Dear Editor,

Macrophages play a key role in maintaining homeostasis and in mounting competent immune responses. Upon stimulation, one of the key effector functions of activated macrophages is production of inflammatory cytokines including interleukin 6 (IL-6) and interleukin 12 (IL-12). IL-6 and IL-12 are important for transition from innate to adaptive immunity, and thus essential for host defense against multiple pathogens (Kishimoto, 2005; Trinchieri, 2003). However, over-production of IL-6 and IL-12 contributes to a number of human autoimmune and inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis (Neurath, 2014). Thus, the production of IL-6 and IL-12 is tightly controlled by various negative regulatory mechanisms at multiple levels targeting upstream signaling pathways, sequence-specific transcription factors, epigenetic modifications, as well as post-transcriptional steps (Lyakh et al., 2008).

Hairy and enhancer of split 1 (Hes1) is a transcription repressor that belongs to a family of basic helix-loop-helix (bHLH) DNA-binding proteins and plays critical roles in the development of multiple organs and cell types (Kobayashi and Kageyama, 2014). The structure of Hes1 is highly conserved among species, which includes bHLH domain, Orange domain and WRPW motif. Functionally, mice globally deficient in the Hes1 gene exhibit embryonic or perinatal lethality and display multiple developmental defects, indicating a critical role for Hes1 in organism development (Kobayashi and Kageyama, 2014). Recently, accumulating evidence has suggested an emerging role of Hes1 in the immune system, including regulation of T and B cell differentiation (Shang et al., 2016b), attenuation of inflammatory responses in macrophages upon LPS stimulation while such inhibitory capacity was compromised in a Hes1 mutant lacking the WRPW motif (Hes1 ΔWRPW) (Fig. 1B), suggesting that the WRPW motif is involved in Hes1-mediated suppression in a gain-of-function system, we assessed the effects of Hes1 on expression of Il6 and Il12b by luciferase assays. Overexpressing of wild-type Hes1 inhibited luciferase activities driven by the Il6 and Il12b promoters in RAW264.7 mouse macrophages upon LPS stimulation while such inhibitory capacity was compromised in a Hes1 mutant lacking the WRPW motif (Hes1ΔWRPW) (Fig. 1B), suggesting that the WRPW motif is required for Hes1-mediated suppression. Together, these data demonstrated that Hes1 suppressed Toll-like receptor (TLR)-mediated inflammatory responses via the WRPW motif that is responsible for recruitment of transcription corepressors.

TLE4 acts as a corepressor of Hes1 to inhibit inflammatory responses in macrophages

Transducin-like enhancer of split 4 (TLE4) is a member of the TLE family of transcription corepressors. TLE proteins are the mammalian homologs of Groucho in Drosophila that is involved in multiple developmental processes including sex determination and neurogenesis (Buscariel and Stifani, 2007; Chen and Courey, 2000). TLE family proteins do not bind to DNA directly but rather interact with a variety of transcription factors such as Hes1, Runx2 and T cell factor/lymphoid enhancer binding factor (TCF/LEF) to form repressor complexes (Buscariel and Stifani, 2007). The Tle gene family consists of four full length Tle genes in mouse and human, which encode four different proteins (TLE1-4) with similar domain structures (Chen and Courey, 2000). Given the potential redundancy imposed by multiple family members, functions of the individual mammalian TLE protein are less well understood than their single Drosophila counterpart. Within the TLE family, TLE1 is the most studied with implicated functions in neuronal differentiation, tumorgenesis and regulation of inflammation (Buscariel and Stifani, 2007; Ramasamy et al., 2016), whereas the functions of TLE4 remain largely unknown, especially in the immune system.

