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Published in:
FEBS Journal

DOI:
[10.1111/febs.15896](https://doi.org/10.1111/febs.15896)

Publication date:
2021

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Document Version
Publisher's PDF, also known as Version of record

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Petrova, T., Zhang, R., Nanda, S., Figueras-Vadillo, C., & Cohen, P. (2021). HOIL-1-catalysed, ester-linked ubiquitylation restricts IL-18 signaling in cytotoxic T cells but promotes TLR signalling in macrophages. *FEBS Journal*, 288(20), 5909-5924. <https://doi.org/10.1111/febs.15896>

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EDITOR'S CHOICE

HOIL-1-catalysed, ester-linked ubiquitylation restricts IL-18 signaling in cytotoxic T cells but promotes TLR signalling in macrophages

Tsvetana Petrova , Jiazhen Zhang, Sambit K. Nanda , Clara Figueras-Vadillo and Philip Cohen 

MRC Protein Phosphorylation and Ubiquitylation Unit, School of Life sciences, University of Dundee, UK

Keywords

HOIL-1; macrophage; myddosome; T cell; ubiquitylation

Correspondence

P. Cohen, MRC Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, UK

Tel: 44-1382-385602

E-mail: p.cohen@dundee.ac.uk

Tsvetana Petrova, Jiazhen Zhang, Sambit K. Nanda made equally important contributions to this study

(Received 2 December 2020, revised 19 March 2021, accepted 19 April 2021)

doi:10.1111/febs.15896

The atypical E3 ligase HOIL-1 forms ester bonds between ubiquitin and serine/threonine residues in proteins, but the physiological roles of this unusual modification are unknown. We now report that IL-18 signalling leading to the production of interferon γ (IFN γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) is enhanced in cytotoxic T cells from knock-in mice expressing the E3 ligase-inactive HOIL-1[C458S] mutant, demonstrating that the formation of HOIL-1-catalysed ester-linked ubiquitin bonds restricts the activation of this pathway. We show that the interaction of IRAK2 with TRAF6 is required for IL-18-stimulated IFN- γ and GM-CSF production, and that the increased production of these cytokines in cytotoxic T cells from HOIL-1[C458S] mice correlates with an increase in both the number and size of the Lys63/Met1-linked hybrid ubiquitin chains attached to IRAK2 in these cells. In contrast, the secretion of IL-12 and IL-6 and the formation of *il-12* and *il-6* mRNA induced in bone marrow-derived macrophages (BMDMs) by prolonged stimulation with TLR-activating ligands that signal via myddosomes, which also requires the interaction of IRAK2 with TRAF6, were not increased but modestly reduced in HOIL-1[C458S] BMDM. The decreased production of these cytokines correlated with reduced ubiquitylation of IRAK2. Our results establish that changes in HOIL-1-catalysed ester-linked ubiquitylation can promote or reduce cytokine production depending on the ligand, receptor and immune cell and may be explained by differences in the ubiquitylation of IRAK2.

Introduction

The linear ubiquitin assembly complex (LUBAC) comprises three proteins, termed HOIL-1 [haem-oxidised IRP2 ubiquitin ligase-1, also called RanBP-

type and C3HC4-type zinc finger-containing protein 1], HOIL-1-interacting protein (HOIP) and Shank-associated RH domain interactor (Sharpin) [1–4].

Abbreviations

ABIN1, A20-binding inhibitor of NF- κ B 1; BMDMs, bone marrow-derived macrophages; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM-CSF, granulocyte-macrophage colony-stimulating factor; HOIL-1, haem-oxidised IRP2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; IFN- γ , interferon γ ; IKK, I κ B kinase; IL, interleukin; IL-18R, IL-18 receptor; IRAK, interleukin receptor-associated kinase; JNK, c-Jun N-terminal kinase; K63-Ub, Lys63-linked ubiquitin; LUBAC, linear ubiquitin assembly complex; M1-Ub, Met1-linked ubiquitin; MAP, mitogen-activated protein; MyD88, myeloid differentiation factor 88; NEMO, NF- κ B essential modifier; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; Sharpin, Shank-associated RH domain interactor; TAK1, TGF β -activated kinase 1; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRAF6, TNF receptor-associated factor 6.

HOIP is an E3 ubiquitin ligase that catalyses the formation of Met1-linked ubiquitin (M1-Ub) oligomers [1,5], also called linear ubiquitin, which are required for the efficient activation of the canonical I κ B kinase (IKK) complex in some cells ([6], reviewed in Ref. [7]). The IKK complex has multiple functions *in vivo*, which include activation of the transcription factors nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) and interferon regulatory factor 5 [8,9]. These proteins are required for the transcription of genes encoding pro-inflammatory cytokines, such as interleukin 6 (IL-6), IL-12 and TNF in macrophages [10,11].

Ligands that activate Toll-like receptors (TLRs) or IL-1 receptor family members (IL-1, IL-18 and IL-33) induce the formation of oligomeric complexes termed 'myddosomes', comprising the ligand-bound TLR, the adaptor protein myeloid differentiation factor 88 (MyD88) and IL-1 receptor-associated kinases (IRAKs) [12,13]. Myddosomes then trigger the activation of several E3 ligases to initiate subsequent signalling events that lead to cytokine production. IRAK1 and IRAK2 interact with TRAF6 inducing its oligomerisation and the activation of its E3 ligase activity [14], while the Pellino family of E3 ligases are activated by IRAK1-catalysed phosphorylation [15–17]. TRAF6 and the Pellinos produce Lys63-linked ubiquitin (K63-Ub) oligomers [18], which activate a 'master' protein kinase TGF β -activated protein kinase-1 (TAK1) [19], also called MAP3K7, and contribute to the recruitment of LUBAC into these signalling complexes [18]. This enables the HOIP component of LUBAC to ubiquitylate pre-formed K63-Ub oligomers, generating hybrid ubiquitin chains containing both K63-Ub and M1-Ub linkages. Some of the K63/M1-linked hybrid ubiquitin chains are attached covalently to components of the myddosome, and most strikingly to IRAK1 and IRAK2 [5,18]. The M1-Ub oligomers interact with NF- κ B essential modifier (NEMO), a component of the canonical I κ B kinase (IKK) complex [20,21], inducing a conformational change [22] that enables TAK1 to phosphorylate IKK α and IKK β , the catalytic subunits of the IKK complex, initiating their activation [23]. TAK1 also activates the mitogen-activated protein (MAP) kinase kinases that activate p38 MAP kinases and c-Jun N-terminal kinases (JNKs). Together, the IKK and TAK1 complexes, and other kinases that they activate, trigger numerous phosphorylation events that culminate in the production and secretion of inflammatory mediators.

The formation of K63/M1-Ub hybrids is thought to facilitate the co-recruitment of the canonical IKK and TAK1 complexes [7,23] and prolong their activation

[24], but they also recruit other ubiquitin-binding proteins, such as A20 and A20-binding inhibitor of NF- κ B 1 (ABIN1), which restrict activation of the TAK1 and IKK complexes, preventing the overproduction of inflammatory cytokines that cause autoimmunity and auto-inflammation [25–29].

Like HOIP, the HOIL-1 component of LUBAC is also a RING-Between-RING E3 ligase family member, but, until recently, the type of ubiquitin linkage that it generated was unknown. We found that HOIL-1 catalyses the attachment of ubiquitin to the hydroxyl side chains of serine and threonine residues in proteins, forming ester bonds [30], instead of the isopeptide bonds formed by linking ubiquitin to the ϵ -amino group of lysine residues, as occurs in nearly all other E3 ubiquitin ligases.

Ubiquitin linked to proteins by ester bonds is cleaved rapidly and specifically by hydroxylamine. By exploiting sensitivity to hydroxylamine and bone marrow-derived macrophages (BMDMs) from knock-in mice expressing the E3 ligase-inactive HOIL-1 [C458S] mutant, we identified several physiological substrates of HOIL-1, most notably IRAK1 and IRAK2. Interestingly some, but not all, of the K63/M1-Ub hybrids attached covalently to IRAK1 and IRAK2 were initiated by HOIL-1-catalysed ester bonds [30]. Thus, the K63/M1 hybrid ubiquitin chains formed when myddosomes are activated are initiated in two different ways.

