63–67). Here, MTX potently inhibited proinflammatory proteases (K_i of $\sim 10^{-9}$ M) such as plasmin, trypsin, and elastase, which likely strengthened its immunosuppressive functions. In addition, the ability of MTX to inhibit multiple proteases may contribute to its high stability (plasma half-life of 16 h in vivo). These features of MTX (i.e., potent inhibitory effects on LTA_4H and multiple proteases, high stability in vivo, and high concentration in the salivary gland) may endow it with superior strength to inhibit host immunity, create immunologically privileged sites, induce immune tolerance, and facilitate pathogen cross-species invasion. As illustrated in *SI Appendix*, Fig. S14, virus infection stimulated LTA_4H-dominant inflammatory responses, while MTX facilitated H1N1 infection and exacerbated pathogenicity.

Given the critical role of LTA_4H in the biosynthesis of LTB_4, an inflammatory mediator (26, 56), LTA_4H inhibition is suggested as a potent strategy for the development of antiinflammatory drugs. At present, however, very few candidates targeting LTA_4H have shown clinical efficacy due to their limited selectivity (13). In this report, MTX was identified as an endogenous LTA_4H inhibitor from an animal. Without affecting the aminopeptidase function of LTA_4H, MTX selectively inhibited the activity of epoxide hydrolase, providing a candidate and/or template for the development of superior and safe antiinflammatory drugs.

Materials and Methods

Ethics Statement. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committees at the Kunming Institute of Zoology, Chinese Academy of Sciences (approval ID: SMKX-20191015-33). All possible efforts were made to reduce sample size and minimize animal suffering. Human blood samples were collected according to the clinical protocols approved by the Institutional Review Board of the Kunming Institute of Zoology (approval ID: SMKX-20191101-197). All human blood samples were collected with informed consent.

Purification and Feature Analysis of MTX. MTX was purified from the bat salivary gland of *M. pilosus* by a Sephadex 200 Increase 10/300 gel filtration combined with resource Q anion exchange and subjected to partial amino acid sequencing by automated Edman degradation. Its cDNA (accession No.: BankIt2509688) was cloned from the cDNA library of the salivary gland using degenerate primers according to the amino acid sequence. The effects of MTX on LTA_4H were studied by coimmunoprecipitation, SPR, native polyacrylamide gel electrophoresis, protein–protein docking, reverse-phase high-performance liquid chromatography combined with ELISA and mass spectrometry. Its effects on enzymatic functions of serine proteases, PLA2, COX1/2, and CYP450, were studied using chromogenic assays and enzyme activity assay kits. Detailed steps are described in *SI Appendix*.

Effects of MTX on LTB_4 Receptor Signaling, Immunity, and H1N1 Infection. The in vitro effects of MTX on LTB_4 receptor signaling, immunity, and H1N1 infection in HUVECs, pDCs, A549 cells, and MRC-5 cells were evaluated using qRT-PCR, Western blot, ELISA, and immunofluorescence analyses to detect LTA_4H, cytokines, NP, BLT1, AKT, ERK, p65, and virus titer. Five-week-old WT and $LTA_4H^{-/-}$ C57BL/6J male mice were used to study the in vivo effects of MTX on immunity and infection through the above techniques combined with H&E and TUNEL staining to detect inflammation, H1N1 virus titer, and histopathological injury. Further details are available in *SI Appendix*.

Data Availability. All study data are included in the article and *SI Appendix*.

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