We have previously showed that Hes1 suppressed expression of key pro-inflammatory cytokines including IL-6 and IL-12 in macrophages (Shang et al., 2016a). To further confirm these observations, we examined the gene expression of Il6 and Il12b in Mx1-Cre (WT) and Hes1fl/− Mx1-Cre (Hes1 KO) bone marrow-derived macrophages (BMDMs) in response to lipopolysaccharide (LPS) stimulation. Deficiency of Hes1 significantly promoted induction of Il6 and Il12b mRNA in BMDMs by LPS in multiple independent experiments (Fig. 1A), supporting that Hes1 indeed acted as a repressor of IL-6 and IL-12. The WRPW motif of Hes1 has been shown to interact with other proteins such as transcription corepressors to mediate gene repression (Kobayashi and Kageyama, 2014). To investigate whether WRPW motif is involved in Hes1-mediated suppression in a gain-of-function system, we assessed the effects of Hes1 on expression of Il6 and Il12b by luciferase assays. Overexpressing of wild-type Hes1 inhibited luciferase activities driven by the Il6 and Il12b promoters in RAW264.7 mouse macrophages upon LPS stimulation while such inhibitory capacity was compromised in a Hes1 mutant lacking the WRPW motif (Hes1ΔWRPW) (Fig. 1B), suggesting that the WRPW motif is required for Hes1-mediated suppression. Together, these data demonstrated that Hes1 suppressed Toll-like receptor (TLR)-mediated inflammatory responses via the WRPW motif that is responsible for recruitment of transcription corepressors.
Given that TLE family proteins are best characterized corepressors of Hes1 (Chen and Courey, 2000) yet there exists scarce knowledge regarding their regulation and function in the immune system, we next sought to examine the expression patterns of TLE family genes in resting and activated macrophages. Upon stimulation of macrophages with multiple TLR ligands including LPS (a TLR4 ligand), Poly(I:C) (a TLR3 ligand), zymosan (a dectin and TLR2 ligand) and R848 (a TLR7 ligand), expression of *Tle1* and *Tle2* was not significantly altered (Fig. S1A, upper panel). In contrast, expression of *Tle3* and *Tle4* was strongly induced by LPS or zymosan and modestly induced by Poly(I:C) or

Figure 1. TLE4 is required for Hes1-mediated suppression of key pro-inflammatory gene expression. (A) Quantitative real-time PCR (qPCR) analysis of *Il6* and *Il12b* mRNA in *Mx1-Cre* (WT) and *Hes1*/*Mx1-Cre* (Hes1 KO) BMDMs stimulated with 10 ng/mL of LPS for 3 h, presented relative to that in WT BMDMs. (B) Luciferase activities in RAW264.7 cells co-transfected with *Il6* or *Il12b* promoter-driven reporter constructs and a Hes1 expression plasmid (pMx-Hes1, wtHes1), a Hes1 mutant expression plasmid (pMx-Hes1ΔWPRW, Hes1ΔWPRW) or control vector (pMx-GFP). 24 h post infection, cells were left untreated or stimulated with 100 ng/mL of LPS for 6 h, and cell lysates were analyzed for luciferase activities. Results are presented as ratio of luciferase activity/total protein concentration, and are normalized to values in the control vector group. (C) Quantitative real-time PCR analysis of *Il6* and *Il12b* mRNA in wild type (WT) and TLE4 KO iBMDMs stimulated with or without LPS (100 ng/mL) for 3 h. (D) Immunoblot analysis of indicated proteins in immunoprecipitated (IP) samples and whole lysates of HEK 293T cells that overexpressed wild-type Hes1 or Hes1 mutants. p38 was used as a loading control. Data are representative of three (C) independent experiments (mean ± s.d. of technical triplicates), or are pooled from four (A) or three (B) independent experiments (mean ± s.d. of biological triplicates). ns, not significant. *P < 0.05, **P < 0.01 (Student’s paired t-test).
LPS stimulation, de
Tle4 (strategies for gene targeting shown in Fig. S2). Upon
Δ lacking the HLH domain (Hes1 mutant of Hes1 (dnHes1), and a truncated Hes1 mutant
WRPW motif by co-immunoprecipitation assay. Indeed,
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Δ de (Fig. 1C), resembling the phenotypes observed in Hes1-
lines that were individually de
gene in immortalized BMDMs (iBMDMs) and generated cell
CRISPR/Cas9 technology, we genetically ablated each
Tle
family members, TLE4 served as the Hes1 corepressor to restrain
and biochemical data demonstrated that among TLE family
Hes1 through the WRPW motif. Together, these functional
in macrophages, we generated TLE4 null mice (Tle4
malities compared with
Tle4
the surviving
R848 (Fig. S1A, lower panel), indicating that TLE3 and TLE4 are likely involved in TLR-induced macrophage activation.
As both TLR2 and TLR4 lead to activation of NF-κB and mitogen-activated protein kinases (MAPKs), we then investi-
gated whether activation of NF-κB and MAPKs was responsible for TLR-mediated induction of Tle3 and Tle4 by
pharmacologically inhibiting these signaling modules. We
found that inhibition of NF-κB or MAPKs p38, JNK and ERK
by specific inhibitors reduced induction of Tle3 and Tle4 by
LPS or zymosan. Among these inhibitors, Bay11-7082 (an
IKK inhibitor) and SP600125 (a JNK inhibitor) were most
effective at dampening Tle expression (Fig. S1B). Collect-
tively, these results demonstrated that among Tle genes,
extion of Tle and Tle4 was upregulated upon macrophage
activation in a manner dependent on the IKK and JNK
signaling pathways.