These unexpected findings raised the question of how ester-linked ubiquitin affects the production of inflammatory mediators. Previous studies aimed at understanding the physiological roles of HOIL-1 employed mice in which the expression of HOIL-1 had been largely [6] or completely ablated [31,32]. However, every functional domain is lost in HOIL-1-deficient cells, and not just its E3 ligase activity, so that the specific effects attributable to loss of just the E3 ligase activity could not be deduced. For example, the absence of HOIL-1 affects the stability of HOIP and drastically decreases its expression, explaining why HOIL-1 KO mice, like HOIP KO mice, display early embryonic lethality [33]. In contrast, mice expressing the E3 ligase-inactive HOIL-1[C458S] mutant are born at normal Mendelian frequencies, develop normally and are of normal size and weight up to 1 year of age when kept under relatively sterile conditions [30]. Here, we have exploited cytotoxic T cells and BMDMs from the HOIL-1[C458S] mice to identify specific roles of the HOIL-1 E3 ligase in regulating myddosome-dependent signalling pathways, which has revealed that HOIL-1 has divergent roles in different immune signalling pathways.

Results

IL-18 signalling and cytokine production is enhanced in cytotoxic T cells from E3 ligase-deficient HOIL-1[C458S] mice

IL-18 signals via myddosomes [34] to stimulate the adaptive immune response and the recruitment of neutrophils to sites of infection [35,36]. We investigated whether the HOIL-1 E3 ligase participates in IL-18 signalling in cytotoxic T cells where it triggers the production and secretion of interferon γ (IFN- γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF). We found that the IL-18-dependent production of the mRNA encoding *ifng* and *csf2* (the gene encoding GM-CSF) (Fig. 1A,B) and the secretion of IFN- γ and GM-CSF (Fig. 1C,D) were increased in cytotoxic T cells from knock-in mice expressing the E3 ligase-inactive HOIL-1[C458S] mutant, suggesting that the E3 ligase activity of HOIL-1 restricts IL-18 signalling at or above the level of gene transcription.

The IL-18-stimulated secretion of IFN- γ and GM-CSF in wild-type (WT) and HOIL-1[C458S] T cells was suppressed by potent and relatively specific small molecule inhibitors of IKK β (BI 605906) [37], p38 MAP kinases (BIRB0796) [38,39] and JNK1/2 (JNK-IN8) [40] (Fig. 1E,F), indicating that the production of these cytokines requires the combinatorial actions of several protein kinases. We therefore compared the IL-18-dependent activation of these protein kinases in WT and HOIL-1[C458S] cytotoxic T cells.

The IL-18-stimulated activation of the canonical IKK complex and MAP kinases occurs rapidly and transiently returning to near basal levels after 2 h (Fig. 1G). Interestingly, we found that the IL-18-dependent phosphorylation of the activation loops of IKK α and IKK β and the phosphorylation of p105/NF- κ B1, a physiological substrate of IKK β [41,42], were enhanced in cytotoxic T cells from HOIL-1[C458S] mice. Additionally, the duration of phosphorylation of IKK and p105/NF- κ B1, as well as the phosphorylation of the activation loops of p38 α and JNKs (which activates these MAP kinase family members), was prolonged in cytotoxic T cells from HOIL-1[C458S] mice (Fig. 1G). These findings are consistent with the increased production of the mRNA encoding *ifng* and *csf2* observed after 1–2 h (Fig. 1A,B).

IRAK ubiquitylation during IL-18 signalling in cytotoxic T cells

The expression of IL-18 receptor 1 (IL-18R1), MyD88, TRAF6 and the three components of LUBAC was

similar in cytotoxic T cells from WT and HOIL-1[C458S] mice (Fig. 1H), indicating that the enhanced IL-18 signalling seen in HOIL-1[C458S] cells is not a consequence of an increase in the expression of these proteins. The minor, more slowly migrating HOIL-1 band present in extracts from WT cytotoxic not HOIL-1[C458S] T cells, is a monoubiquitylated species arising from HOIL-1-catalysed autoubiquitylation [30].

To investigate why IL-18-dependent signalling pathways might be enhanced and prolonged in cytotoxic T cells from HOIL-1[C458S] mice, we next studied ubiquitylation events that occur ‘upstream’ of the activation of IKK β and MAP kinases. The ubiquitylation of IRAK1 peaked after 20 min and then declined rapidly, while the IRAK2 ubiquitylation was sustained for up to 6 h (Fig. 2A).

Strikingly, the size of the ubiquitin chains attached to IRAK1 and IRAK2 that formed during IL-18 signalling was larger and the extent of their ubiquitylation was higher in cytotoxic T cells from HOIL-1[C458S] mice (Fig. 2A), indicating that the HOIL-1-catalysed formation of ester-linked ubiquitin restricts the size that IRAK-linked ubiquitin chains can attain.

The cleavage of ester-linked ubiquitin with hydroxylamine reduced the size of the ubiquitin chains attached to IRAK1 and IRAK2 in cytotoxic T cells from WT mice and reconverted some of the IRAK1 and IRAK2 to the fully deubiquitylated proteins (Fig. 2B). These observations imply that some but not all of the ubiquitin chains attached covalently to IRAK1 and IRAK2 are initiated by ester-linked monoubiquitylation, similar to observations made in BMDM [30]. In contrast, and as expected, the size of the ubiquitin chains attached to IRAK1 and IRAK2 was unaffected by hydroxylamine in T cells from HOIL-1[C458S] mice and no reversion to the deubiquitylated proteins took place (Fig. 2B). This result establishes that, similar to our previous findings in BMDM [30], HOIL-1 is the E3 ligase that catalyses the ester-linked monoubiquitylation of IRAK1 and IRAK2 in cytotoxic T cells.

Otulin, a deubiquitylase that cleaves M1-Ub linkages exclusively [43,44], decreased the size of the ubiquitin chains attached to IRAK1 and IRAK2 in both WT and HOIL-1[C458S] T cells, but without any conversion to the monoubiquitylated or deubiquitylated species (Fig. 2C). These results indicate that, as previously found in other cells [5], the first ubiquitin molecule attached to IRAK1 and IRAK2 is initially elongated by another type of ubiquitin linkage, presumably a K63-Ub linkage, prior to the introduction of M1-Ub linkages to form the K63/M1-Ub hybrid

