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Tissue factor Deficiency Increases Alveolar Hemorrhage and Death in Influenza A Infected Mice

Silvio Antoniak[*], **Kohei Tatsumi**[*], **Yohei Hisada**[*], **J. Justin Milner**[†], **Scott D. Neidich**[†], **Ciara M. Shaver**[‡], **Rafal Pawlinski**[*], **Melinda A. Beck**[†], **Julie A. Bastarache**[‡], and **Nigel Mackman**[*]

[*]Department of Medicine, Division of Hematology and Oncology, UNC McAllister Heart Institute, University of North Carolina at Chapel Hill, 111 Mason Farm Road Campus Box 7126, Chapel Hill, North Carolina, USA

[†]Department of Nutrition, UNC Gillings School of Global Public Health, University of North Carolina at Chapel Hill, 135 Dauer Drive Campus Box 7461, Chapel Hill, North Carolina, USA

[‡]Department of Medicine, Division of Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University School of Medicine, T-1218 MCN, Nashville, TN, USA

Abstract

Background—Influenza A virus (IAV) infection is a common respiratory tract infection that causes considerable morbidity and mortality worldwide.

Objective—To investigate the effect of a genetic deficiency of tissue factor (TF) in a mouse model of influenza A infection.

Methods—Wild-type mice, low tissue factor (LTF) mice and mice with the TF gene deleted in different cell types were infected with a mouse-adapted A/Puerto Rico/8/34 H1N1 strain of IAV. TF expression was measured in the lungs, and bronchoalveolar lavage fluid (BALF) was collected to measure extracellular vesicle TF, activation of coagulation, alveolar hemorrhage and inflammation.

Results—IAV infection of wild-type mice increased lung TF expression, activation of coagulation and inflammation in the BALF, but also led to alveolar hemorrhage. LTF mice and mice with a selective deficiency of TF in lung epithelial cells had low basal levels of TF and failed to increase TF expression after infection; these two strains of mice had more alveolar hemorrhage and death compared with controls. In contrast, deletion of TF in either myeloid cells or endothelial cells and hematopoietic cells did not increase alveolar hemorrhage or death after IAV infection. These results indicate that TF expression in the lung, particularly in epithelial cells, is required to maintain alveolar hemostasis after IAV infection.

Address for correspondence: Nigel Mackman, PhD, University of North Carolina at Chapel Hill, 111 Mason Farm Road Campus Box 7126, Chapel Hill, North Carolina, 27599, Telephone: (919) 843 3961, Fax: (919) 966 7639, nigel_mackman@med.unc.edu.

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Authorship.

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Conclusion—Our study indicates that TF-dependent activation of coagulation is required to limit alveolar hemorrhage and death after influenza A infection.

Keywords

coagulation; hemorrhage; hemostasis; tissue factor; influenza virus A

Introduction

Influenza A virus (IAV) infection is a common cause of respiratory tract infection worldwide resulting in considerable mortality every year, especially in the elderly.(1) Viral infections in general are often associated with activation of the coagulation system as part of the innate immune response.(2–8) In mouse models of lethal IAV subtype H1N1 infection there is both pulmonary and systemic activation of coagulation.(9–11) In humans, acute IAV infection is associated with an increase in the incidence of thrombosis-related cardiovascular events, such as myocardial infarction and stroke.(12) Despite the activation of the coagulation system in the airspace, alveolar hemorrhages are observed in patients with IAV pneumonia and in lungs of H1N1 IAV-infected mice.(13–15)

Tissue factor (TF) plays an essential role in hemostasis but aberrant expression can contribute to thrombosis.(16) TF expression is induced in different cell types after viral infections. For instance, TF is induced in monocytes in HIV patients, is induced in circulating blood cells in monkeys infected with Ebola, and is induced in endothelial cells infected with herpes simplex virus or Dengue virus.(3–5, 8, 17, 18) Importantly, inhibition of the TF/FVIIa complex reduced mortality in a monkey model of Ebola hemorrhagic fever.(17) Therefore, the prevailing view is that TF contributes to the pathology of viral infections.

TF is expressed in a tissue-specific manner.(19) We have proposed that TF expression in the lung is required to maintain hemostasis in this vital organ.(19) It is expressed by a variety of cell types, including bronchiolar, bronchial and alveolar epithelial cells, alveolar macrophages and fibroblasts.(20–25) Low TF [LTF] mice express ~1% levels of TF (26) and when crossed more than 6 generations onto a C57Bl/6 background exhibit spontaneous hemorrhages in their lungs.(27) Moreover, we found that intra-tracheal instillation of LPS into the lungs of LTF mice increased levels of cell-free hemoglobin in bronchoalveolar lavage fluid (BALF), which indicated alveolar hemorrhage.(25) Finally, $Tf^{+/-}$ mice have decreased levels of the coagulation activation marker thrombin-antithrombin complexes (TATc) in BALF after ventilator-induced acute lung injury (ALI).(28) These results demonstrate that TF plays a central role in lung hemostasis under basal conditions and after lung injury.

TF also contributes to lung pathology. Indeed, excessive TF-dependent activation of coagulation leads to intra-alveolar fibrin deposition associated with endotoxemia and sepsis.(29) In a mouse endotoxemia model, we observed increased total *Tf* mRNA expression in the lung and increased expression in alveolar epithelial cells but not bronchiolar epithelial cells.(20, 30) Similarly, Drake and colleagues found increased TF protein expression in alveolar epithelial cells in the lungs of septic baboons.(23) BALF from healthy individuals contains TF activity in small membrane vesicles called extracellular vesicles (EVs).(31) In

addition, patients with acute respiratory distress syndrome (ARDS) or ALI have increased TF expression in alveolar epithelial cells and alveolar macrophages.(24) Interestingly, EV TF activity is increased in the airspace in patients with ALI/ARDS and appears to originate from alveolar epithelium.(24, 32, 33) Finally, LPS stimulation of alveolar macrophages increases TF expression.(34)

In this study, we used a genetic approach to examine the role of TF-dependent activation of coagulation on alveolar hemorrhage and survival in mice after IAV infection.

