Unphosphorylated STAT1 Represses Apoptosis in macrophages during *Mycobacterium Tuberculosis* Infection

Kezhen Yao¹,²†, Qi Chen¹,²†, Yongyan Wu¹,², Fayang Liu¹,², Xin Chen¹,², Yong Zhang¹,²,*

¹College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi, China
²Key Laboratory of Animal Biotechnology, Ministry of Agriculture, Northwest A&F University, Yangling, Shaanxi, China

† These authors contributed equally to this work.

*Corresponding author
Tel.: +86 29 87080092
Fax: +86 29 87080092
E-mail: zhy1956@263.net
Abstract

In murine macrophages infected with *Mycobacterium tuberculosis (Mtb)*, the level of phosphorylated STAT1 (PSTAT1) driving the expression of many pro-apoptosis genes, increases quickly but then declines over a period of hours. On the contrary, there is a continued increase of unphosphorylated STAT1 (USTAT1) that persists for several days. Similar trend is discovered between USTAT1 level and the intracellular bacterial burden during late infection. To investigate the significance of high level of USTAT1, we increased its concentration exogenously, and the apoptosis rate induced by *Mtb* is sufficiently decreased. Further experiment confirms USTAT1 affects the expression of several immune-associated gene, and lessens sensitivity of macrophages to CD95-mediated apoptosis during *Mtb* infection. Furthermore, we characterized 149 USTAT1-interacting proteins and the interactome network. The cooperation between USTAT1 and STAT3 results in downregulation of CD95 expression. Additionally, a competitive binding reactions between USTAT1 and IFIT1 to eEF1A was verified. Together, our data firstly show the USTAT1 differs from the PSTAT1, and represses apoptosis in macrophages to promote immune evasion during *Mtb* infection.

Keywords: Stat1 gene, *Mycobacterium tuberculosis*, cell apoptosis, Protein interactome; immune evasion
**Introduction**

*Mycobacterium tuberculosis* (*Mt*) is a spirited and infectious bacterial pathogen. Macrophages are the main host cells for *Mt*, and they exert their effects via immune modulation and antigen presentation by "programmed" cell death or autophagy (Cooper and Khader, 2008, Cooper, 2009). However, *Mt* is able to survive and proliferate via reducing apoptosis and sensitivity of macrophages (Tiwari et al., 2009, Vergne et al., 2005, Behar et al., 2010). The study on the mechanisms of immune evasion by *Mt* and the immunological role of macrophages in tuberculosis infection is important for insights into the anti-tuberculosis immune mechanisms in the host and design of new anti-tuberculosis vaccines.

Cell signaling could be triggered by *Mt* leading to apoptosis of macrophages, and one of main signaling pathways is JAK/STAT pathway, which is mediated by the phosphorylation of JAKs and STATs (Rojas et al., 2002a, Lim et al., 2016, Rhee et al., 2003). The phosphorylated STAT1 (PSTAT1) has been described as a pathogen suppressor because of its function as an immunosurveillance mediator (Najjar and Fagard, 2010, Lim et al., 2016). The pro-apoptosis activity of PSTAT1 seems to be significantly important at the onset of *Mt* infection and is supposed to result in the elimination of infected cells by the innate and adaptive immune system (Rojas et al., 2002a). At the cellular level, PSTAT1 can exert function by upregulating the expression of many pro-apoptosis genes, including TNF-α (Gifford and Lohmann-Matthes, 1987, Scuderi et al., 1987, Collart et al., 1986), caspases (Shakhov et al., 1996, Chin et al., 1997), CD95 (Xu et al., 1998) and nitric oxide synthase (NOS2) (Gao et al., 1997). The expression of these genes can trigger cell responses to *Mt* infection and lead to macrophages activation and apoptosis.

PSTAT1 also induced STAT1 expression to increase unphosphorylated STAT1 (USTAT1) accumulation (Cheon and Stark, 2009). As PSTAT1 only lasts a few hours, whereas USTAT1 can persist for several days, raising the possibility that increased concentration of USTAT1 might play an important role during the late *Mt* infection. The function of PSTAT1 in regulation of *Mt* infected macrophages is well-established (Rojas et al., 2002b). However, the role of USTAT1 is not well-defined. Previous study
showed that both PSTAT1 and USTAT1 are transcription factor (Chatterjee-Kishore et al., 2000b, Cheon and Stark, 2009) so that they may be both involved in the cell apoptosis induced by *Mtb* strain H37Ra. In the present study, we have shown that the similar trend between USTAT1 expression level and the intracellular bacterial burden in H37Ra-infected macrophages, which suggested that USTAT1 is a negative regulator to the host cell during H37Ra infection. Indeed, H37Ra infection induced apoptosis was severely compromised in USTAT1 overexpressed macrophages compared to control. USTAT1 inhibited CD95 signaling resulted in activation of anti-apoptotic effectors, and decrease of active Caspase-3 and cytosolic translocation of Cytochrome c. Furthermore, we have characterized the USTAT1 protein interaction network with an efficient proteomic approach, and 149 high-confidence, USTAT1-interacting proteins were identified. In addition, we have fully verified USTAT1/STAT3 complex is involved in repressing CD95 expression in macrophages, and a competitive binding reactions between USTAT1 and IFIT1 to eEF1A was verified which is associated with the inhibition of apoptosis. Thus our data firstly indicated that differing from the pathogen suppressor PSTAT1, the USTAT1 shows anti-apoptosis activity, and promotes immune evasion during *Mtb* infection.
Result

H37Ra increase STAT1 expression in macrophages

Pathogen infection activates JAK/STAT pathway and that its activation is related to apoptosis in macrophages (Najjar and Fagard, 2010, Rojas et al., 2002b, Lim et al., 2016). Thus, we investigated whether *Mtb* strain H37Ra could affect STAT1 signaling in mice bone marrow derived macrophages (BMDMs). As shown in Fig. 1A, the concentration of phosphorylated STAT1 increased within 1 h, and then decreased over the next several hours. As PSTAT1 decreased, there was a reciprocal increase in the concentration of USTAT1 (The monoclonal C-terminal anti-STAT1 reagent used detects only U-STAT1 and not P-STAT1 (Cheon and Stark, 2009)), beginning at about 6 h, which persisted for at least 2 days. At each time points after infection, the viability of H37Ra was determined by CFU assay and the intracellular bacterial burden shows similar trend with the level of USTAT1 expression in a time dependent manner during the late infection (Fig. 1B).