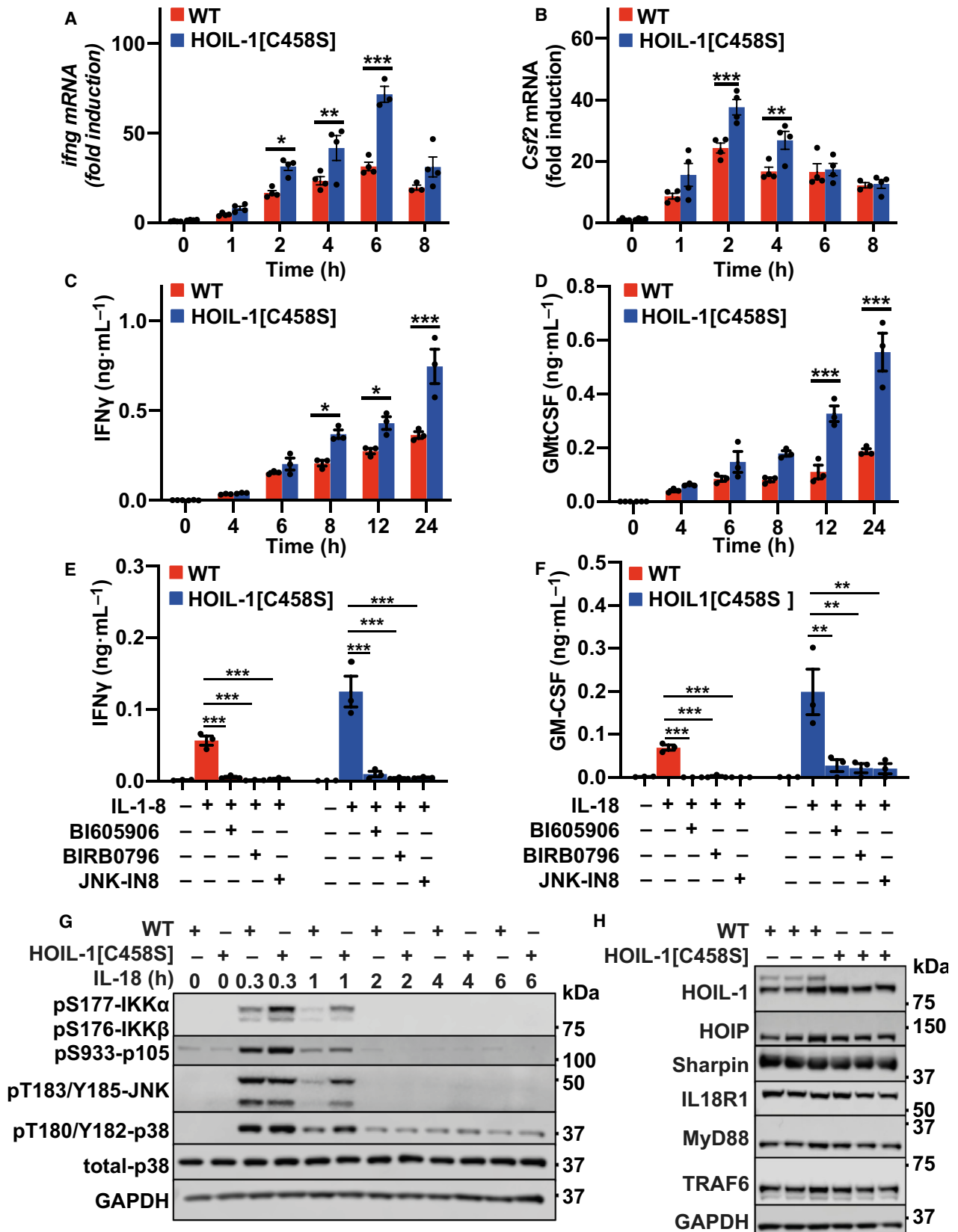


Fig. 1. The HOIL-1 E3 ligase is a negative regulator of IL-18 signalling and cytokine production in CD8⁺ cytotoxic T cells (CTLs). (A, B) CTLs from WT and HOIL-1[C458S] mice were stimulated for the times indicated with IL-18 (20 ng·mL⁻¹). The increase in *ifnγ* mRNA encoding IFN-γ and *csf2* mRNA encoding GM-CSF is plotted as fold increase in mRNA relative to that determined in unstimulated cells. Error bars represent the mean ± SEM for experiments with 3–4 mice of each genotype. (C, D) As in A, except that plots show the concentrations of IFN-γ and GM-CSF secreted into the culture medium following stimulation. Error bars represent the mean ± SEM for experiments with three mice of each genotype. Similar results were obtained in three independent experiments. (E, F) As in C, except that CTLs were incubated for 1 h without (–) or with (+) the IKKβ inhibitor BI605906 (1 μM), the p38 MAPK inhibitor BIRB0796 (1 μM) or the JNK inhibitor JNK-IN8 (10 μM), prior to stimulation for 8 h with IL-18 (20 ng·mL⁻¹). The IFN-γ (E) and GM-CSF (F) secreted into the culture medium were then measured. The graphs show the results for three biological replicates of each genotype ± SEM. * denotes $P < 0.05$, ** $P < 0.01$ and *** denotes $P < 0.001$. Statistical significance was calculated using two-way ANOVA and Sidak's multiple comparisons test (A–D) or one-way ANOVA followed by Dunnett's multiple comparisons test (E, F). (G) As in A, except that after stimulation with IL-18, the CTLs were lysed and cell extracts (20 μg protein) were subjected to SDS/PAGE followed by immunoblotting with the indicated antibodies (p, phosphorylated). Similar results were obtained in three different experiments. (H) Cell extracts prepared from cytotoxic T cells from 3 WT and 3 HOIL-1[C458S] mice were denatured in 1% (w/v) SDS, subjected to SDS/PAGE and immunoblotted with the indicated antibodies. Each lane represents an individual biological replicate.

chains (see [Introduction](#)). These results also established that the formation of M1-Ub chains and their attachment to IRAK1 and IRAK2 was not suppressed in T cells expressing the E3 ligase-inactive HOIL-1[C458S] mutant and is consistent with the normal level of expression of HOIP in T cells from HOIL-1[C458S] mice (Fig. 1H).

The interaction of IRAK2 with TRAF6 is critical for IL-18 signalling in cytotoxic T cells

The expression of IRAK1 was decreased drastically during prolonged IL-18 signalling, but the expression of IRAK2 did not and increased slightly (Fig. 2D), consistent with the transient ubiquitylation of IRAK1 and sustained ubiquitylation of IRAK2 (Fig. 2A). Similar results have been observed previously in BMDM following stimulation with TLR-activating ligands, the rapid disappearance of IRAK1 presumably explaining why IRAK2 becomes rate limiting for cytokine production during prolonged TLR signalling [45,46]. We have reported that the TLR-dependent production of pro-inflammatory cytokines is greatly reduced in BMDM from knock-in mice expressing the IRAK2[E525A] mutant that is unable to interact with TRAF6 [46]. Similar to BMDM, we found that the IL-18-dependent secretion of IFN-γ and GM-CSF was reduced by 77% and 72%, respectively, in cytotoxic T cells from IRAK2[E525A] mice (Fig. 2E). These results establish that the interaction of IRAK2 with TRAF6 has a critical role in IL-18-stimulated cytokine production in cytotoxic T cells and raised the question of whether the differences in the number, size and topology of the ubiquitin chains attached to IRAK2 in cytotoxic T cells from HOIL-1[C458S] mice underlies the enhanced production of IFN-γ and GM-CSF in these cells.

The HOIL-1 E3 ligase enhances myddosome-dependent cytokine secretion by TLR ligands in BMDM

To investigate whether the HOIL-1 E3 ligase has a similar role in BMDM to that observed in cytotoxic T cells, we stimulated BMDM with Pam3CSK4 (an activator of the TLR1/2 heterodimer) or R848 (an activator of the TLR7/8 heterodimer). Similar to IL-18, these ligands signal exclusively via myddosomes. Unexpectedly, and in contrast to IL-18 signalling in T cells, we found that the Pam3CSK4- or R848-stimulated secretion of several pro-inflammatory cytokines was not increased, but modestly reduced in BMDM from HOIL-1[C458S] mice (Fig. 3A–F). The secretion of IL-6 (Fig. 3A,D) and IL-12 (Fig. 3B,D) only became marked after stimulation for more than 4 h. In contrast, TNF secretion (Fig. 3C,F) was already significant by 4 h, but no reduction in TNF secretion in HOIL-1[C458S] BMDM was seen at this time point. The reduction in TNF secretion in HOIL-1[C458S] BMDM only became statistically significant after stimulation for 8 h and was less than that seen in IL-6 and IL-12 secretion.

We next investigated whether the TLR-dependent production of these cytokines might be regulated at the transcriptional level by measuring mRNA. Stimulation with Pam3CSK4 or R848 induced an increase in *il6*, *il12* and *tnf* levels. In BMDM from HOIL-1[C458S] mice, there was a statistically significant decrease in *il6* mRNA 8 h after stimulation with Pam3CSK4. There also appeared to be a small reduction in *il6* after stimulation with R848, which did not reach statistical significance (Fig. 3G,J). There was also a statistically significant reduction in *il12* mRNA levels, at most time points between 2 and 12 h after stimulation with either Pam3CSK4 or R848 (Fig. 3H,