Material and Methods

We used the mouse-adapted strain of influenza A/Puerto Rico/8/1934 (H1N1, PR/8) (ATCC, Manassas, VA). The virus was propagated in 10–12 day old embryonated chicken eggs and purified as described previously.(7) Viral titers were determined using a hemagglutination assay.(7) For virus inoculation, mice were anesthetized with isoflurane and infected intra-nasally with IAV at the dose of 0.02 hemagglutination units (HAU) in 50 μL PBS. Lungs and BALF were collected at various times after infection. Mice were euthanized if they had ≥25% loss of initial body weight as instructed by our animal protocol.

Mice

We used male wild-type (WT) C57Bl/6, LTF mice and mice with deletion of the TF gene on different cell types. LTF mice have been described (26, 27). $Tf^{fl/fl}$ mice were crossed with mice expressing the Cre recombinase in different cell types to generate mice with cell type-specific deletion of the TF gene.(35) Mice with the TF gene deleted in myeloid cells using the lysosomal M promoter ($Tf^{fl/fl}$,LysM-Cre) have been described and will be referred as TFΔMy mice.(36) We used the Tie2 promoter to delete the TF gene in both endothelial cells and hematopoietic cells ($Tf^{fl/fl}$,Tie2-Cre) and will refer to these mice as TFΔEc.(36) Mice with the TF gene deleted in lung epithelial cells using the surfactant protein C promoter ($Tf^{fl/fl}$,SPC-Cre) have been described and will be referred to as TFΔEp mice.(37) We used $Tf^{fl/fl}$ littermates as controls. We also used factor IX (FIX) null mice.(38) All mice were backcrossed onto the C57Bl/6 background and were 8–10 weeks of age. All experimental protocols were approved by the University of North Carolina-Chapel Hill's Institutional Animal Care and Use Committee.

Bronchoalveolar lavage

Mice were euthanized by cervical dislocation under isoflurane anesthesia after the terminal blood draw, and then BALF or tissue samples were prepared from non-perfused lungs as described below. BALF was collected from infected and uninfected mice by postmortem lavage using 3 × 900 μL of ice-cold PBS.(7) BALF were centrifuged (500 × g, 20 minutes, 4°C), and the supernatant and cell pellets separated. Cell pellets were re-suspended in 200 μL of PBS. WBC and hemoglobin levels in re-suspended cell pellets were determined using a Hemavet 950 (Drew Scientific, Miami Lakes, FL).

TF immmunostaining

Paraffin-embedded lung sections were de-paraffinized with antigen retrieval by standard procedures. Slides were blocked by 2% normal rabbit serum (Vector Lab, Burlingame, CA) in 0.4% triton X-100/PBS for 15 min at room temperature. Then, slides were incubated overnight at 4°C with 1 μg/mL anti-human TF goat polyclonal antibody (R&D Systems, Minneapolis, MN), rinsed and incubated with 1.5 μg/mL biotinylated rabbit anti-goat secondary antibody (Vector Lab) for 20 min at room temperature. Slides were developed color with DAB (Vector Lab) for 1 min and counterstained with hematoxylin (Thermo Fisher Scientific, Waltham, MA) for 1 min.(39)

RNA isolation and RT-PCR

Total lung RNA was isolated by TRIzol method (Life Technologies, Carlsbad, CA).(40, 41) One microgram of RNA was reverse transcribed into cDNA using the iScript™ RT Supermix (Bio-Rad Laboratories, Hercules, CA). The levels of H1N1 PR/8 genomes and TF mRNA were measured by real-time PCR using SSoFast™ Probes Supermix (Bio-Rad) in a realplex^2 Mastercycler (Eppendorf, Hamburg, Germany) as described.(7) The housekeeping gene *Rpl4* was used to correct for variations in input RNA and reaction efficiency.(42) Real-time PCR primer/probes for H1N1 PR/8, murine *Tf*, and murine *Rpl4* were purchased from Integrated DNA Technologies (Coralville, IA): H1N1 PR/8 forward, 5'-GGACTGCAGCGTAGACGCTT-3'; H1N1 PR/8 reverse, 5'-CATCCTGTTGTATATGAGGCCCAT-3'; H1N1 PR/8 probe, 5'-/56-FAM/CTCAGTTATTCTGCTGGTGCACTTGCCA/36-TAMSp/-3'; *Tf* forward, 5'-TTTGGCAAGGACTTGGGTTA-3'; *Tf* reverse, 5'-GCTTACTCCTTCTTCCACATCA-3'; *Tf* probe, 5'-/56-FAM/CCGTGCTTG/ZEN/AGCCTTTCCGATAAGT/3IABkFQ/-3'; *Rpl4* forward, 5'-TGGTGGTTGAAGATAAGGTTGA-3'; *Rpl4* reverse, 5'-CTTGCCAGCTCTCATTCTCTG-3'; *Rpl4* probe, 5'-/56-FAM/CTGAACAGC/ZEN/CTCCTTGGTCTTCTTGTA/3IABkFQ/-3'.

Measurement of TF activity

For measurement of TF activity, lung tissues were homogenized in 15 mM n-Octyl-β-D-glucopyranoside in 25 mM HEPES/saline buffer at the volume of 1.5 mL per 10 mg of tissue. Samples were incubated at 37°C for 15 minutes.(26, 35) Homogenized samples were diluted 20 times with 25 mM HEPES/saline buffer. For measurement of TF activity in the BALF cells, cell pellets were homogenized in 200 μL of 15 mM n-Octyl-β-D-glucopyranoside in 25 mM HEPES/saline buffer. The homogenized samples were diluted 5 times with 25 mM HEPES/saline buffer. Twenty-five μL of the diluted samples were mixed with an equal amount of pooled normal mouse plasma and 20 mM $CaCl_2$ and the clotting time was determined using a STart4 coagulation analyzer. EV TF activity was measured from 10 μL of BALF supernatant as described.(43) Serially diluted recombinant human TF (Innovin, Dade Behring) was used to generate a standard curve. TF activity was normalized to the protein concentration obtained by BCA assay as described.(7)

Measurement of TATc and cytokines/chemokines

Levels of TATc in BALF supernatant were quantified by ELISA (TAT Enzygnost Micro Kit; Dade Behring, Deerfield, IL).(7, 36) The level of various cytokines/chemokines in the BALF supernatant was measured using commercial ELISAs (Duo-Set, R&D Systems, Minneapolis, MN).(7)

Statistics

All statistical analyses were performed using either GraphPad Prism (version 5.0; GraphPad Software Inc., La Jolla, CA). Data are represented as mean ± SEM. For 2-group comparison of continuous data, 2-tailed Student's *t* test was used. For multiple-group comparison, data were analyzed by 1- or 2-way ANOVA and were Bonferroni corrected for repeated measures over time. A *P* value less than 0.05 was considered significant.