Increased USTAT1 represses apoptosis in RAW264.7 cell during H37Ra infection

To investigate the role of H37Ra-induced USTAT1, we transduced mouse macrophage cell line RAW264.7 lentiviruses encoding USTAT1 (Y701F, S727E double mutant) with FLAG tag to establish a cell line stably overexpressed USTAT1 (RAW-USTAT1); RAW264.7 cells transduced with empty lentiviruses were used as control (RAW-CT). The immunoblot results confirmed that USTAT1 was expressed stably in RAW-USTAT1 cells (Fig. 2A). Next, we tested PSTAT1 (701p) and USTAT1 levels at each infected time points on both RAW-USTAT1 and RAW-CT cells. As showed in Fig. 2B, the results is similar as we detected in BMDMs. Both immunoblot and immunofluorescence experiments showed USTAT1 was distributed in the whole cell, and the accumulation of USTAT1 within the nuclear compartment had been increased in response to H37Ra infection (Figs. 2C&2D). Next, we analyzed the apoptotic rates of the infected RAW-CT or RAW-USTAT1 cells with H37Ra. At indicated post-infection time points, the Annexin-V staining assay results showed that the cell apoptosis rate declined gradually with the prolonging of infected time. In addition, the H37Ra-infected RAW-USTAT1 cells had a significantly lower apoptotic rate than that
of infected RAW-CT cells (Figs. 2E&2F). Next, we analyzed the intracellular bacterial burden of the H37Ra-infected RAW-CT and RAW-USTAT1 cells. As expected, the bacterial burden of the RAW-USTAT1 cells increased significantly in comparison with the RAW-CT cells (Fig. 2G). It has been shown that USTAT1 directly regulates the expression of several constitutive genes (Chatterjee-Kishore et al., 2000b, Cheon and Stark, 2009). q-PCR analysis revealed that the expression levels of Lmp2, Mcl1, CIIta were up-regulated and CD95, Hsp70, Jak1 were down-regulated in RAW-USTAT1 cells. Surprisingly, we also noticed that infection with Mtb strain H37Ra regulated those genes in similar trend (Fig. 2H).

**USTAT1 reduced susceptibility in macrophages to CD95L induced apoptosis**

The viability of intracellular Mtb in macrophages is often associated with CD95/CD95-ligand (CD95L) mediated apoptosis (Oddo et al., 1998, Kornfeld et al., 1999). As USTAT1 down-regulated the mRNA expression of CD95 (Fig. 2F), we suggest that USTAT1 may associate with CD95/CD95L apoptotic pathway in H37Ra-infected macrophages. To test this hypothesis, we first analyzed the cell surface protein level of CD95 by flow cytometry. The mean fluorescence intensity (MFI) results indicated overexpression USTAT1 in RAW264.7 cells expressed lower level of cell surface CD95 protein as compared with the control cells (Figs. 3A&3B). Secondly, we tested the apoptosis rate of CD95L-mediated apoptosis in RAW-USTAT1 cell. While RAW-CT cells were sensitive to CD95L induced apoptosis, and the proportion of apoptotic cells in RAW-USTAT1 cell was diminished compared with that of RAW-CT cell (Figs. 3C&3D). Next, we evaluated the mRNA expression level of CD95L in H37Ra infected RAW-CT and RAW-USTAT1 cells. The qPCR results showed the expression of CD95L was elevated in H37Ra infected cell but such change doesn’t seem to be caused by USTAT1 (Fig. 3E).

In order to further validate and strengthen our observation, we detected cytosolic pro-apoptosis and anti-apoptosis effectors expression of RAW-USTAT1 cells and infected with H37Ra. The loss of integrity of mitochondrial outer membrane is one of the hall marks of apoptosis, a significant event resulting of Cytochrome c that culminates in the activation of caspases (Liu et al., 1996, Bratton et al., 2001). As shown in Fig. 3F, the
H37Ra infection induced apoptosis was severely compromised in RAW-USTAT1 cells compared to RAW-CT cells as analyzed by the expression levels of pro-apoptosis genes release of Cytochrome c and activation of Caspase-3, with concomitant increase in anti-apoptosis gene Mcl-1 expression.