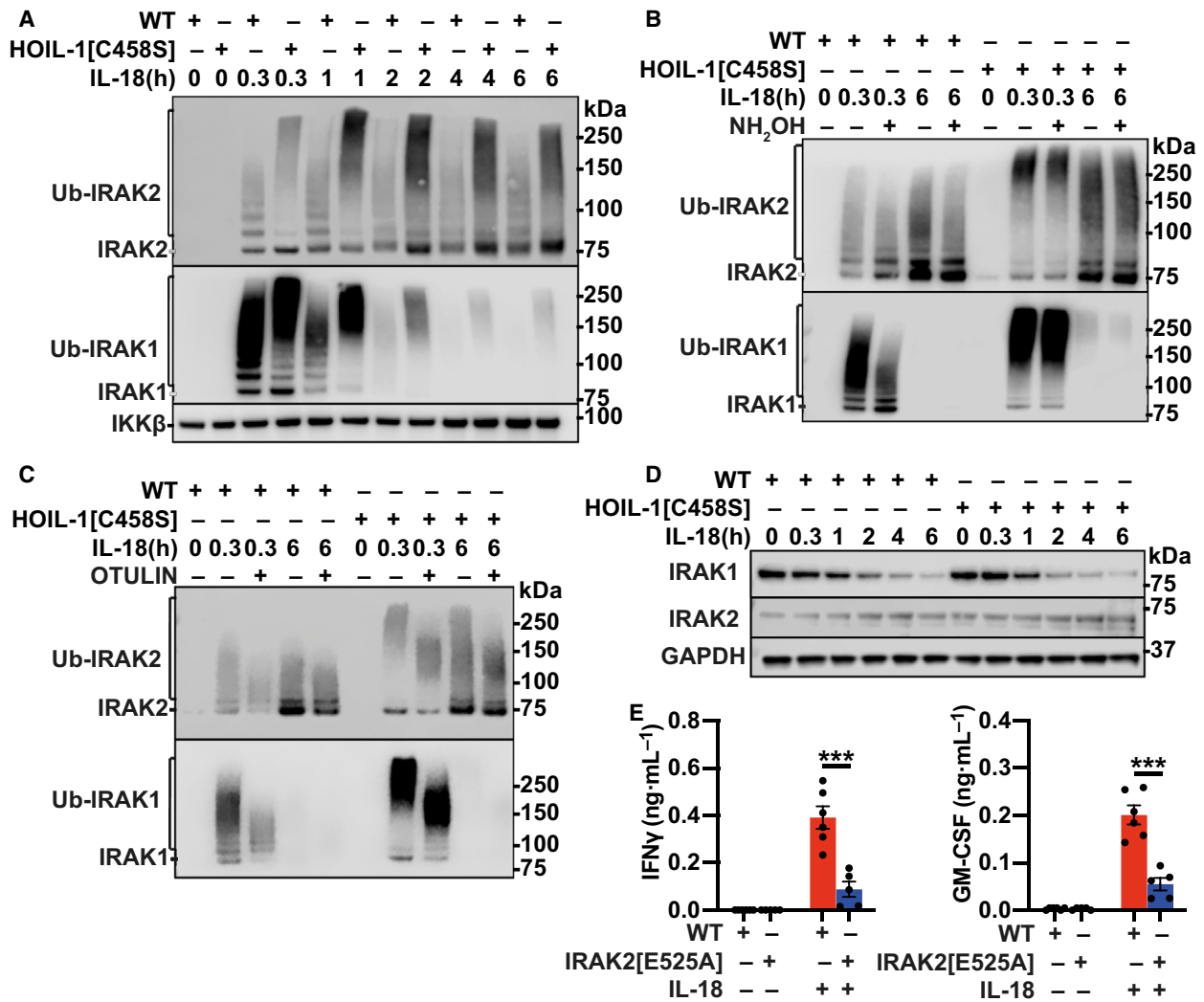
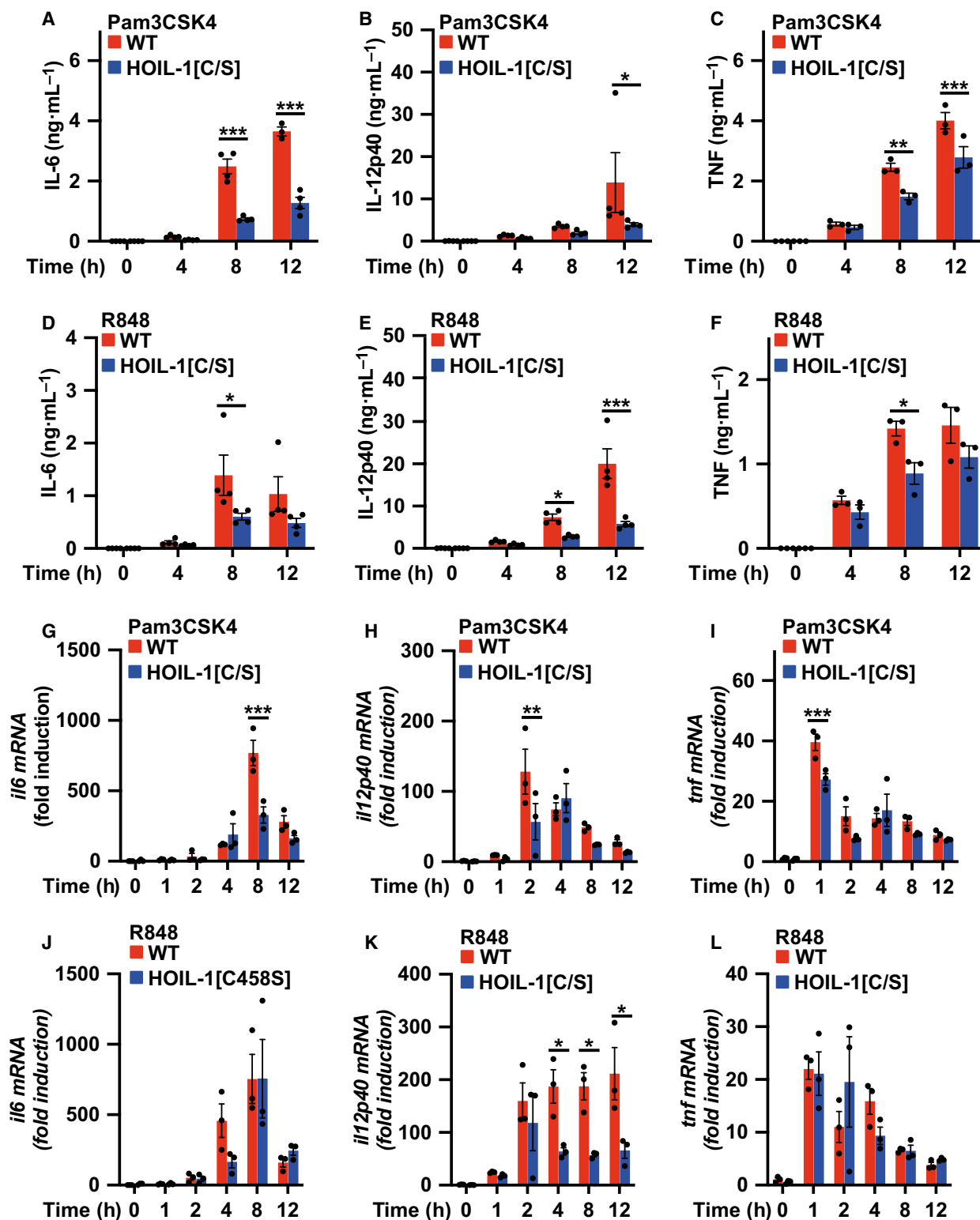


Fig. 2. IL-18 stimulates IRAK1 and IRAK2 ubiquitylation in cytotoxic T cells. (A) CTLs from WT or HOIL-1[C458S] mice were stimulated for the times indicated with IL-18 (20 ng·mL⁻¹) and ubiquitylated forms of IRAK1 and IRAK2 captured from the cell extracts with Halo-NEMO beads, and then treated and immunoblotted as described in [Materials and methods](#). (B, C) As in A, except the Halo-NEMO beads were incubated for 60 min without or with 0.5 M hydroxylamine pH 9.0 (B) or with Otulin (1 μM) (C). (D) The expression levels of IRAK1 and IRAK2 in CTLs were investigated during prolonged stimulation with IL-18. Cells were lysed in the absence of phosphatase and deubiquitylase inhibitors and the extracts incubated for 60 min with both λPPase and USP2 to dephosphorylate and deubiquitylate these proteins (see [Materials and methods](#)) prior to SDS/PAGE and immunoblotting. (E) CTLs from WT or IRAK2[E525A] mice were stimulated for 8 h with (+) or without (-) IL-18 (20 ng·mL⁻¹), and IFN-γ and GM-CSF in the culture medium were then measured. Error bars represent the mean ± SEM for experiments with six WT and 5 IRAK2[E525A] knock-in mice. Statistical significance between the genotypes was calculated using two-way ANOVA and Sidak's multiple comparisons test; *** denotes $P < 0.001$.

Fig. 3. Changes in cytokine secretion and cytokine mRNA production during TLR signalling in BMDM from WT and HOIL-1[C458S] mice. BMDM from WT or HOIL1[C458S] mice were stimulated for the times indicated with 1 μg·mL⁻¹ Pam3CSK4 (A-C) or 250 ng·mL⁻¹ R848 (D-F). The concentrations of IL-6 (A, D) and IL-12p40 (B, E) secreted into the culture medium were measured by Bio-Plex and TNF (C, F) by ELISA. (G-L) As in (A-F), except that RNA was isolated from WT and HOIL-1[C458S] BMDM that had been stimulated with 1 μg·mL⁻¹ Pam3CSK4 (G-I) or 250 ng·mL⁻¹ R848 (J-L) for the times indicated. The *il6*, *il12p40* and *tnf* mRNA results are plotted as fold increase in mRNA relative to the level determined in unstimulated cells. Error bars represent the mean ± SEM for experiments with 3–4 mice of each genotype. Statistical significance between the genotypes was calculated using two-way ANOVA and Sidak's multiple comparisons test; *denotes $P < 0.05$, ** $P < 0.01$ and *** denotes $P < 0.001$. Similar results were obtained in three independent experiments.