Results

Influenza A virus infection of mice increases TF expression, activation of coagulation, alveolar hemorrhage and inflammation

To determine the effect of IAV infection on TF expression in the lung, WT mice were infected with IAV and lung and BALF samples were collected at 0, 1, 3, 4, 7 and 14 days post infection (dpi). We detected IAV genomes in the lungs at 3, 4 and 7 days (Figure 1A). We measured lung TF activity as well as EV TF activity and TATc as a marker of the activation of coagulation in BALF. IAV infection led to a time-dependent increase in lung TF activity and EV TF activity with maximal levels 4 dpi (Figure 1B and C). IAV infection also increased TATc in the BALF at 4, 7 and 14 dpi (Figure 1D). We measured levels of hemoglobin in the BALF as a marker of alveolar hemorrhage. Levels of hemoglobin increased in a time-dependent manner with maximal levels at 7 dpi (Figure 1E). Levels of WBC in the BALF also increased after infection (Figure 1F). We measured levels of different cytokines/chemokines in the BALF of mice before and 7 dpi. IAV infection significantly increased IL-1β and Ccl2 (Figure 1G and H), and Ccl5 (37.6±14.9 pg/mL vs. 327.3 ± 25.7 pg/mL, N=4, $P<0.05$) and Cxcl10 (0±0.0 pg/mL vs. 5882.7±255.5 pg/mL, N=4, $P<0.05$) but not TNF-α or Cxcl2 (data not shown). These data indicate that IAV infection of WT mice increases TF expression, activation of coagulation, alveolar hemorrhage and inflammation.

Influenza A infection increases TF expression in lung epithelial cells

We examined the effect of a global deficiency of TF on the induction of TF and activation of coagulation after IAV infection. LTF mice had significantly lower basal levels of lung TF activity than control mice (Figure 2A). In addition, we did not observe any increase in lung TF activity after IAV infection in LTF mice (Figure 2A). Levels of EV TF activity in BALF were also significantly lower in LTF mice after IAV infection compared with controls (Figure 2B). As expected, IAV-infected LTF mice had significantly lower levels of TATc in the BALF compared with IAV-infected control mice (Figure 2C).

To examine the contribution of lung epithelial cell TF to total TF expression in the lung and activation of coagulation after IAV infection, we used TFΔEp mice that have a selective

deletion of the TF gene in lung epithelial cells. We recently showed that these mice have lower basal levels of lung TF compared with controls.(37) Uninfected TFΔEp mice had significantly lower levels of lung TF mRNA and activity compared to $Tf^{fl/fl}$ littermate controls (Figure 2D and E). Importantly, we did not observe any increase in lung TF mRNA or activity in TFΔEp mice after IAV infection (Figure 2D and E). Moreover, there were significantly lower levels of EV TF activity in BALF in TFΔEp mice compared with controls (Figure 2F). TFΔEp mice also had lower levels of TATc in the BALF compared with control mice but the difference was not statistically significant (Figure 2G). Next, we analyzed TFΔMy mice to examine the role of myeloid cell TF in IAV infection. We have shown that levels of TF are significantly decreased in circulating myeloid cells in these mice.(36) Deletion of TF in myeloid cells did not affect TF expression in the lung or EVs in the BALF, or activation of coagulation after IAV infection (Figure 2H–K). Similar results were observed with TFΔEc mice that have a deficiency of TF in endothelial cells and hematopoietic cells (data not shown).

We analyzed TF protein in the lung of uninfected and IAV infected mice by immunohistochemistry. In uninfected WT mice we observed strong TF signal in adventitial cells surrounding larger blood vessels and in bronchial epithelial cells as well as weaker signal in alveolar epithelial cells (Figure 3B and E). The TF signal was increased in all cell types after IAV infection (Figure 3C and F). We did not observe TF signal in endothelial or blood cells. In IAV infected TFΔEp mice, we observed TF antigen in adventitial cells surrounding blood vessel but no signal in bronchial epithelial cells and alveolar epithelial cells (Figure 3G–I). Interestingly, adventitial cells surrounding bronchioles expressed TF in TFΔEp mice (Figure 3H). These results indicate that lung epithelial cells are a major source of basal TF expression in the lung and are the primary source of inducible TF in the lungs of IAV infected mice.

Reduced TF expression leads to increased alveolar hemorrhage in mice after influenza A virus infection

We determined the role of TF in maintaining lung hemostasis in mice after IAV infection. We assessed alveolar hemorrhage by measuring the level of hemoglobin in BALF. There were no signs of alveolar hemorrhage in uninfected mice. However, BALF from IAV-infected control mice was light pink and had increased levels of hemoglobin compared with uninfected mice at 7 dpi (Figure 4A–D). BALF of LTF mice was bright red and had a significantly higher level of hemoglobin compared with control mice at 7 dpi (Figure 4A). BALF of infected TFΔEp mice also had increased levels of hemoglobin in the BALF at 7 dpi compared with controls (Figure 4B). In contrast to the results with the LTF and TFΔEp mice, we did not observe an increase in alveolar hemorrhage in TFΔMy or TFΔEc mice (Figure 4C and D). Interestingly, FIX null mice did not exhibit increased alveolar hemorrhage after IAV infection (data not shown). These data indicate that either a global a deficiency of TF or a selective deficiency of TF in lung epithelial cells is associated with increased alveolar hemorrhage after IAV infection.

A deficiency of TF does not affect inflammation or levels of virus

In an endotoxemia model LTF mice had significantly lower levels of IL-6 compared with controls.(44) Therefore, we determined if a deficiency of TF was associated with reduced expression of inflammatory mediators in the BALF at 7 dpi. Levels of IL-1β, Ccl5 and Cxcl10 were not significantly reduced in LTF mice after IAV infection but there was a significant increase in Ccl2 (Supplementary Figure 1A, C, E and G). We did not observe any differences in the expression of inflammatory mediators in TFΔEp mice (Supplementary Figure 1B, D, F and H). In addition, either a global deficiency of TF or cell type-specific deletion of TF did not affect the level of IAV genomes in the lungs 7 dpi (Supplementary Figure 2A–D).