**Characterization of the USTAT1 protein interactome**

Since USTAT1 showed to function as a novel regulator to regulate expression of several genes and inhibit cell apoptosis (Cheon and Stark, 2009, Zimmerman et al., 2012, Chatterjee-Kishore et al., 2000b), but till now, its binding proteins have been little known even for its human homology. To map the USTAT1 interactome in macrophages, a one-step pull-down procedure with biotinylated (Bio) tag by streptavidin was taken in RAW264.7 cells, which possesses high-affinity without affecting protein function (Blumert et al., 2013). We constructed a multi-cistronic lentiviral construct encoding a Bio tag fused to the C terminus of the USTAT1 protein and Escherichia coli biotin ligase (BirA) separated by a self-cleaving 2A peptide sequence (P2A) derived from porcine teschovirus-1 (Kim et al., 2011) (Fig. 4A). The lentiviral construct encoding BirA alone was used as a control. RAW264.7 cells were transduced with these lentiviral constructs to establish cell lines stably overexpression BirA-P2A -BioUSTAT1 (RAW-Bio-USTAT1) or BirA (RAW-Bio-CT) (Fig. 4B). The Bio-USTAT1 protein complexes were isolated from whole-cell extracts streptavidin-mediated affinity purification. RAW-Bio-CT cells were used as negative controls. After purification, the protein complexes were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Staining the gel with Commissie blue, many protein bands were visible in the RAW-Bio-USTAT1 lane, but such band was seldom visible in the RAW-Bio-CT lanes (Fig. 4C). Individual protein bands were excised, in-gel digested with trypsin, and then sequenced by high-performance liquid chromatography-electrospray tandem mass spectrometry. A total of 149 known or predicted proteins were identified, which were repeatedly quantified in at least twice of three independent experiments in RAW-Bio-USTAT1 cells, but not detected or at relatively low abundance in RAW-Bio-CT cells (Table S1). Among them are the closely related factor STAT3 that forms heterodimers or the histone as previous studies suggested (Buro et al., 2010, Blumert et al., 2013). Interaction of USTAT1 with
STAT3, eEF1A, Rps3, Anxa2, Ybx1 and Bag2 were confirmed by a further IP and immunoblotting experiment, which indicating such interaction occurs in vivo (Fig. 4D). A network map of the identified USTAT1 interactome was constructed using the STRING software (Szklarczyk et al., 2015). The network clusters associated with ribosome, antigen processing, virus infection and presentation, endoplasmic reticulum were readily identified in the map (Fig. S1). To better clarify the functions and signaling pathways of USTAT1-interacting proteins, we used the DAVID software package (Huang da et al., 2009) to classify all identified proteins according to their molecular functions and biological processes (Fig. 4E). The substantial enrichment in translation, protein transport, macromolecular assembly, macromolecular complex subunit organization and generation of precursor metabolites energy indicated several unexplored functions of USTAT1. KEGG pathway analysis revealed that USTAT1-interacting proteins were enriched in ribosome, oxidative phosphorylation, antigen processing, and glycolysis/ gluconeogenesis (Fig. 4F). The results confirm that we successfully isolated the USTAT1 protein complex from RAW264.7 cells and identified its interacting proteins. The interactome data suggest that USTAT1 relates to transcriptional regulation, histone modification, and immunological stress, and shuttles both in nuclear and cytoplasm.

**Decrease of USTAT1/STAT3 complex Rescues CD95 Expression in H37Ra infected macrophages**

We verified that USTAT1 and STAT3 could form complex. It has been reported STAT3 is an oncogene, regulates many anti-apoptotic proteins [57, 58] and elicits suppression of CD95 by cooperating with other transcription factor(s) (Ivanov et al., 2001). Hence, we predicted USTAT1/STAT3 complex suppresses CD95 transcription. To validate our hypotheses, we silenced USTAT1 and STAT3 expression in RAW 264.7 cells (Fig. 5A) and analyzed the CD95 expression both in mRNA level and cell surface protein level. A clear increase in the expression level of CD95 was observed in either USTAT1 or STAT3 silenced cells. Similarly, silencing USTAT1 and STAT3 coinstantaneously showed most remarkable increase in CD95 expression compared with control (Figs. 5B, 5C&5D). Furthermore, we tested the apoptosis rate of the USTAT1 or STAT3 silenced
cells with infection of H37Rα. Compared with control, silencing uSTAT1 and STAT3 dramatically increased macrophages sensitivity to H37Rα-induced apoptosis in vitro (Figs. 5E&5F). This finding points to an inverse correlation between USTAT1/STAT3 complex and CD95 expression, suggesting that the role of such complex in inhibition of CD95 mediated apoptosis in H37Rα infected macrophages.

Previous studies have shown that the -(460-230) sequence (-(-360-130) for mouse) in CD95 promoter is the key region containing GAS, AP1 and NF-κB sites which represents the activities seen by the full-length 1.7 kb promoter region (Chan et al., 1999). So we considered this region may be the binding site of USTAT1/STAT3 complex. Further confirmation for USTAT1 and STAT3 binding to the CD95 promoter was investigated in vivo via ChIP assays on chromatin samples from H37Rα infected RAW264.7 cell (Fig. 5G). Both USTAT1 and STAT3 were found in association with CD95 promoter in infected for 12 h cell, consistent with the notion that both transcription factors are bound to CD95 promoter under condition in which CD95 expression is suppressed. Decreased binding of USTAT1 to CD95 promoter was seen as early as 6 h and more so in uninfected cells. The change in USTAT1 and STAT3 binding to CD95 promoter sequences after H37Rα infection, which mediates the inhibition of apoptosis through lessened CD95 expression.