K). The increase in TNF mRNA occurred much more rapidly than with IL-6 and IL-12, peaking after only 1 h. However, there was not a significant difference

between *tnf* mRNA levels in BMDM from HOIL-1 [C458S] and WT mice at any time point, apart from a modest reduction after 1h with Pam3CSK4 only

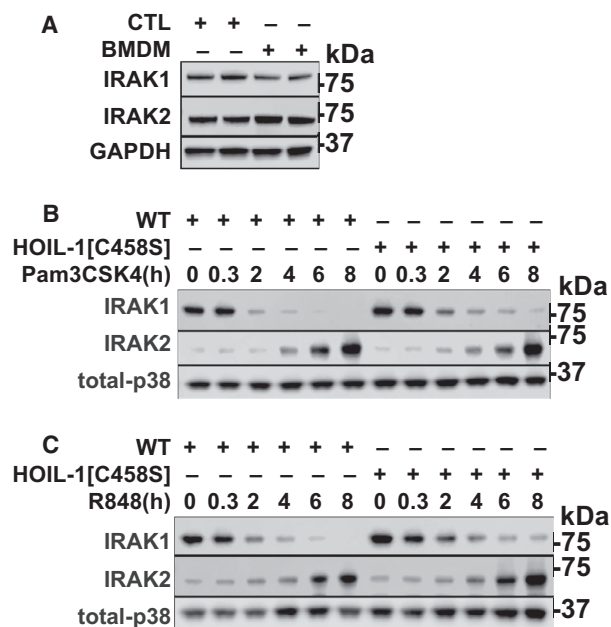


Fig. 4. IRAK1 and IRAK2 expression in cytotoxic T cells and BMDMs from WT and HOIL1[C458S] knock-in mice. (A) Cell extracts were prepared from CTLs not stimulated with IL-18 and from BMDM not stimulated with a TLR ligand, subjected to SDS/PAGE and immunoblotted with the indicated antibodies. (B, C) BMDM from WT or HOIL-1[C458S] mice was stimulated for the times indicated with $1 \mu\text{g}\cdot\text{mL}^{-1}$ Pam3CSK4 (B) or $250 \text{ ng}\cdot\text{mL}^{-1}$ R848 (C) and cells were lysed in the absence of phosphatase and deubiquitylase inhibitors. The cell extracts were incubated with λPPase and USP2 to completely dephosphorylate and deubiquitylate IRAK1 and IRAK2 (see [Materials and methods](#)), subjected to SDS/PAGE and immunoblotted with the antibodies indicated.

(Fig. 3I,L). It is well established that TNF production is regulated at both the translational and post-translational levels [47,48], and so the reduction in TNF secretion in HOIL-1[C458S] BMDM may arise from a post-transcriptional role(s) of the HOIL-1 E3 ligase activity.

To investigate possible reasons for the reduced TLR-dependent production of IL-6 and IL-12 in BMDM and why this differs from the increased cytokine production seen in IL-18 stimulated cytotoxic T cells, we next studied IRAK2 and its ubiquitylation. The expression of IRAK2 in BMDM was a little higher than in cytotoxic T cells (Fig. 4A), while the expression of IRAK1 was slightly lower (Fig. 4A). Stimulation of BMDM from either WT or HOIL-1[C458S] mice with either Pam3CSK4 or R848 greatly increased the expression of IRAK2 (Fig. 4B,C), and induced the disappearance of IRAK1, as expected. The ubiquitylation of IRAK2 in WT BMDM was increased considerably during prolonged stimulation,

presumably as a consequence of its increased expression over this period. IRAK1 ubiquitylation had almost disappeared by 4 h (Fig. 5A,B), consistent with its rate of degradation (Fig. 4B,C). Strikingly, and in contrast to WT BMDM, there was little increase in IRAK2 ubiquitylation in HOIL-1[C458S] BMDM during prolonged TLR ligation (Fig. 5C,D), suggesting that the increased ubiquitylation of IRAK2 during prolonged signalling in WT BMDM might be explained by increased formation of ester-linked ubiquitin chains during prolonged TLR ligation. Consistent with this notion, treatment with hydroxylamine caused a marked decrease in IRAK2 ubiquitylation between 2 and 8 h, especially during stimulation with Pam3CSK4 (Fig. 5E,F). The reduced ubiquitylation of IRAK2 in HOIL-1[C458S] BMDM from 2 to 8 h correlated with the decreased production of *il6* and *il12* mRNA and secretion of these cytokines.

Similar to IL-18 signalling in cytotoxic T cells, the size of the ubiquitin chains attached to IRAK1 and IRAK2 was increased in BMDM from the HOIL-1[C458S] mice during the first hour of Pam3CSK4 or R848 stimulation (Fig. 5A,B) [30]. However, in contrast to IL-18 signalling in T cells, the extent of ubiquitylation was not enhanced and nor was the increased size of the ubiquitin chains attached to IRAK2 prolonged. After 4–8 h, the size of the ubiquitin chains attached to IRAK2 became similar in WT and HOIL-1[C458S] BMDM.

Despite the increased size of the ubiquitin chains attached to IRAK2 and IRAK1 in BMDM from HOIL-1[C458S] mice during the first hour of TLR ligation, no consistent increase was seen in the phosphorylation of $\text{p105/NF-}\kappa\text{B1}$ or the rate of degradation of $\text{I}\kappa\text{B}\alpha$ in these cells, which are readouts of $\text{IKK}\beta$ activity. Moreover, the rate of reappearance of $\text{I}\kappa\text{B}\alpha$ after 45–60 min, a readout of $\text{NF-}\kappa\text{B}$ -dependent gene transcription, was similar in BMDM from HOIL-1[C458S] and WT mice (Fig. 6A,B). The similar level of *tnf* mRNA 1 h after stimulating BMDM from WT and HOIL-1[C458S] mice with Pam3CSK4 (Fig. 3I) or R848 (Fig. 3L) is consistent with this finding. There was also no significant difference in the activation of $\text{p38}\alpha$ MAP kinase or JNK1/2 in WT and HOIL-1[C458S] BMDM during the first hour of TLR signalling (Fig. 6A,B), implying that the activities of ‘upstream’ activators of these pathways, such as TAK1, were also unaffected.

LPS-induced cytokine production is similar in BMDM from WT and HOIL-1[C458S] mice

LPS interacts with TLR4, which is unique among TLRs in signalling via TRIF and myddosomes. TRIF-