Effect of TF deficiency on mortality of mice after influenza A virus infection

We used a dose of IAV that caused ~20% mortality in control mice. The body weight of control mice decreased after infection and then partially recovered by day 14. We analyzed the effect of either global deficiency in TF or deletion of the TF gene in different cell types on the mortality of mice after IAV infection. Infected LTF mice had a significantly higher mortality compared with infected control mice but surviving mice had similar weight changes as control mice (Figure 5A and B). Similarly, TFΔEp mice also exhibited an increase in mortality but no change in weight loss after infection compared to control mice (Figure 5C and D). TFΔMy and TFΔEc mice had a similar mortality and weight change as control mice (Figure 5E–H). FIX null mice exhibited similar survival to controls (data now shown). These results indicate that either a global deficiency of TF or a deletion of TF in epithelial cells is associated with increased mortality after IAV infection.

Discussion

Patients infected with IAV commonly have alveolar hemorrhage and this is a complication of influenza pneumonia particularly with the H1N1 strain.(13, 45–50) Importantly, retrospective studies showed an association between alveolar hemorrhages and increased mortality.(51, 52) Although alveolar hemorrhage is a feature of influenza pneumonia, the mechanisms of hemorrhage are unknown. Furthermore, it is unclear why severe alveolar hemorrhage is well described in influenza pneumonia but is a less common feature in other infectious pneumonias.(15) Our studies show that the lung epithelium, the very cells infected and killed by influenza infection, is the primary source of TF in the airspace. Importantly, either a global deficiency in TF or a genetic deficiency of TF in lung epithelial cells is associated with a significant increase in alveolar hemorrhage after IAV infection. These results indicate that lung epithelial TF helps limit alveolar hemorrhage in IAV pneumonia.

We found that IAV infection increases TF expression in the lungs of WT mice and this induction is abolished in mice with either a global deficiency in TF or a selective deficiency of TF in lung epithelial cells, but not in myeloid cells or endothelial cells and hematopoietic cells. Similarly, we recently found that induction of TF expression in the lung by intra-tracheal LPS is abolished in these TFΔEp mice but not in mice with a deficiency of TF in myeloid cells.(37) These studies indicate that epithelial cells are a major source of basal TF expression and the primary source of induced TF expression in the lung. However, TFΔEp

mice had higher levels of TF than LTF mice suggesting that other cell types in the lung express TF, such as fibroblasts. Indeed, we observed TF expression in adventitial cells surround blood vessels and bronchioles in TFΔEp mice that are likely to be fibroblasts. We did not see any deteectableTF expression in endothelial or blood cells. The increase in TF expression in infected WT mice was associated with activation of coagulation in the BALF. This is consistent with a previous study showing that levels of TATc were increased in the BALF of mice 4 days after IAV infection.(11) A global deficiency of TF was associated with a significant reduction in TATc in the BALF, which indicated that TF regulates coagulation in the lung after IAV infection.

We observed a low level of alveolar hemorrhage in WT mice after IAV infection, which indicates that the infection represents a hemostatic challenge to the lung. This finding is consistent with other studies of IAV infection in mice.(9–11) Mice with either a global deficiency in TF or a selective deficiency of TF in lung epithelial cells, but not mice lacking TF in myeloid cells or endothelial cells and hematopoietic cells or FIX null mice, exhibited increased alveolar hemorrhage after IAV infection. Similarly, we found that LTF mice and TFΔEp mice exhibited increased alveolar hemorrhage after intra-tracheal LPS.(25, 37) LTF mice exhibited more alveolar hemorrhage than TFΔEp mice indicating that cell types in addition to epithelial cells, such as fibroblasts, contribute to lung hemostasis after lung injury.

We observed a significant increase in mortality in infected LTF mice compared with controls. Similarly, TFΔEp mice were more susceptible to IAV infection compared with controls but this phenotype was not as dramatic as the LTF mice. At present, we can only speculate about the reasons for the increased mortality in LTF and TFΔEp mice. Increased alveolar hemorrhage is likely to contribute to the increased death but a deficiency of TF may also impacts other processes, such as vascular permeability. Interestingly, a deficiency of TF was not associated with a reduction in inflammatory mediators. In fact, LTF mice had increased levels of Ccl2. Finally, a reduction of TF did not affect levels of IAV genomes at 7 dpi.

Inhibition of TF using a monoclonal antibody has been shown to attenuate the coagulopathy, reduce ALI and decrease mortality in an *Escherichia coli* baboon model of septic shock.(53) Similarly, systemic inhibition of the TF/FVIIa complex with site-inactivated recombinant factor VIIa (FFR-FVIIa), which acts as a competitive inhibitor of FVIIa, attenuated the coagulopathy and reduced lung injury in an *Escherichia coli* baboon model of septic shock. (54) These studies led to a clinical trial to determine if FFR-FVIIa would be beneficial in mechanically ventilated patients with ALI and ARDS. However, there were no beneficial effects of FFR-FVIIa on morbidity and clinical outcome but there was a trend towards an increased risk of serious bleeding and higher mortality with the higher dose of FFR-FVIIa. (55, 56) These results support the notion that TF plays a critical role in lung hemostasis.

In summary, we demonstrate a critical protective role for TF in the lung in a clinically relevant model of severe influenza pneumonia. Our results suggest that impairment of hemostasis may contribute to the pathology associated with influenza A pneumonia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
BALF	bronchoalveolar lavage fluid
dpi	days post infection
IAV	influenza A virus
H1N1/PR8	hemagglutanin type 1 and neuraminadase type 1/Puerto Rico strain 8/34
HAU	hemagglutination units
Hb	hemoglobin
LTF	low tissue factor
Rbc	red blood cells
SPC	surfactant protein C
TATc	thrombin-antithrombin complexes
TF	tissue factor
TFΔEp	$Tf^{fl/fl}$,SPC-Cre
TFΔMy	$Tf^{fl/fl}$,LysM-Cre
TFΔEc	$Tf^{fl/fl}$,Tie2-Cre