Competitive binding reactions between USTAT1 and IFIT1 to eEF1A

Another USTAT1 interacting partner, eukaryotic elongation factor 1A (eEF1A), is reported to be involved in apoptosis in several cell types (Duttaroy et al., 1998, Chen et al., 2000, Lamberti et al., 2004, Condeelis, 1995, Kato, 1999, Kato et al., 1997). Recent study pointed out that the interaction of interferon-induced protein tetratricopeptide repeats-1 (IFIT1) and eEF1A complex involve in processes of macrophages apoptosis induced by TNF-α (Li et al., 2010). In order to assess whether or not IFIT1 and eEF1A protein levels in RAW264.7 cells are affected by H37Rα infection, the immunoblots were performed and confirmed by densitometric analysis of band intensities standardized with internal control. As displayed by immunoblot band densitometries, the time-course kinetics of IFIT1 increase is similar with that of eEF1A. The IFIT1 and eEF1A protein expressions are altogether induced after initiation of H37Rα infection.
Both IFIT1 and eEF1A protein levels achieved the peak elevation within 6 h, and do not return to normal levels, but have been persisting with high levels to 48 h (Figs. 6A&6B). Since USTAT1 and eEF1A could also form complex, it implies that there may exit competitive binding reactions between USTAT1 and IFIT1 to eEF1A. To validate our hypotheses, we transfected an expression vector encoding 3×FLAG-tagged IFIT1 into RAW-Bio-USTAT1 cell or RAW-Bio-CT cell. Cellular extracts were co-immunoprecipitated with anti-FLAG antibody. eEF1A protein were precipitated with FLAG-IFIT1 in both RAW-Bio-USTAT1 cell or RAW-Bio-CT cell, but the band of eEF1A in RAW-Bio-USTAT1 from the coimmunoprecipitation experiment had lower intensity than the band of RAW-Bio-CT (Fig. 6C), suggesting that USTAT1 affects the interaction affinity between eEF1A and IFIT1. These observations establish the interaction of IFIT1 and eEF1A is disassociated with the accumulation of USTAT1 (Fig. 6D).
Discussion

Following *Mtb* binding to macrophages by Toll-like receptor (TLRs), there is activation of intracellular cell signaling events leading to macrophages apoptosis or necrosis. Previous reports have documented the JAK/STAT signaling pathway is dependent on a variety of adapter proteins, such as IFNγRs (Dale et al., 1989) and TLRs (Rhee et al., 2003, Kovarik et al., 1999). Besides, the JAK/STAT signaling pathway played an important role in the *Mtb* phagocytosis-independent way to induce macrophage apoptosis (Rojas et al., 2002b, Lim et al., 2016). Selective gene deletion of STAT1 in mice leads to rapid death from severe infections, including intracellular bacteria and viruses, demonstrating its major role in the response to pathogens (Meraz et al., 1996, Dupuis et al., 2003). However, our study shows the phosphorylated STAT1 form is transient in H37Ra infected mice macrophages, while the expression of STAT1 is substantially increased and the USTAT1 is reciprocally accumulated in consequence, which persists for at least two days. The represses expression of Jak1 regulated by USTAT1 may lead to the inhibition of STAT1 phosphorylation, which terminated the PSTAT1 signaling pathway. Analogical negative feedback mechanism is also found in several other immune cells (Zimmerman et al., 2012, Cheon and Stark, 2009). Interestingly, there is a similar trend between USTAT1 expression level and the intracellular bacterial burden in H37Ra-infected macrophage. The bacillary viability was associated with the apoptotic death of infected macrophages (Oddo et al., 1998).

Additionally, the inhibition activation of Caspase-3, Cytochrome c translocation and increase of Mcl-1 in USTAT1 overexpressed macrophages, lead to a severely obstruction in H37Ra infection-induce apoptosis. Overall, our finding thereby suggested that other than PSTAT1, USTAT1 inhibit *Mtb*-induced macrophages apoptosis.

Previous studies extensively presented the localization of USTAT1 in many cell lines, finding that its nuclear export independently of phosphorylation, is in a cell type-specific manner (Cheon and Stark, 2009, Meyer et al., 2002). Our results indicted the U-STAT1 induced by *Mtb* moves into nuclei, where it can function as a novel transcription factor to increase the expression of immune regulatory genes. It has been
shown that USTAT1 directly regulates the expression of several constitutive genes in several types of cells (Zimmerman et al., 2012, Chatterjee-Kishore et al., 2000b), and similar results were validated in mice macrophage cell line RAW264.7. These genes take biology processes in immune responses, antigen presentation and programmed cell death: Lmp2 and CIIta mediate IFN induced antiviral, antitumor and immunomodulatory (Muhlethaler-Mottet et al., 1998, Chatterjee-Kishore et al., 1998). Mcl1 is an anti-apoptosis member of the B-cell lymphoma 2 (Bcl-2) family (Thomas et al., 2010). Jak1 is the key kinase that involved in the phosphorylation of STAT1 (Yeh and Pellegrini, 1999). Hsp70 is associated with the CD95-induced apoptotic cell death (Liossis et al., 1997). Interestingly, those USTAT1-regulated genes are also induced by H37Ra infection (the result is consistent with the previous finding by microarray experiments on murine macrophages (Zhang, 2013)), raising the possibility that the H37Ra-induced gene expression may partly due to the accumulation of USTAT1 in macrophages. It provides evidence for the fact that USTAT1 may be as a novel transcription factor, involving in the expression of a wide variety of genes and regulation of cell destiny in H37Ra infected macrophages.

It is observed that the accumulation of USTAT1 signaling participates in resistance cell apoptosis (Bowman et al., 2000, Kao et al., 2013). Indeed, the aberrant expression of USTAT1 is closely associated with inflammation caused by pulmonary infection (Sampath et al., 1999, Lee et al., 2000). In this study, we showed that increasing USTAT1 concentration exogenously lead to significantly lower apoptosis rates of H37Ra or CD95L-initiated cell compared with control. Hence, it indicated that the accumulation of USTAT1 decreases the sensitivity of RAW264.7 cell to CD95/CD95L mediated apoptosis during H37Ra infection. The CD95/CD95L system is often associated with pathogen infection (Oddo et al., 1998, Zhang et al., 2005). Infected macrophages showed a reduced susceptibility to CD95L-induced apoptosis correlating with a reduced level of CD95 expression in both our and other studies (Oddo et al., 1998, Zhang et al., 2005). CD95 itself does not initiate apoptosis. CD95L is the physiologic ligand of CD95 and expressed in the surface of cytotoxic T lymphocytes (CTLs) (Kilinc et al., 2009, Fritzsching et al., 2005) and macrophages (Zhang et al.,
Up-regulated CD95L proteins in H37Ra infected macrophages cells may interact with each other to induce apoptosis (Xu et al., 1998). Overall, our results suggested the USTAT1 might confer macrophages resistance to CD95-mediated apoptosis to escapes the CTLs or macrophages mediated pathogen suppression, which represent a pathogen strategy for survival in the host.