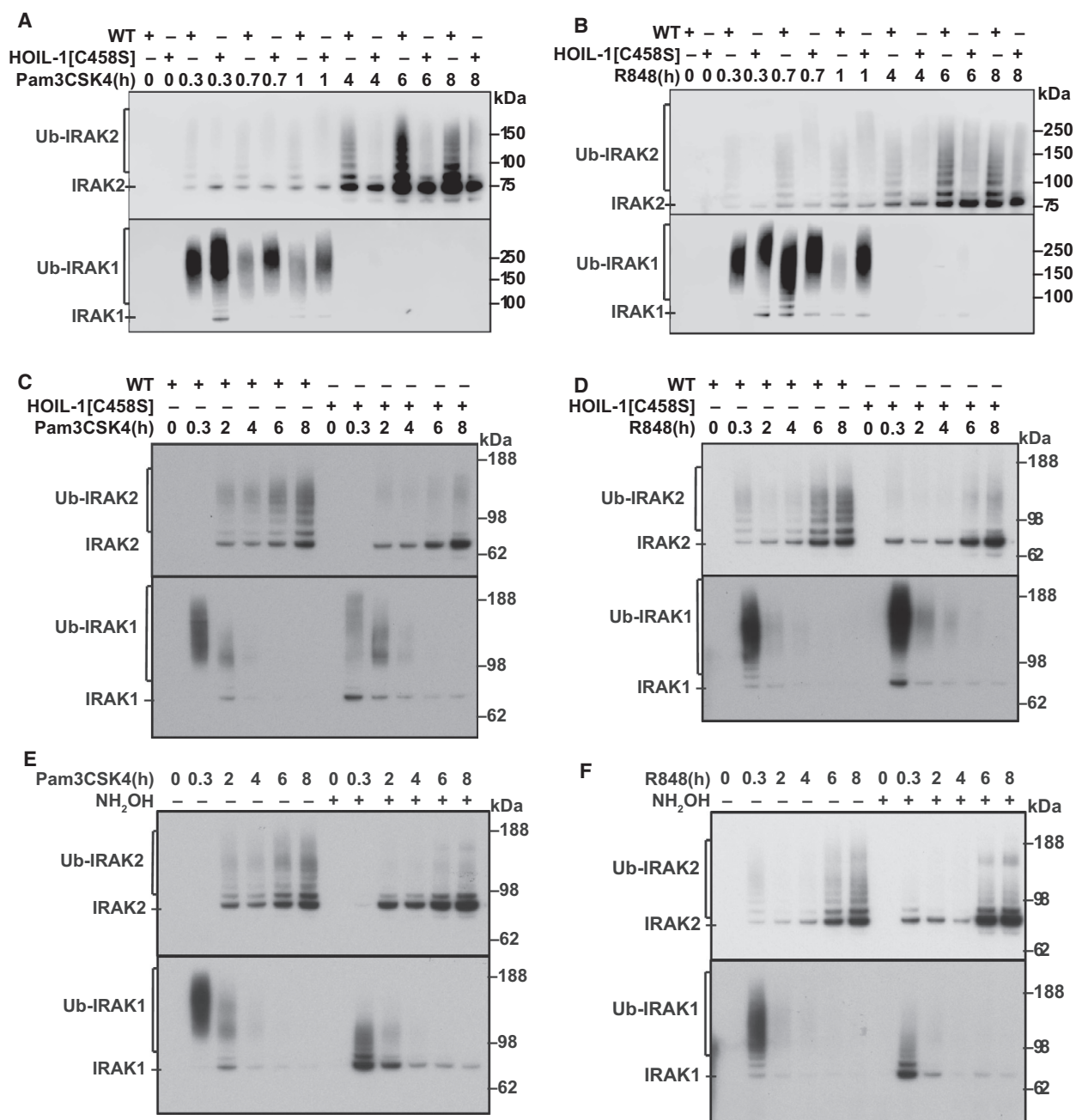


Fig. 5. IRAK1 and IRAK2 ubiquitylation during prolonged TLR signalling. (A–D) BMDM from WT or HOIL-1[C458S] mice were stimulated for the times indicated with $1 \mu\text{g}\cdot\text{mL}^{-1}$ Pam3CSK4 (A, C) or $250 \text{ ng}\cdot\text{mL}^{-1}$ R848 (B, D). The ubiquitylated forms of IRAK1 and IRAK2 were captured from the cell extracts with Halo-NEMO beads, then treated and immunoblotted as in [Materials and methods](#). (E, F) The experiment was carried out as in C and D, except that Halo-NEMO beads were incubated without (–) or with (+) 0.5 M hydroxylamine at pH 9.0 to hydrolyse ester-linked ubiquitin, and immunoblotted for IRAK1/2. Similar results were obtained in a least two independent experiments.

dependent signalling is essential and rate limiting for cytokine secretion during prolonged TLR4 signalling in mouse BMDM, while IRAK2 and its interaction with TRAF6 are not [46]. LPS-stimulated secretion of

IL-6 and IL-12 was similar in BMDM from WT and HOIL-1[C458S] mice from 4- to 12 h although a small but statistically significant increase, in IL-6, was noticed after 8 h and a small decrease in IL-12 after

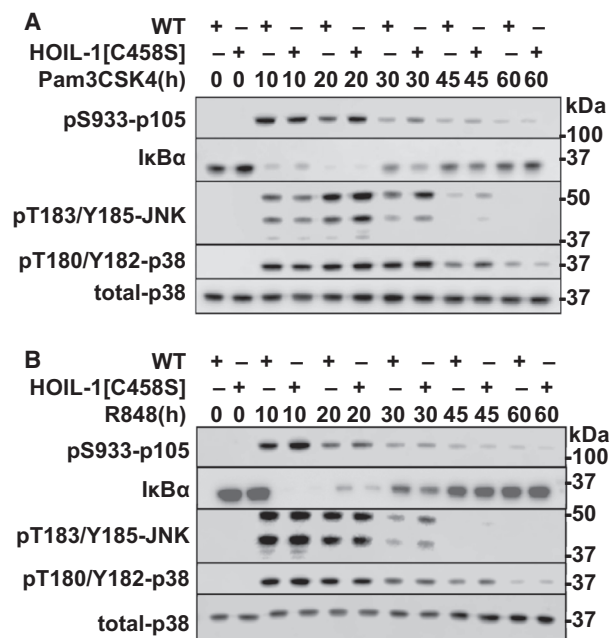


Fig. 6. The first hour of Pam3CSK4 and R848 signalling in BMDM from HOIL-1[C458S] and WT mice. BMDM from WT or HOIL-1[C458S] mice were stimulated with $1 \mu\text{g}\cdot\text{mL}^{-1}$ Pam3CSK4 (A) or $250 \text{ ng}\cdot\text{mL}^{-1}$ R848 (B) for the times indicated. The cells were lysed and cell extract protein (15 μg) was denatured in 1% (w/v) SDS, subjected to SDS/PAGE and immunoblotted with the antibodies indicated. Total p38 α MAP kinase was used as a loading control. Similar results were obtained in two independent experiments.

12 h (Fig. 7A,B). LPS-stimulated TNF secretion was similar in WT and HOIL-1[C458S] BMDM (Fig. 7C). Taken together, these observations indicate that HOIL-1 E3 ligase activity is not critical for the TRIF-dependent secretion of these pro-inflammatory cytokines.

Nevertheless, it was of interest to study the LPS-stimulated ubiquitylation of IRAK1 and IRAK2. We found that, similar to stimulation by Pam3CSK4 and R848, the size of the ubiquitin chains attached to IRAK2 during the first hour of LPS stimulation was increased in BMDM from HOIL-1[C458S] mice, but the extent of ubiquitylation was not increased (Fig. 7D). The LPS-stimulated phosphorylation of p105/NF- κ B1, the degradation of I κ B α and the activation of MAP kinases over this period were also similar in BMDM from WT and HOIL-1[C458S] mice (Fig. 7E).

In contrast to Pam3CSK4 and R848, the LPS-stimulated increase in the size of the ubiquitin chains attached to IRAK2 and the extent of ubiquitylation were both maintained for up to 8 h in BMDM from

WT and HOIL-1[C458S] mice (Fig. 7D). However, the significance of these findings is unclear since TRIF, and not IRAK2, is rate limiting for LPS-induced cytokine production during prolonged TLR4 ligation in BMDM from HOIL-1[C458S] mice.

Discussion

Although the importance of ubiquitin chains in initiating innate immune signalling has been recognised for 20 years [19,49], only recently has the complexity of the ubiquitin oligomers that are formed begun to be fully appreciated. These chains, which can contain up to three ubiquitin linkage types, namely Lys63-linked, Met1-linked and ester bonds, are formed during signalling by IL-1 family members and ligands that activate TLRs and are attached covalently to proteins that include components of myddosomes, such as IRAK1 and IRAK2. The ubiquitin chains attached to IRAK1 and IRAK2 are not only initiated by HOIL-1-catalysed ester bonds but also by initiated by isopeptide bonds formed by the action of 'conventional' E3 ligases, implying that there are at least two polyubiquitin chains attached to each of these proteins [30]. In principle, every protein-linked ubiquitin chain could operate as a distinct signalling node, so that the phosphorylation events widely used as readouts of innate immune signalling pathways may be averaging the outputs of multiple polyubiquitin-triggered events.

The present study has established that the HOIL-1-catalysed formation of ester-linked ubiquitin has important roles in controlling Myddosome-dependent signalling processes, and revealed that their effects vary with the ligand, receptor and cell type. We found that the presence of ester-linked ubiquitin restricts the strength and duration of IL-18 signalling in cytotoxic T cells, because failure to produce these linkages enhanced both early signalling events and subsequent events leading to the production and secretion of IFN- γ and GM-CSF. We found that IRAK2 and its interaction with TRAF6 were critical for the production of IFN- γ and GM-CSF during prolonged IL-18 signalling and that stimulation with IL-18 increased both the number and size of the ubiquitin chains attached to IRAK2 in cytotoxic T cells expressing the E3 ligase-inactive HOIL-1[C458S] mutant. The NEMO component of the canonical IKK complex binds with greater avidity to longer M1-Ub and K63-Ub oligomers than to shorter ubiquitin oligomers [25], and so the increased size of the ubiquitin chains attached to IRAK1 and IRAK2 might contribute to the enhanced and more prolonged activation of the IKK complex seen in HOIL-1 E3 ligase-deficient cytotoxic T cells.

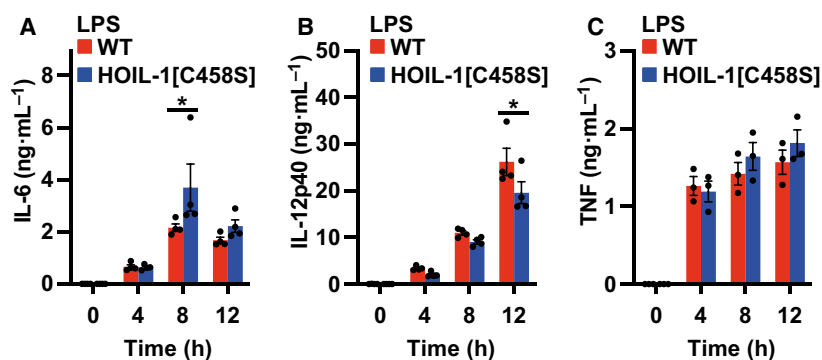
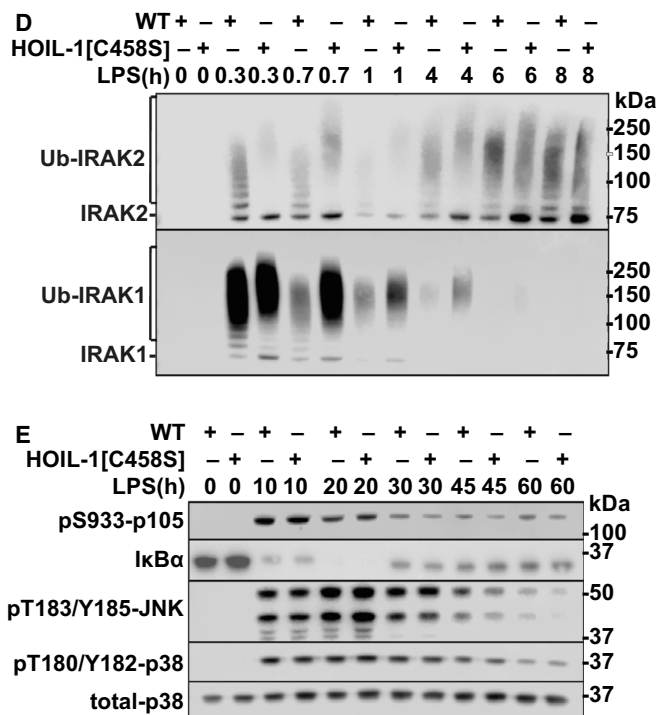