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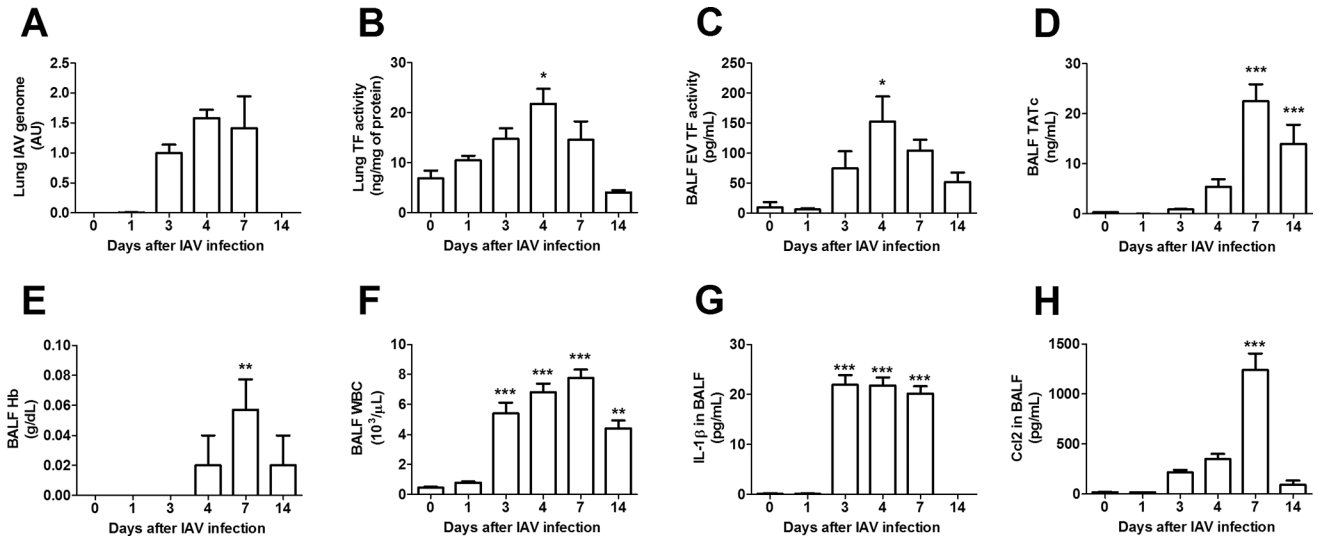


Figure 1. Influenza A infection increases TF expression and inflammation in the mouse lung

Wild-type mice were infected with influenza A virus (IAV) and samples were collected before and 1, 3, 4, 7 and 14 days after infection. (A) IAV genome levels in the lungs were quantified by real-time PCR and normalized to *Rpl4* mRNA levels. Data are shown relative to day 3. (B) TF activity levels in the lung were measured using a one-stage clotting assay. (C) TF activity levels of extracellular vesicles (EV) isolated from the bronchoalveolar lavage fluid (BALF) were measured using a two-stage FXa generation assay. (D) Levels of thrombin-antithrombin complexes (TATc) in the BALF. (E) Levels of hemoglobin (Hb) in the BALF. (F) Levels of white blood cells (WBCs) in the BALF after IAV infection. (G) Levels of IL-1β in the BALF after IAV infection. (H) Levels of Ccl2 in the BALF after IAV infection. Data (mean ± SEM; n = 3–13 per group) were analyzed by 1-way ANOVA. Statistical significances are shown as **P*<0.05, ***P*<0.01 and ****P*<0.001 versus day 0.

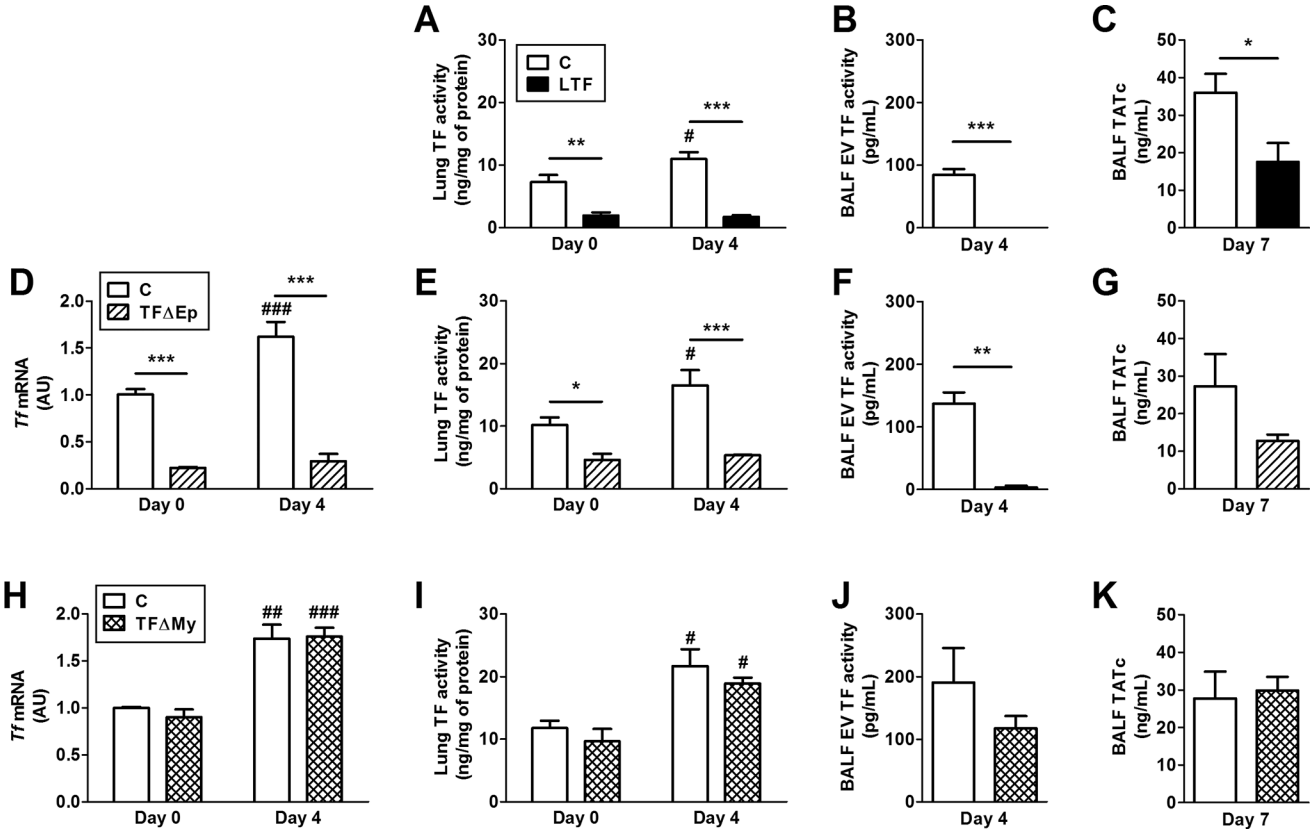


Figure 2. Levels of TF expression in TF deficient mice before and after influenza A infection