Previous study shows that unphosphorylated STAT family protein plays role as active transcription factors: USTAT3 cooperates with NF-κB to induce RANTES gene expression (Yang et al., 2007, Yang et al., 2005). USTAT6 forms a complex with p300 to up-regulated cyclooxygenase-2 gene (Cui et al., 2007). To describe the transcriptional pattern and the complicated cellular biological processes of USTAT1, exploring its binding partners and networks of protein is necessary. In the present study, we used streptavidin-mediated affinity purification approach to comprehensively analyze composition of USTAT1 interactome in RAW264.7 cell, and the USTAT1/STAT3 complex seems mostly associated with the mechanism of regulation ability of USTAT1. Previous study suggested that USTAT1 with other STATs could form homo or heter dimer which bind to overlap GAS element to regulate gene expression (Chatterjee-Kishore et al., 2000a). Our ChIP data demonstrated that USTAT1/STAT3 complex inhibit the expression of CD95 by binding on the key region of CD95’s promoter. Following Mtb infection, there is an increase in USTAT1 and an enhanced binding to CD95 promoter in vivo, which coincides with decreased CD95 transcriptional expression and concomitant CD95L dependent apoptosis, which provides important support for a dynamic regulation by recruitment of USTAT1/STAT3 complex to CD95 promoter under relevant infection.

On the other hand, the cytoplasm localization USTAT1 may also play an anti-apoptosis role. Our results show that the USTAT1 affects the interaction affinity between eEF1A and IFIT1. The eEF1A has been characterized as a very complicated role not only in the protein elongation step of translation (Browne and Proud, 2002), but also in the process of stress-induced cell apoptosis (Duttaroy et al., 1998, Chen et al., 2000, Lamberti et al., 2004, Condeelis, 1995, Kato, 1999, Kato et al., 1997). IFIT1 is acts as an infection resistor by suppressing protein translation (Guo et al., 2000, Hui et al.,
2005), and LPS or TNF-α stimulation rapidly activates IFIT1 transcription (Smith and Herschman, 1996, Wathelet et al., 1987). Previous study shows that coupling with IFIT1 result in a change of eEF1A activity as for subsequent stressed cell apoptosis, suggested such complex which may accumulate in the early infection involves in processes of cell apoptosis (Li et al., 2010). We observed the levels of IFIT1 and eEF1A in RAW264.7 cells infected by H37Ra. Their protein levels elevating (within 6 h) appears in the early infection, suggesting that the interaction between IFIT1 and eEF1A may augment the apoptosis process of infected cells. The USTAT1 affects the interaction affinity between eEF1A and IFIT1, and give a further inferring that during the late infection, increased cytoplasm USTAT1 involved the competitive binding reactions may inhibit the apoptosis processes mediated by eEF1A/IFIT1 complex. In order to characterize the detail function of USTAT1-eEF1A interaction, more support of evidences are required, and further experimental studies are also needed to confirm how USTAT1 and interactive partners affect H37Ra-induced apoptosis in macrophages.

In conclusion, we propose that STAT1 may play a double-edged sword role during H37Ra infection in macrophages (Fig. 7). On the one hand, exposure of macrophages to H37Ra induces rapid phosphorylation STAT1 formation following with production of TNF-α and NOS2, caspase activation enhance macrophages sensitivity to apoptosis induction, which is linked to good response for host to resistance to pathogens. On the other hand, after short-term activation of PSTAT1, USTAT1 starts to accumulate after PSTAT1 start to degrade. The sustainable high USTAT1 level suppresses Jak1 to terminate the PSTAT1 signaling in a feedback inhibition manner. The USTAT1 recruit STAT3 forming complex, further represses CD95 to inhibit macrophages apoptosis resistance to Mtb, which takes bad impact on the outcome of infection. In addition, the featured binding partners of USTAT1 might play roles as alternative signaling responsible for involving in the formation of transcriptional complex and recruitment of this complex to the apoptotic gene promoters. These observations might eventually contribute to a deeper understanding into the immune evasion mechanism and elevate therapeutic implications of the role of USTAT1 functions during Mtb infection in macrophages.
Material and Method

Ethic statements
The animal experiments were approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University. Adult C57BL/6 mice (4–6 weeks old, weighing 20–25 g) were purchased from the experimental animal center of the Fourth Military Medical University (Xi’an, Shaanxi, China) and maintained on a 14-/10-h light/dark cycle with free access to food and water in strict accordance with the Guidelines for the Care and Use of Animals of Northwest A&F University. Every effort was made to minimize animal pain, suffering and distress and to reduce the number of animal used.

Mice and Cell culture macrophage differentiation and polarization
The BMDMs of C57BL/6 mice were isolated as described previously (Lim et al., 2016). Briefly, BMDMs were generated by flushing bone marrow cells from femurs and tibias, and culturing for 2 days in RPMI1640 supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), streptomycin (100 μg/mL), 25 ng/ml granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN), or 25 ng/ml macrophage colony-stimulating factor (R&D Systems). The mouse macrophage cell line RAW264.7 were grown in RPMI1640 supplemented with 10% fetal bovine serum. All cells were cultured at 37°C in 5% CO₂ in humidified incubator.