Having shown that four TLE family members exhibited
distinct expression patterns in macrophages, we then sought
to identify the TLE family member(s) that served as Hes1
corepressor to inhibit Il6 and Il12b expression. With the
CRISPR/Cas9 technology, we genetically ablated each Tle
gene in immortalized BMDMs (iBMDMs) and generated cell
lines that were individually deficient in Tle1, Tle2, Tle3 or
Tle4 (strategies for gene targeting shown in Fig. S2). Upon
LPS stimulation, deficiency of TLE1, TLE2 or TLE3 did not
promote Il6 and Il12b expression (Fig. S3), indicating that
TLE1-3 were not the corepressors of Hes1 to suppress
inflammatory responses in macrophages. In contrast, abla-
tion of Tle4 resulted in enhanced expression of Il6 and Il12b
(Fig. 1C), resembling the phenotypes observed in Hes1-
deficient macrophages (Fig. 1A). These results implied that
functionally, TLE4 may act as the corepressor of Hes1 to
regulate inflammatory responses. Given the WRPW motif of
Hes1 is responsible for corepressor recruitment, we then
examined whether TLE4 interacted with Hes1 through
WRPW motif by co-immunoprecipitation assay. Indeed,
TLE4 interacted with wild-type Hes1, a dominant-negative
mutant of Hes1 (dnHes1), and a truncated Hes1 mutant
lacking the HLH domain (Hes1ΔHLH), but not the truncated
Hes1 mutant lacking the WRPW motif (Hes1ΔWRPW)
(Fig. 1D), indicating that TLE4 physically interacted with
Hes1 through the WRPW motif. Together, these functional
and biochemical data demonstrated that among TLE family
members, TLE4 served as the Hes1 corepressor to restrain
inflammatory responses in macrophages.

To further corroborate the above observations in primary
macrophages, we generated TLE4 null mice (Tle4
−/−) that
were globally deficient in the Tle4 gene. Global deletion of
Tle4 led to partial lethality by 4 weeks after birth, plausibly
due to growth defects and skeletal abnormalities, consistent
with the previous reports (Wheat et al., 2014). Nevertheless,
the surviving Tle4−/− mice showed no observable abnor-
malities compared with Tle4+/+ littermates (data not shown)
and thus were used for the following experiments. After
confirmation of efficient deletion of Tle4 in Tle4−/− and Tle4−/+ cells (Fig. 2A), we generated BMDMs from Tle4+/+, Tle4−/−
and Tle4−/+ littermates and stimulated these cells with LPS.
Quantitative real-time PCR analysis showed that TLE4 inhibited Il6 and Il12b expression in primary macrophages in
a gene-dosage dependent manner (Fig. 2B). Upregulation of
Il6 and Il12b upon TLE4 deficiency was highly consistent
among multiple independent experiments (Fig. 2C). More-
over, protein levels of IL-6 and IL-12p40 were elevated in the
supernatants of TLE4 KO BMDMs compared with those of
WT BMDMs (Fig. 2D). To exclude the possibility that TLE4
may impair immune cell development, we examined popu-
lations of monocytes and lymphocytes in TLE4 KO bone
marrows, and found no significant differences between WT
and TLE4 KO animals (Fig. S4 and data not shown),
implying a role of TLE4 in regulation of myeloid cell function
instead of development. Collectively, the above genetic evi-
dence demonstrated that TLE4 acted as a critical negative
regulator of IL-6 and IL-12 in primary macrophages.

In order to specifically study the role of TLE4 in myeloid
cells and to exclude the potential secondary effects from
other cell types, we generated TLE4 myeloid-specific condi-
tional knockout mice. Firstly, CRISPR/Cas9 technology
was applied to knock in two loxp sites surrounding exon2 of
the Tle4 gene to obtain Tle4lox/lox mice that were further bred
with Lyz2-Cre mice to yield Tle4lox/lox Lyz2-Cre (TLE4 cKO)
mice, which specifically depleted TLE4 in myeloid cells
(Fig. S5A). After confirmation of efficient deletion of Tle4 in
TLE4 cKO BMDMs (Fig. 2E), we performed flow cytometry
analysis and found that the percentages of monocyte pop-
hulations in bone marrows of TLE4 cKO mice were identical to
those in WT animals (Fig. S5B), indicating that TLE4 defi-
ciency did not affect myeloid cell development. Compared to
the control cells, expression of IL-6 and IL-12 was increased
at both mRNA and protein levels in TLE4 cKO BMDMs in
response to LPS stimulation (Fig. 2F–H), further supporting
the notion that TLE4 functioned as a suppressor of inflam-
matory responses.

Having identified that TLE4 repressed IL-6 and IL-12
expression in macrophages in vitro, we next sought to
investigate the functions of TLE4 in vivo in a LPS-induced
septic shock model. Upon challenge with a sub-lethal dose
of LPS, TLE4 cKO mice succumbed more easily (Fig. 2I)
and exhibited more severe body weight loss than Lyz2-Cre mice
(Fig. 2J), suggesting that myeloid cell intrinsic TLE4 pro-
tected mice from LPS-induced septic shock. Thus, our
results demonstrated that TLE4 suppressed expression of
key immune cytokines and protected host from acute
inflammation.