(A–C) Low TF (LTF) mice, (D–G) $TF^{fl/fl}$,SPC-Cre (TFΔEp) and (H–K) $TF^{fl/fl}$,LysM-Cre (TFΔMy) mice and their respective control mice (referred as C) were infected with influenza A virus (IAV) and the lungs and bronchoalveolar lavage fluid (BALF) were collected. Levels of lung *Tf* mRNA (D and H) and TF activity (A, E and I), TF activity of BALF extracellular vesicles (EV) (B, F and J), and BALF thrombin-antithrombin complexes (TATc) (C, G and K) are shown. Controls are shown in white bars and experimental mice are shown in black (LTF), hatched (TFΔEp), or cross-hatched (TFΔMy) bars. Levels of *Tf* mRNA in the lungs were quantified by real-time PCR before and 4 days after IAV infection. Data were normalized to *Rpl4* mRNA levels. Levels of the uninfected controls were set to 1. Data (mean ± SEM; n = 3–7 for day 0, n =3–10 for day 4, and n = 3–8 for day 7) were analyzed by 2-way ANOVA or Student's *t*-test. Statistical significance is shown as $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ between groups or $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ versus uninfected control of the respective genotypes.

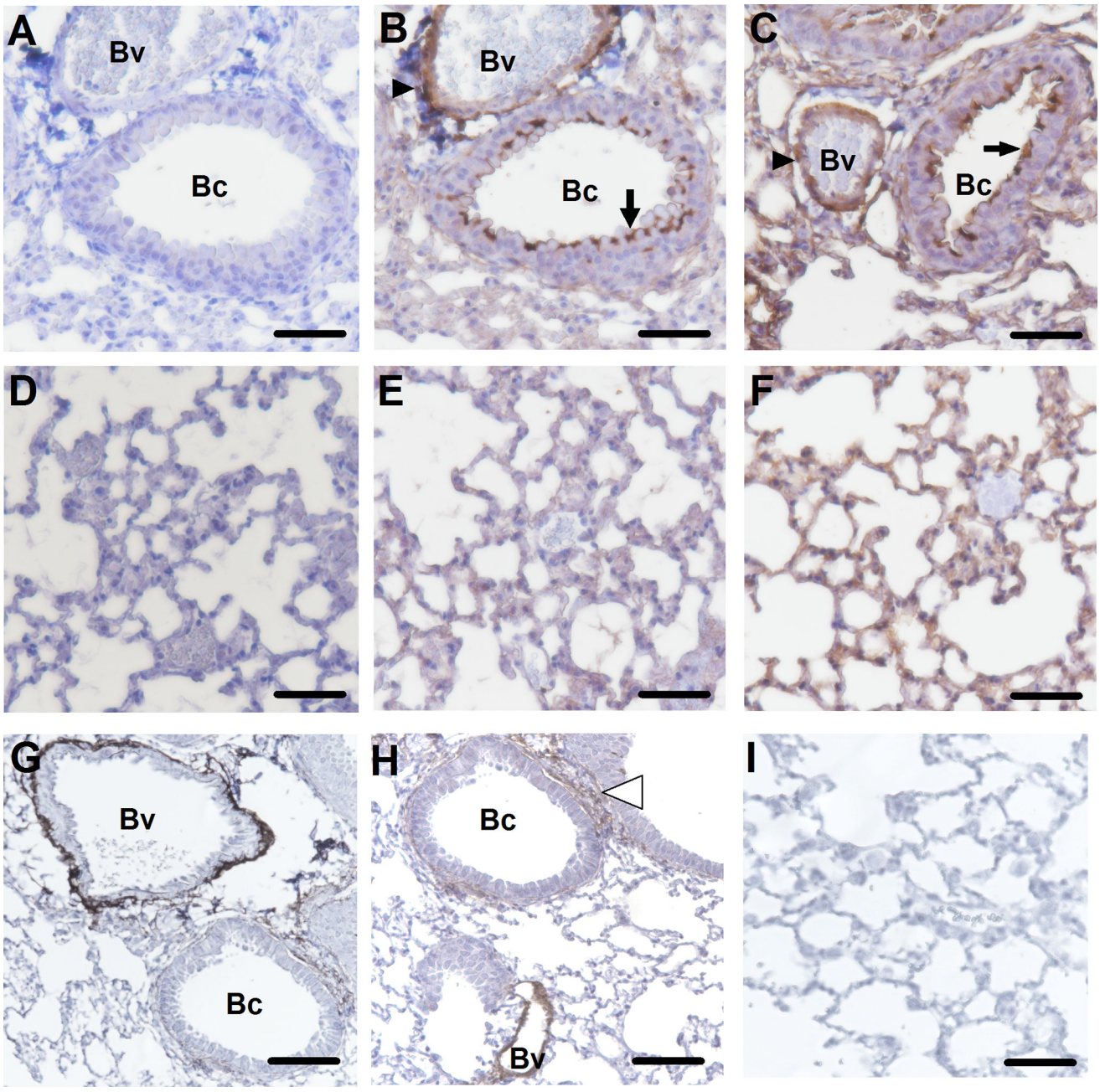


Figure 3. Influenza A infection increases TF protein expression in the lung

We analyzed TF antigen expression in the lungs of uninfected (B, E) and infected (7 dpi) wild-type mice (C, F), and infected TFΔEp mice (G–I) by immunohistochemistry. Tissue sections were incubated with (B, C, E, F–I) or without (A and D) a goat anti-mouse TF polyclonal antibody. The black arrows indicate TF expression in the epithelium of bronchi (Bc). The arrowheads indicate TF staining in adventitial cells of blood vessels (Bv). The white arrow indicates TF expression in adventitial cells surrounding a bronchiole. Original magnification x200. Scale bar is 100 μm.