Bacteria infection and CFU assay
*M. tuberculosis* strain H37Ra (ATCC 25177) was cultured in Middlebrook 7H9 broth medium supplemented with 10% OADC (Becton, Dickinson and Company, Franklin Lakes, NJ). BMDMs or RAW264.7 cells were infected at a multiplicity of infection of 5 bacteria per cell (MOI 5:1). After 1 h, the infected cells were washed with RPMI1640 and new medium added. To assay intracellular bacterial burden of macrophages, the infected cells were incubated for the indicated time (Timing starts when medium was refreshed) and then lysed in PBS with 0.05% SDS, and intracellular bacteria were plated on Middlebrook 7H10 agar plates supplemented with OADC and incubated for 3 weeks at 37 °C to determine the colony number.
Constructs and Gene Transfection

The STAT1 coding sequence was amplified by PCR using cDNA from C57BL/6 mouse lung. The coding sequence of biotin ligase BirA was amplified from genomic DNA of Escherichia coli BL21 (DE3). The STAT1 site directed mutagenesis (Y701F -S727E) were introduced using QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). To construct the lentiviral constructs USTAT1, Bio-USTAT1-P2A-BirA or Bio-P2A-BirA, the expression cassettes with FLAG tag, Biotin tag, P2A, USTAT1 and BirA ORF sequences were assembled according to their relative location by overlap extension PCR, and then cloned into the lentiviral vector pCDH-MCS-T2A-Puro-MSCV respectively. The full length coding cDNA fragment of mouse IFIT1 was obtained using cDNA from C57BL/6 mouse lung, then cloned into p3×FLAG-CMV-10 vectors. Primer sequences for the plasmids construction were listed in Table S2. All the constructs were confirmed by DNA sequencing. To produce infectious virus, each construct was transfected into 293FT packaging cells by using Lipofectamine®2000 (Invitrogen, Carlsbad, CA). The supernatant medium, collected once each day, was applied to infect cells. To select stably transfected cells, they were treated with 5 μg/mL puromycin for more than two weeks. Proteins or RNAs were extracted and purified from these cell pools for Western blot or qPCR analyses.

Nuclear extract preparation

Nuclear extracts of RAW264.7 cells prepared as described (Rojas et al., 2002a). Briefly, macrophages were harvested and resuspended in 1.5 ml cold PBS. The cell pellets were resuspended in 400μl lysis buffer (10mM Hepes–KOH, pH 7.9, at 4 °C, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF), incubated on ice for 10 min and then vortexed for 10 s. Samples were centrifuged for 10 s at 15,000g at room temperature and the supernatants were used to detect cytoplasmic component by Western blot. The pellet was resuspended in 100μl of cold nuclear lysis buffer (20mM Hepes–KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and incubated on ice for 20 min. Nuclear debris was removed by centrifugation at 15,000g for 10 s, and the supernatants were used to detect nuclear component.
**Immunofluorescence and Confocal Microscopy Analysis**

Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min, incubated with DAPI (Beyotime, Jiangsu, China) to visualize the nuclei, washed with PBS, and mounted onto microscope slides. Exogenous FLAG-tagged STAT1 stained with anti-FLAG antibody (1:500, Sigma, Saint Louis, MO), diluted in blocking solution, was applied. After incubation at 4°C overnight, the cells were incubated with Alexa555-conjugated goat anti-mouse antibodies (1:500, Beyotime) diluted in blocking solution for 2 h. After washing twice with PBS, the Slides were examined and images acquired using a Nikon ECLIPSE TE2000 confocal microscope.

**Apoptosis assays and CD95 assays**

Cells apoptosis was stained with Alexa Fluor 488-conjugated Annexin V and propidium iodide (PI) (Molecular Probes, Eugene, OR), and then analyzed by flow cytometry (BD Biosciences, San Jose, CA). CD95 Ligand protein was purchased from Sigma. Cells were stained with FITC-conjugated anti-CD95 mAb (Biorbyt, Berkeley, CA) and analyzed by flow cytometry. CD95 protein level is quantified by mean fluorescence intensity. Flow cytometry acquired viable cells which were further gated on the basis of forward versus side scatter and then analyzed after staining with conjugated dye. In each analysis, appropriate negative or positive controls were used to define gate settings.

**Quantitative PCR**

Total RNA was extracted from RAW264.7 cells using Trizol regent (Invitrogen), and then 1μg of RNA was reverse transcribed to cDNA using SYBR PrimeScript RT reagent Kit (Takara, Dalian, China). The qPCR was performed using SYBR Premix ExTaq II (Takara) on a StepOne Plus PCR system (Applied Biosystems, Foster City, CA). The comparative CT method was employed for quantification of target mRNA expression, and the relative expression of mRNA was normalized to GAPDH expression. Primer sequences for the qPCR were listed in Table S2.

**Cytosolic subcellular fractionation (mitochondria isolation)**

The fractionation of cytosolic extract was followed by using a previously described procedure (Ghorpade et al., 2012). In brief, the cells were harvested and gently resuspended in Lysis and Extraction Buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1
mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM PMSF). Cell membranes were disrupted with 10% NP-40 after incubation on ice for 15 min. Cytosolic extract was finally separated from nuclei and mitochondria by centrifugation at 13,000 rpm for 15 min at 4°C.

**Purification and Mass-spectrometric (MS) analysis of USTAT1 complexes**

The purification of USTAT1 protein complexes was followed by a described protocol (Kim et al., 2009). The affinity purification protein sample was separated by 4-20% gradient SDS-PAGE (Bio-Rad), and then stained with Acqua stain protein gel dye (Bulldog Bio Inc., Portsmouth, NH). Bands were excised from the gel and digested with trypsin. The digested extraction was analyzed using liquid chromatography coupling electrospray ionization tandem mass spectrometry (LC/MS/MS) as reported before (Wang et al., 2016). According to the obtained amino acids sequence information, the blast (n) was carried out at the NCBI database.