Fig. 7. LPS-stimulated cytokine secretion, IRAK1/2 ubiquitylation and the first hour of signalling in BMDM from HOIL-1[C458S] mice. (A-C) BMDMs from WT or HOIL-1[C458S] mice were stimulated for the times indicated with 100 ng·mL⁻¹ LPS. The concentrations of IL-6 (A) and IL-12p40 (B) were measured by Bio-Plex and TNF (C) by ELISA. Error bars represent the mean \pm SEM for experiments with 3–4 mice of each genotype. Statistical significance between the genotypes was calculated using two-way ANOVA and Sidak's multiple comparisons test; *denotes $P < 0.05$. The experiments were repeated at least three times. (D) As in A-C, except that the cells were lysed and ubiquitylated proteins captured from the cell extracts with Halo-NEMO beads, treated with λ PPase and immunoblotted for IRAK1 or IRAK2. (E) As in D, except that the cell extract protein (15 μ g) was denatured in 1% (w/v) SDS, subjected to SDS/PAGE and immunoblotted with the antibodies indicated (p, phosphorylated protein).



Similarly, stronger interactions between larger K63/M1-Ub hybrids and the ubiquitin-binding subunits of TAK1 complexes, TAB2 and TAB3, might enhance TAK1 activation and account for the increased activation of p38 MAP kinase and JNKs.

Taken together, our results suggest the following working hypothesis to account for the increased production of cytokines in cytotoxic T cells from HOIL-1[C458S] mice during prolonged IL-18 signalling. The IRAK2-TRAF6 interaction activates the E3 ligase activity of TRAF6, enabling TRAF6 to produce K63-linked ubiquitin oligomers that are attached to IRAK2 by isopeptide linkages, thereby activating TAK1 (in contrast to IRAK1, IRAK2 is a catalytically inactive pseudokinase, which is unable to phosphorylate and activate the Pellino family of E3 ligases—see

Introduction). The K63-Ub chains attached to IRAK2 interact specifically with the Npl4 zinc finger domain of HOIP [5] recruiting LUBAC to the signalling complex and enabling the HOIP component of LUBAC to convert the K63-Ub oligomers attached to IRAK2 to K63/M1-Ub hybrids, thereby maintaining a low level of TAK1 and IKK activation needed to sustain cytokine production during prolonged IL-18 signalling. The HOIL-1 component of LUBAC monoubiquitylates a serine or threonine residue(s) on IRAK2 initiating the formation of distinct ester-linked ubiquitin chains. We speculate that the presence of both ester-linked and isopeptide-linked ubiquitin chains attached to IRAK1 and IRAK2 restricts the size that each type of ubiquitin chain can attain, perhaps due to competition between the chains for the limiting amounts of

TRAF6 and LUBAC that are available. In cytotoxic T cells expressing the HOIL-1[C458S] mutant, ester-linked ubiquitin chains are not formed permitting the isopeptide-linked ubiquitin chain(s) to become larger, and enhancing and prolonging the activation of TAK1 and IKK to increase the production of cytokines.

Although HOIL-1 can initiate ubiquitin chain formation *de novo* by monoubiquitylating IRAK2 and other proteins, which are subsequently elongated and branched by other E3 ligases [5,30], it can also catalyse the formation of ubiquitin dimers *in vitro* in which the C terminus of one ubiquitin is linked by an ester bond to the hydroxyl side chain of Thr12 of another ubiquitin molecule [30,50]. If such linkages are formed in cells, which has yet to be established, then HOIL-1 may not only initiate ubiquitin chain formation but also incorporate ester linkages within the hybrid ubiquitin chains. The presence of such linkages could have positive or negative effects on the strength and duration of signalling depending on where, when and if these linkages are introduced. For example, Thr12-linked ubiquitin dimers present within a hybrid polyubiquitin chain might help (or hinder) the recruitment of specific binding proteins that restrict the strength of signalling, such as ABIN1.

In contrast to cytotoxic T cells, we found that the TLR1/2- and TLR7/8-stimulated secretion of the pro-inflammatory cytokines IL-6, IL-12 and TNF was not increased but modestly reduced in BMDM from HOIL-1 [C458S] mice, and this correlated with modestly reduced levels of the mRNAs encoding IL-6 and IL-12. Interestingly, the decreased secretion of these pro-inflammatory cytokines also correlated with a marked decrease in the ubiquitylation of IRAK2 during prolonged TLR signalling in HOIL-1[C458S] BMDM. Since IRAK2 and its interaction with TRAF6 are critical for the production of these pro-inflammatory cytokines [45,46], we speculate that it is the decreased ubiquitylation of IRAK2 during prolonged TLR signalling that underlies the reduced production of IL-6 and IL-12 in BMDM from HOIL-1 [C458S] mice. However, the possibility cannot be excluded that the reduced ubiquitylation of other proteins modified by HOIL-1-catalysed ester-linked ubiquitylation, such as MyD88 [30], also contributes to the decreased cytokine production. An interesting possibility that is worth exploring in future work is whether the loss of ester-linked ubiquitylation permits the serine/threonine residues to which they were attached to undergo another post-translational modification, such as phosphorylation or O-GlcNacylation, that affects signalling and cytokine production.

Although we speculate that the increased size of the ubiquitin chains attached to IRAK1/2 may underlie

the enhanced and more sustained IL-18 signalling in cytotoxic T cells from HOIL-1[C458S] mice, a significant increase in TLR signalling was not observed in BMDM from HOIL-1[C458S] mice during the first hour of signalling (Fig. 6), even though the size of the ubiquitin chains attached to these IRAKs was enhanced similarly to that seen in cytotoxic T cells (Fig. 5A). However, in contrast to cytotoxic T cells, the extent of ubiquitylation of IRAK1/2 was not increased. It may therefore be that it is the increased extent of IRAK1/2 ubiquitylation, rather than the increased size of the ubiquitin chains, that is responsible for enhancing IL-18 signalling in cytotoxic T cells.

Myddosomes are complexes between the liganded receptor, MyD88 and IRAK family members. Moreover, the relative amounts of different IRAK family members can vary from cell to cell and with the stimuli to which they have been exposed. This plasticity in the composition of myddosomes adds to the difficulty in elucidating the molecular basis for the diverse effects on cytokine production caused by the loss of ester-linked ubiquitylation in different immune cells. The present study has not only highlighted the potential importance of IRAK2 ubiquitylation but also begun to explain why loss of the E3 ligase activity of HOIL-1 can cause immunodeficiency and auto-inflammation [51,52].

Materials and methods

Hydroxylamine (50% wt/vol) was obtained from Sigma-Aldrich (Merck Group, Munich, Germany), the protein phosphatase from bacteriophage λ (λ PPase) from New England Biolabs (Hertfordshire, UK) and the TLR-activating ligands Pam3CSK4 and R848 (Resiquimod) from InvivoGen. LPS (lipopolysaccharide) was from Enzo Life Sciences (Lausen, Switzerland), recombinant mouse IL-18 from R&D Systems (Minneapolis, MN, USA), Halo-Link Resin from Promega (Fitchburg, WI, USA), Protein G-Sepharose from Expedeon (Cambridge, UK), reagents for cell culture from Gibco Thermo Fischer Scientific (Cambridge, MA, USA), Immobilon-P PVDF membranes from Merck-Millipore (Merck Group) and Brefeldin A (420601) from BioLegend (San Diego, CA, USA). Other solvents and reagents were obtained from Sigma-Aldrich (Merck Group) or VWR International (Radnor, PA, USA). The deubiquitylases USP2 and Otulin were expressed and purified as described [53].