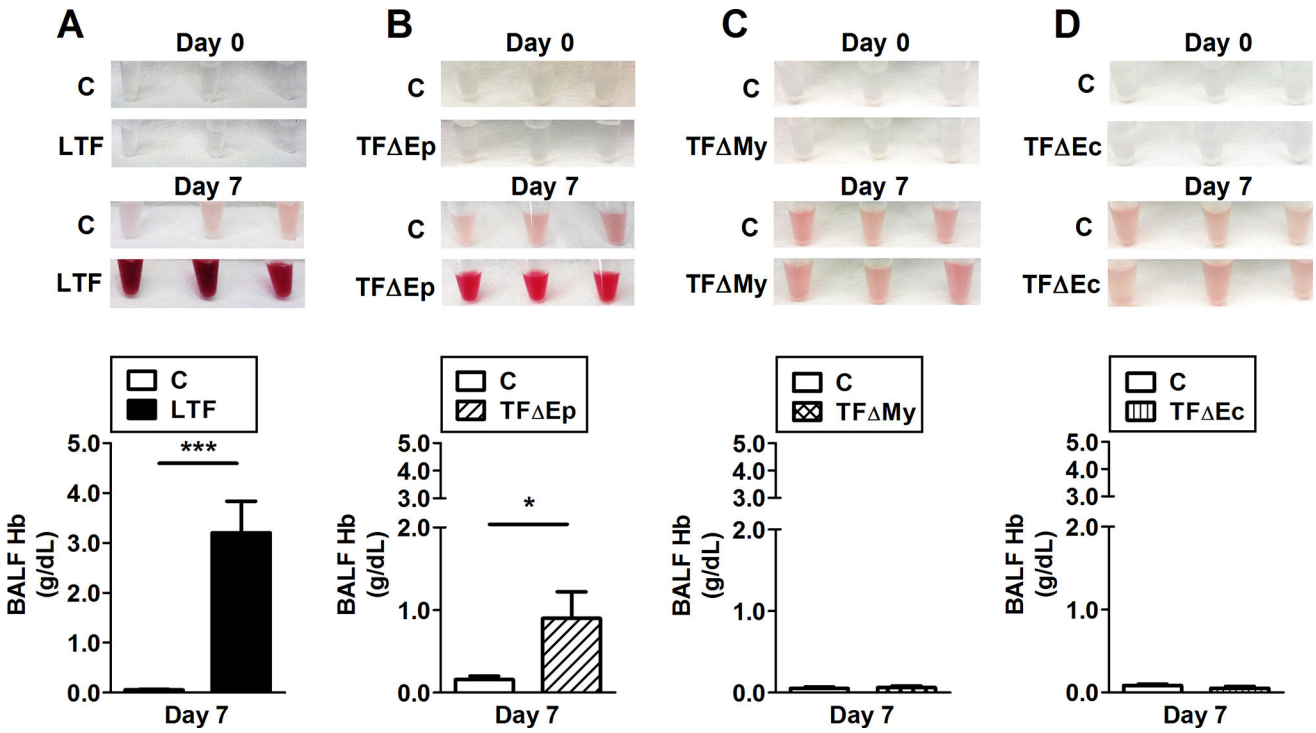
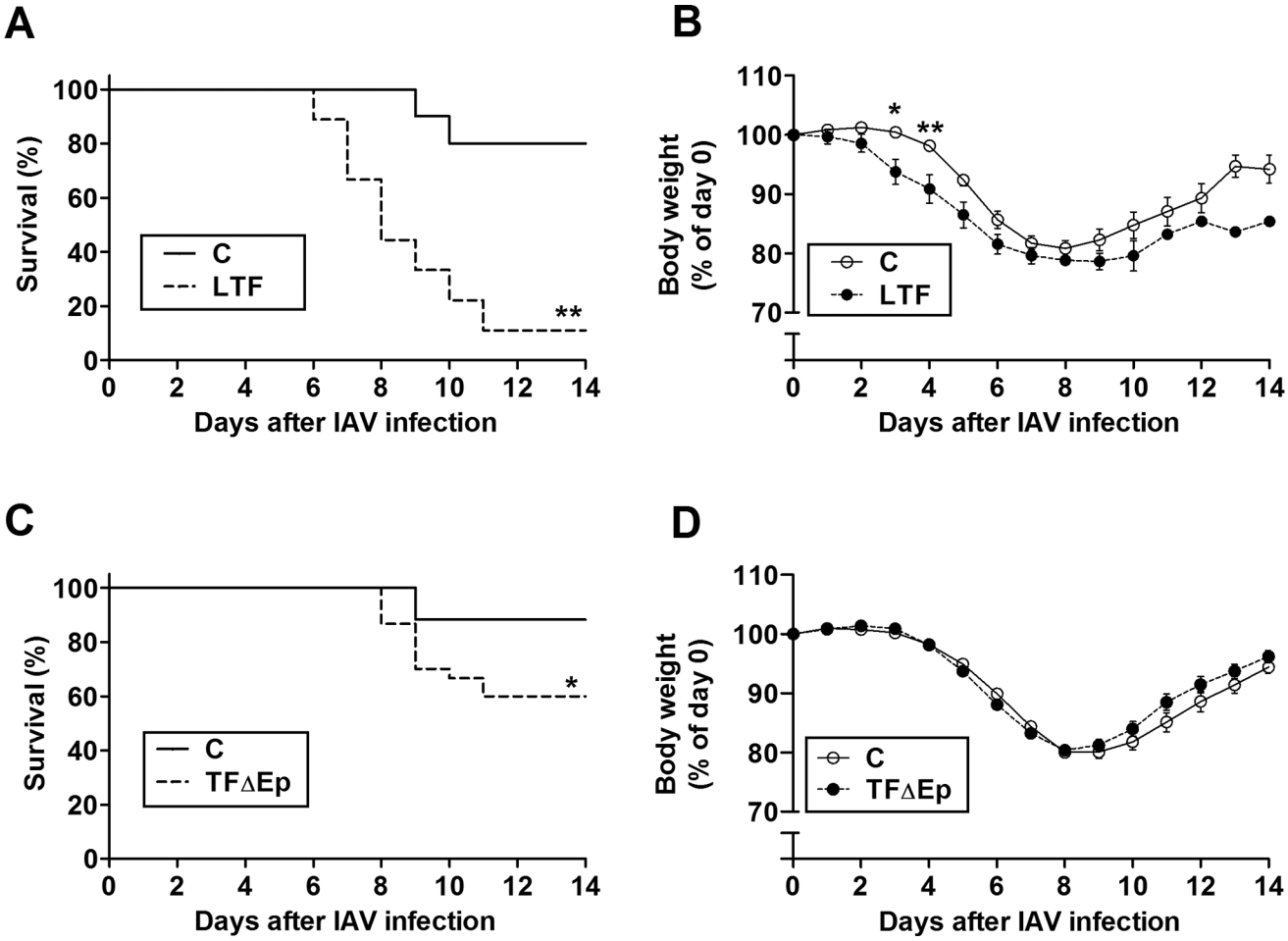