**Co-immunoprecipitation and Western blotting**

Co-immunoprecipitation was carried out using Co-IP Kit (Pierce, Rockford, IL), following manufacturer’s instructions. Immunoprecipitated protein sample was resolved in a 12% SDS-PAGE gel and transferred to a PVDF membrane. Membranes were blocked with 10% non-fat dry milk diluted in TBST for 3h, probed with Primary antibodies (1:1000) over night at 4°C, and subsequently incubated with HRP-labeled goat anti-mouse antibodies (1:1000, Beyotime, Jiangsu, China). Finally, blots were developed with ECL chemiluminiscence reagent (Beyotime). Primary antibodies used were pStat1 (Tyr701), pStat1 (Ser727), Caspase 3, Mcl-1, Cytochrome c, STAT3, eEF1A, Anxa2, Ybx1 and Bag2 purchased from Cell Signaling Technology (Danvers, MA), monoclonal antibody against STAT1 (C-terminal) purchased from BD Transduction Laboratories (Biocompare, CA), IFIT-1 (Abcam, Cambridge, MA), FLAG (Sigma), Actin (TransGen Biotech, Beijing, China), PCNA, Cox IV and HRP-Streptavidin (Beyotime).
**RNA interference**

siRNA targets mouse STAT1, STAT3 and negative control siRNA were purchased from GenePharma (Shanghai, China). siRNA sequences were as follows, si-Stat1: GCUGAACUAUAACUUGAAA; si-Stat3: GGGUCUGGCUAGACAAUAUTT; si-control: UUCUCCGAACGUGUCACGU. RAW264.7 cells were transfected with 50 nM of indicated siRNAs overnight using Lipofectamine 2000 Reagent (Thermo Scientific, Rockford, IL).

**Chromatin Immunoprecipitation assays**

The Chromatin Immunoprecipitation (ChIP) was carried out using ChIP Kit (Pierce, Rockford, IL). RAW264.7 cells (2.5×10^6 cells per 10 cm diameter plate) were infected with *Mtb* H37Ra strain and cells were fixed by 1% (wt/vol) formaldehyde at indicated time points. The reaction subsequently quenched with 125 mM glycine. Genomic DNA was isolated and sheared to average lengths of 500-1000 bp by ultrasonic. 50μg of purified chromatin samples were immunoprecipitated with 1 μg of anti-USTAT1, anti-STAT3 or IgG. ChIP enrichment was performed by qPCR (17 cycles of 1 min at 94℃, 53℃ and 72℃) using primers (Forward: TGAGCAGCAGGCAGAAAAAAATCTCACTTGAC and Reverse: TGTCACTTTTTTTTTTGGGGACCAAACACAAT ) that amplify the fragment containing key region of CD95 promoter.

**Statistical analysis**

The data were represented as the mean ± SD and were analyzed using the Student’s t-test. A value of p<0.05 was considered significant.

**CONFLICTS OF INTEREST**

The authors have declared that no competing interests exist.

**FUNDING**

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Fig 1. H37Ra increase the expression of USTAT1.

(A) BMDMs were infected with H37Ra. The amounts of PSTAT1 and USTAT1 were measured by the Western Blotting method. (B) Correlation analysis between STAT1 protein level and bacterial burden in H37Ra infected cell. The bar graph shows intracellular mycobacterial burden at the time points indicated by a CFU assay (Y axis on the left). The curves are mean relative density measurements of the PSTAT1 and USTAT1 bands normalized for the density of their corresponding Actin band (Y axis on the right). Each data are shown as the mean ± SD (error bars) of triplicate independent experiments.
Fig 2. USTAT1 confers RAW264.7 cell resistance to H37Ra-induced apoptosis

(A) RAW264.7 cells were infected with lentiviruses expressing mutant-STAT1 (RAW-USTAT1), or with empty vector (RAW-CT). The U-STAT1 protein expression levels were measured by the Western blotting method. (B) RAW-CT and RAW-USTAT1 were infected with H37Ra, and the PSTAT1 and USTAT1 expression was determined by Western Blotting method. (C-D) The location of exogenous USTAT1 was determined
by immunoblot and immunocytochemistry. PCNA or Actin is used as control for normalization in nucleus or cytoplasm. Nuclei were stained with DAPI. (E-F) RAW-CT and RAW-USTAT1 cells were infected with H37Ra at pointed time. Apoptotic cells were evaluated by Annexin-V staining followed by flow cytometry. (G) RAW-CT and RAW-USTAT1 cells were infected with H37Ra for 18 h. Intracellular mycobacterial burden was determined indicated by a CFU assay. (H) RAW-CT and RAW-USTAT1 cells were infected with H37Ra for 18 h. Expression of Lmp2, Mcl1, CIIta, CD95, Hsp70 and Jak1 was determined by qPCR. Data are normalized to the uninfected RAW-CT. Each data are shown as the mean ± SD (error bars) of triplicate independent experiments. *p<0.05; **p<0.01; ***p<0.001, compared with controls.
Fig 3. USTAT1 reduced susceptibility in RAW264.7 cell to CD95L induced apoptosis

(A-B) Cell surface CD95 protein levels were measured by staining cells with FITC-conjugated CD95 mAb and analyzed by flow cytometry. CD95 protein level is quantified by MFI. (C-D) RAW-CT and RAW-USTAT1 cells were incubated in the absence or presence of CD95L (250 ng/mL) for 18 h. Apoptotic cells were evaluated by Annexin-V staining followed by flow cytometric. (E) RAW-CT and RAW-USTAT1 cells were infected with H37Ra for 18 h. Expression of CD95L was determined by qPCR. Data are normalized to the uninfected RAW-CT. (F) Cyosolic translocation of Cytochrome c, activation of Caspase-3 and expression of Mcl-1 in RAW-CT and RAW-USTAT1 were assayed using western blotting after infection with H37Ra. Actin or Cox IV is used as control for normalization in cytoplasm or mitochondria. Each data are shown as the mean ± SD (error bars) of triplicate independent experiments. *p<0.05; **p<0.01; ***p<0.001, compared with controls.
Fig 4. Characterization of the USTAT1 protein interactome by affinity purification and mass spectrometry.