Production of inflammatory mediators is negatively con-
trolled by multiple mechanisms to prevent toxicity and
maintain immune homeostasis. We have previously reported
that TLR stimulation induced expression of Hes1 that in turn
inhibited inflammatory cytokine expression, which consti-
tuted a negative feedback loop restraining TLR-mediated
inflammatory responses in macrophages (Hu et al., 2008;
Shang et al., 2016b). To mediate gene repression, Hes1
recruits transcription corepressors such as TLEs via the
TLE4 negatively regulates macrophage cytokine production

**A** Relative expression (mRNA/Gapdh mRNA) of Tle4

**B** Relative expression (mRNA/Gapdh mRNA) of Il6

**C** Relative mRNA expression of Il6 and Il12b

**D** IL-6 (ng/mL) and IL-12p40 (ng/mL) levels after LPS treatment

**E** Relative expression (mRNA/Gapdh mRNA) of Lps

**F** Relative mRNA expression of Il6 and Il12b

**G** Relative mRNA expression of Il6 and Il12b

**H** IL-6 (ng/mL) and IL-12p40 (ng/mL) levels after LPS treatment

**I** Survival (%) after LPS treatment

**J** Body weight (% of initial weight) after LPS treatment
C-terminal WPRW motif (Kobayashi and Kageyama, 2014; Chen and Courey, 2000). However, little is known about the expression patterns and functions of TLE family proteins in the immune system. In this study, we showed that expression of TLE family members TLE3 and TLE4, but not TLE1 and TLE2, was inducible in response to stimulation of macrophages with multiple TLR ligands, suggesting that TLE3 and TLE4 may play important roles in innate immune responses. Consistent with this notion, we found that TLE4 physically interacted with Hes1 and inhibited expression of IL-6 and IL-12, two key pro-inflammatory cytokines, in a pattern highly similar to inhibitory action of Hes1. Therefore, our findings identified TLE4 as a corepressor of Hes1 to repress expression of key inflammatory genes, highlighting the importance of TLE4 in regulation of TLR-mediated inflammatory responses.

The mammalian TLE family proteins have been mainly studied in developmental processes (Chen and Courey, 2000). Although few reports have linked TLE1 with regulation of inflammatory responses (Ramasamy et al., 2016), the exact roles of TLE family members in immune cells remain largely unclear. In this study, we found that TLE4, but not the other three TLE members, inhibited expression of IL-6 and IL-12 in macrophages. According to the established paradigm, TLEs interact with Hes1 via a conserved WDR domain with little specificity (Chen and Courey, 2000). Nevertheless, our findings suggested that Hes1 specifically recruited TLE4 to repress expression of IL-6 and IL-12. One plausible explanation for such specificity may be the different expression levels of TLEs in macrophages. In fact, compared to the other three TLEs, the expression level of TLE4 was relatively high in macrophages (Fig. S1A), raising the possibility that Hes1 preferentially interacted with TLE4 to suppress inflammation in macrophages. In addition, interaction of Hes1 with different TLE proteins may lead to formation of different transcription repressive complexes by recruiting additional proteins (Buscarlet and Stifani, 2007). Further studies are needed to gain comprehensive understanding of the precise molecular mechanisms underlying Hes1-TLE interaction in the context of inflammatory responses. Moreover, TLE4 has been reported to interact with transcription factors other than Hes family proteins such as TCF/LEF involved in Wnt signaling that regulates development and functionality of multiple immune cell lineages (Staal et al., 2008). Thus, we speculate that TLE4 may also interact with TCF/LEF to regulate macrophage activation, which demands future investigation. In summary, through biochemical and genetic approaches, we uncovered a critical and previously unidentified role of TLE4 in suppressing macrophage inflammatory responses and in protecting host from acute inflammation.

FOOTNOTES

This research was supported by Ministry of Science and Technology of China National Key Research Projects 2015CB943200 (X. Hu), National Natural Science Foundation of China grants 31725010 (X. Hu), 81422019 (X. Hu), 81571580 (X. Hu), 91642115 (X. Hu) and 8166130161 (X. Hu), Shandong Provincial Natural Science Foundation of China grant ZR2017MC021 (Y. Shang), funds from Tsinghua-Peking Center for Life Sciences (X. Hu) and funds from Shandong “Double Top” Program 564013 (Y. Shang).

Xiang Zhang, Xiaoyu Li, Fei Ning, Yingli Shang and Xiaoyu Hu declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13238-018-0554-3) contains supplementary material, which is available to authorized users.