Figure 4. Effect of genetic deficiency of TF on alveolar hemorrhage in mice after influenza A infection

(A) Low TF (LTF) mice, (B) $Tf^{fl/fl}$,SPC-Cre (TFΔEp), (C) $Tf^{fl/fl}$,LysM-Cre (TFΔMy) and (D) $Tf^{fl/fl}$,Tie2-Cre (TFΔEc) mice and their respective control mice (referred as C) were infected with influenza A virus (IAV), and the bronchoalveolar lavage fluid (BALF) was collected before and 7 days after infection. Gross appearance of BALF and levels of hemoglobin (Hb) in BALF before and after IAV infection are shown. Controls are shown as white bars and experimental mice are shown as black bars (LTF), hatched (TFΔEp), cross-hatched (TFΔMy), or vertical-striped (TFΔEc) bars. Data (mean ± SEM; 3– 11) were analyzed by Student's *t*-test. Statistical significance is shown as $*P<0.05$ between groups.



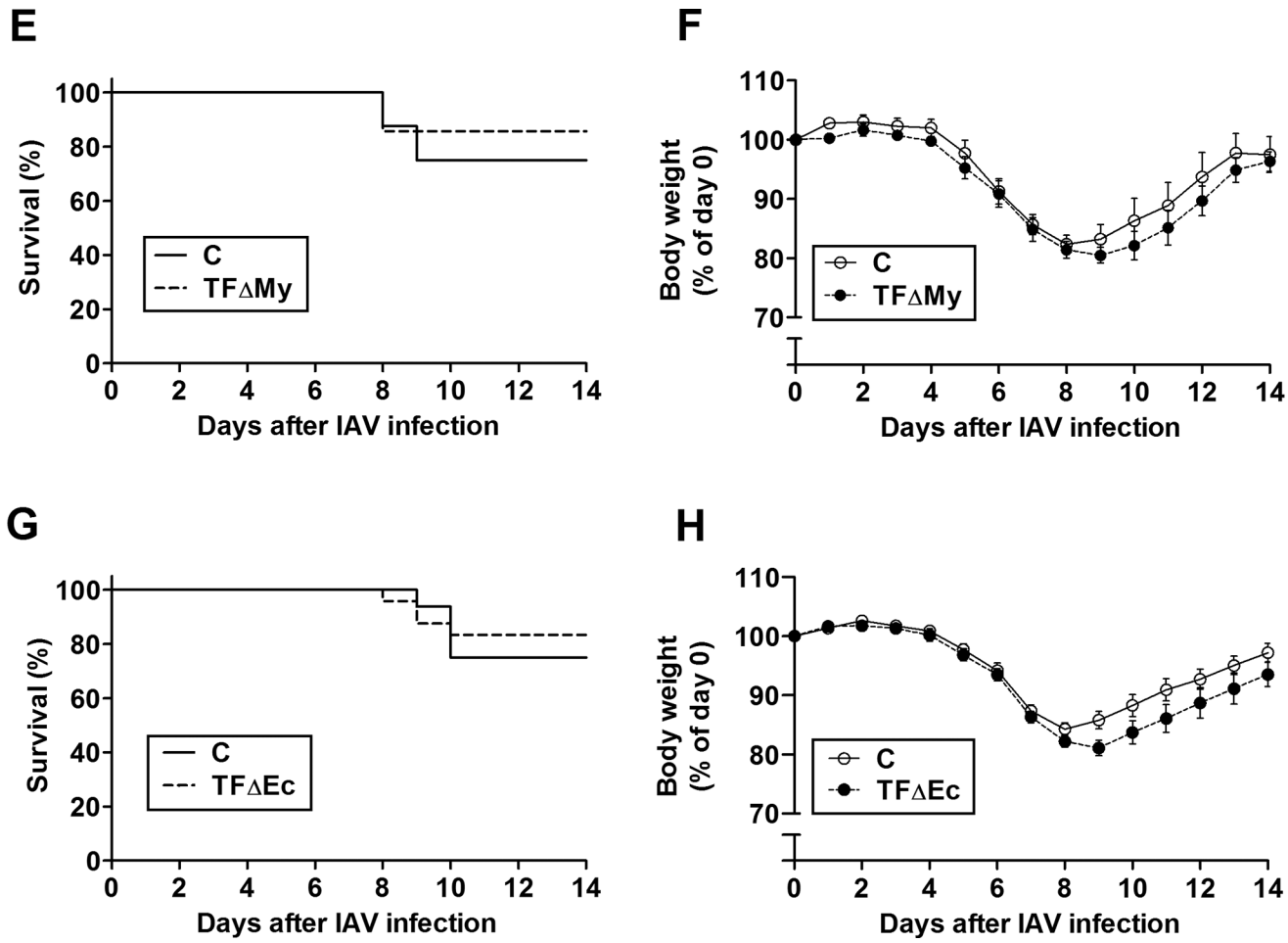


Figure 5. Effect of a global or cell type-specific deficiency of TF on mortality of mice after influenza A infection

(A and B) Low TF (LTF), (C and D) $Tf^{fl/fl}$,SPC-Cre (TFΔEp), (E and F), $Tf^{fl/fl}$,LysM-Cre (TFΔMy) and (G and H) $Tf^{fl/fl}$,Tie2-Cre (TFΔEc) mice and their respective control mice (referred as C) were infected with influenza A virus (IAV) and observed for 14 days. Survival rates (A, C, E and G) and changes in body weights of infected mice (B, D, F and H) are shown. Body weights before infection were set to 100% and did not differ significantly between genotypes. Data (mean ± SEM; n = 9–10 for A and B, n = 17–30 for C and D, n = 7–8 for E and F, and n = 14–24 for G and H) were analyzed by log-rank test (A, C, E and G) or by 2-way ANOVA (B, D, F and H). Statistical significance is shown as *P<0.05 and **P<0.01 between groups.