(A) Schematic representation of the structure of plasmids used for constructing the stably-transfected RAW-Bio-USTAT1 cells. (B) Verification of biotinylated USTAT1 by immunoblotting using streptavidin-coupled horse radish peroxidase (HRP). (C) The affinity-purified proteins were eluted from the beads by SDS sample buffer and separated by SDS-PAGE. The gel was stained with colloidal Coomassie. (D) The presence of the USTAT1 protein partner was detected by immunoblotting using specific
antibodies. (E) GO annotation of USTAT1-interacting proteins. The top 15 GO terms (biological process) ranked according to the protein counts are plotted. (F) KEGG pathway analysis of USTAT1-interacting proteins. Each data shown are representative of three independent experiments.
Fig 5. Cooperation between USTAT1 and STAT3 Suppresses CD95 Transcription

(A) Silence of USTAT1 and STAT3 upregulates CD95. RAW264.7 cells were transfected with siRNA targets STAT1, STAT3 and control siRNA overnight. The knockdown efficiency of STAT1 and STAT3 was examined by immunoblotting. (B) The siRNA–transfected cells were infected with H37Ra for 18 h, the mRNA expression of CD95 was determined by qPCR. (C-D) Cell surface CD95 protein levels were measured by staining cells with FITC-conjugated CD95 mAb and analyzed by flow cytometry. CD95 protein level is quantified by MFI. (E-F) The siRNA–transfected cells were infected with H37Ra for 18 h. Apoptotic cells were evaluated by Annexin-V staining followed by flow cytometric. (G) ChIP assays were carried out on chromatin
that was immunoprecipitated with antibodies to USTAT1 or STAT3 from control or H37Ra infected cells at the indicated time points. Shown is agarose gel electrophoresis of a CD95 promoter fragment amplified by PCR from each of the ChIP samples. Control IP was performed with anti-IgG. Amplification of genomic DNA is shown (gDNA). Each data are shown as the mean ± SD (error bars) of triplicate independent experiments. *p<0.05; **p<0.01; ***p<0.001, compared with controls.
Fig 6. Competitive binding reactions between USTAT1 and IFIT1 to eEF1A

(A-B) A kinetic study of IFIT1 and eEF1A proteins following infection of RAW264.7 cultures with H37Ra at indicated time points was performed. The curves are mean relative density measurements of the IFIT1 and eEF1A bands normalized for the density of their corresponding Actin band. (C) Lysates of RAW-BioUSTAT1 and RAW-Bio-CT cells expressing p3×FLAG-IFIT were immunoprecipitated (IP) with anti-FLAG antibody respectively, and detected by western blot (WB) with anti-eEF1A antibody. The data are representative of three independent experiments. (D) Schematic representation of a competitive binding reactions between USTAT1 and IFIT1 to eEF1A. Each results shown is representative of three independent experiments.
Fig 7. Two-sided effect of STAT1 during *Mtb* infection. Schematic representation of a proposed model to illustrate the cross-talk network of PSTAT1 and USTAT1 during *Mtb* induced apoptosis.
**Fig. S1** The protein-protein interaction network of USTAT1. The identified USTAT1 interactome was constructed using the STRING software.

**Table S1**

Click here to Download Table S1
**Table S2 Primer sequences used in this study.** (A) Primer sequences used for plasmids construction. (B) Primer sequences used for qPCR analysis.

### A

**Primer sequences for plasmids construction**

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<th>Plasmids</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<tr>
<td>STAT1</td>
<td>ATGCAGCGCGCCACCACCTGACTCAAAG</td>
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<td>ACGATGACGACAAGATGACTAAAAGCA</td>
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<td>CAGAGGAGACCTGATGGAAGAACCCTCATGAC</td>
<td>CTCAGACTACGCAGG</td>
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<td>P2A-BioST</td>
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<td>AGAGCGGCCGCTTTT</td>
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<tr>
<td>AT1</td>
<td>TCC AGCTCTGAAAGCCAGTGAAAGGAGACGGTTC</td>
<td>GCCACTGACCTTTT</td>
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<td></td>
<td>GGGAGGAGACCCCTGGGCTACTCAGT</td>
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<tr>
<td>IFIT1</td>
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<td>CCGGATCTCAAGAATG</td>
</tr>
<tr>
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<td>TGAC</td>
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### B

**Primer sequences for qPCR analysis**

<table>
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<th>Gene name</th>
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<td>Lmp2</td>
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<td>TCATCGTAGAATTTTGCACTGT</td>
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<td>Mcl1</td>
<td>CGACTCCGAGACAGACGAC</td>
<td>CCAATTTAAACAGGCTTTTGTA</td>
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<td>Ciita</td>
<td>TGCGTGCTGGAATGCTCAATCAG</td>
<td>CCAAAGGGGATAGTGGTGT</td>
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<td>CD95</td>
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<td>ACAACCAGATGCCATTTTCG</td>
</tr>
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<td>Hsp70</td>
<td>TCTCGGTCCACACCCTACTCTC</td>
<td>CCAGGACCGAGATCCCTTT</td>
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<tr>
<td>Jak1</td>
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<td>TTGGTAAAGGTAAACCTCATCG</td>
</tr>
<tr>
<td>CD95L</td>
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<td>GGGGTTTCCTGGTAAAATG</td>
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<tr>
<td>Gapdh</td>
<td>GTGTGCTTACCCCAATGTGTG</td>
<td>ATGGCTATACCAGGAAATGAG</td>
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(A) Primer sequences used for plasmids construction. (B) Primer sequences used for qPCR analysis.