Antibodies

Antibodies that recognise phospho-S176 of IKK α and phospho-S177 of IKK β (#2078), phospho-Thr180/Tyr182

of p38 α (#9211), phospho-Thr183/Tyr185 of JNK1/2 (#9251), phospho-Ser933 of p105/NF- κ B1 (#4806), IRAK1 (#4505), p38 α (#9212), GAPDH (#2118), MyD88 (#4283) and TNF (#11948) and HRP-coupled secondary antibodies against mouse (#7076) and rabbit (#7074) IgG were purchased from Cell Signalling Technology (Danvers, MA, USA), rabbit polyclonal antibodies for immunoblotting of mouse IRAK2 were obtained from Abcam (#62419) (Cambridge, UK), IL-18R1 antibody was purchased from Thermo Fisher Scientific (#PA5-102647) and an antibody recognising α -tubulin (66031-1) was obtained from Protein-Tech Group (Rosemont, IL, USA). The antibodies used to detect HOIP, HOIL-1 and Sharpin have been described [30].

HOIL-1[C458S] knock-in mice

The mice were generated as reported [30] and back-crossed at least five times. In contrast, to the embryonic lethality of HOIL-1 KO mice [31,32], E3 ligase-inactive HOIL-1[C458S] knock-in mice were born at normal Mendelian frequencies [30]. Mice were maintained on a C57BL/6J (Charles River, Harlow, UK) background and provided with free access to food (R&M3 pelleted irradiated diet) and water. Animals were kept in individually ventilated cages at 21 °C, 45–65% relative humidity and a 12-h/12-h light/dark cycle under specific pathogen-free conditions in accordance with UK and EU regulations. Experiments on mice were approved by the University of Dundee ethical review board under a UK Home Office project license.

Stimulation of bone marrow-derived macrophages (BMDMs)

BMDMs were obtained by differentiating bone marrow from the femur and tibia using L929 preconditioned medium [46]. Adherent BMDMs were replated in fresh culture medium in 24-well (0.25×10^6 cells) or 6-well (1×10^6 cells) tissue culture plates or 15-cm tissue culture plates (10×10^6 cells), and stimulated with TLR ligands for up to 12 h at the concentrations specified in figure legends.

Generation and stimulation of cytotoxic CD8⁺ T cells (CTLs) with IL-18

Spleens were mashed through a 70 μ m cell strainer (#10788201; Falcon) in 5 mL of RPMI 1640 medium from Gibco Thermo Fisher Scientific. Erythrocytes were eliminated by treatment for 2 min at 21 °C with red blood cell lysis buffer (Sigma-Aldrich, Merck Group). Lysis was stopped by adding 10 mL of ice-cold PBS and centrifugation for 5 min at 524 *g*. Cell pellets were resuspended in complete RPMI medium (RPMI-1640 containing 10%

heat-inactivated FBS, 50 U·mL⁻¹ penicillin–streptomycin, 5 mM L-glutamine, 10 mM HEPES buffer pH 7.5, 1 mM sodium pyruvate and 50 μ M 2-mercaptoethanol). Cell suspensions (5 mL and containing 2.5×10^6 cells per mL) were added to 6-well plates, and splenocytes activated with 0.5 μ g·mL⁻¹ anti-CD3 monoclonal antibody (#145.2C11, eBioscience/Thermo Fisher Scientific) and 20 ng·mL⁻¹ IL-2 (Peprotech, Rocky Hill, NJ, USA). After 48 h, the cells were washed and cultured for 5 days in fresh RPMI medium containing 20 ng·mL⁻¹ IL-2. The cells were counted daily, and their concentrations were adjusted to 1×10^6 cells/mL. Cytotoxic T cells were rested for 3 h prior to stimulation with IL-18 (20 ng·mL⁻¹).

Preparation of cell extracts

After stimulation with ligands, BMDM and cytotoxic T cells were washed with ice-cold PBS and lysed in 50 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 270 mM sucrose, 10 mM sodium 2-glycerophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 μ g·mL⁻¹ aprotinin and 1 μ g·mL⁻¹ leupeptin. Iodoacetamide (100 mM) was added to inactivate deubiquitylases when IRAK1 and IRAK2 ubiquitylation was studied. Cell lysates were centrifuged at 16 000 *g* for 10 min at 4 °C and protein concentrations in the supernatants (termed cell extracts) measured by the Bradford assay.

Dephosphorylation and deubiquitylation of IRAK1 and IRAK2 in cell extracts

Cells were stimulated, washed and lysed as described above except that EDTA and the protein phosphatase inhibitors sodium fluoride, sodium pyrophosphate and sodium orthovanadate were omitted, and the deubiquitylase inhibitor iodoacetamide was also excluded. Cell extract (50 μ g protein) was incubated in a total volume of 50 μ L containing 50 mM HEPES pH 7.5, 100 mM NaCl, 2 mM DTT, 0.01% (wt/vol) Brij-35, 1 mM MnCl₂, λ PPase (100 units per reaction) and 1 μ M USP2. After incubation for 60 min at 37 °C, the samples were denatured in SDS plus 2.5% (vol/vol) 2-mercaptoethanol, subjected to SDS/PAGE, transferred to PVDF membrane and immunoblotted as described [30,54].

Capture of ubiquitylated proteins, treatment with hydroxylamine and deubiquitylases, and immunoblotting

Ubiquitylated proteins containing M1-Ub oligomers were captured from cell extracts using Halo-NEMO beads and treated with hydroxylamine or deubiquitylases [30], then

denatured in LDS containing 2.5% (vol/vol) 2-mercaptoethanol and subjected to SDS/PAGE and immunoblotted for IRAK1 and IRAK2.

Cytokine measurements

After stimulation with ligands, the cell culture medium was removed, clarified by centrifugation (10 min at 14 000 *g*) frozen in liquid nitrogen and stored in aliquots at -80°C . The levels of IL-6, IL-10 and IL-12 in the cell culture medium were measured using the Bio-Plex Pro Assay multiplex system from Bio-Rad Laboratories (Hercules, CA, USA) and the level of TNF by ELISA (R&D Systems) according to the supplier's instructions. When TNF was measured using the Bio-Plex assay, an apparent decrease in the level of TNF was measured in the culture medium between 8 and 12 h of stimulation. The reason is unclear, but it is possible that the antibody used in the Bio-Plex assay does not recognise a modified species of TNF that is formed in the culture medium during prolonged stimulation with TLR ligands. Such a situation has been described for IL-33, where oxidation of cysteine residues prevents its detection by some antibodies, but not by others [55]. The cell culture medium was diluted to ensure that the cytokine measurement was made within the linear range of the standard curve.

Isolation of mRNA and quantitative real-time PCR

RNA was isolated from cytotoxic T cells using the MicroE-lute Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturers' instructions and reverse transcription of RNA to cDNA was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) using 300 ng total RNA. Quantitative RT-PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad Laboratories). The primers for measuring mouse *ifng* mRNA were as follows: forward 5'-AGCCAAGACTGTGATTGC-3', reverse 5'-TTATTGGTCAGTGAAGTAAAGG-3', and for *csf2* were as follows: forward 5'-CTCACCCATCACTGTCACCC-3', reverse: 5'-TGAAATTGCCCGTAGACCC-3'. The primers for measuring *il6*, *il12* and *tnf* mRNA were described previously [46]. Normalisation and quantitation were performed using *gapdh* RNA and the $\Delta\Delta\text{Ct}$ method.

Statistical analysis of the data

Graphs were generated using GRAPHPAD PRISM 9 (San Diego, CA, USA), Microsoft PowerPoint (WA, USA) and Adobe Illustrator 2021 (CA, USA). Statistical analysis was done in GRAPHPAD PRISM 9 using one-way ANOVA (analysis of variance) followed by Dunnett's multiple comparisons test or two-way ANOVA followed by Sidak's multiple comparisons test.

Acknowledgements

This study was supported by Wellcome Trust Investigator Award 209380/Z/17/Z and MRC Programme Grant MR/R021406/1 (to PC).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

TP, SKN and JZ performed the experiments and analysed the data. CF performed experiments and mouse genotyping. PC conceived the study and PC, TP, SKN and JZ wrote the paper.

Peer Review

The peer review history for this article is available at <https://publons.com/publon/10.1111/febs.15896>.

References

- 1 Kirisako T, Kamei K, Murata S, Kato M, Fukumoto H, Kanie M, Sano S, Tokunaga F, Tanaka K & Iwai K (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J* **25**, 4877–4887.
- 2 Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, Webb AI, Rickard JA, Anderton H, Wong WW *et al.* (2011) Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature* **471**, 591–596.
- 3 Tokunaga F, Nakagawa T, Nakahara M, Saeki Y, Taniguchi M, Sakata S, Tanaka K, Nakano H & Iwai K (2011) SHARPIN is a component of the NF-kappaB-activating linear ubiquitin chain assembly complex. *Nature* **471**, 633–636.
- 4 Ikeda F, Deribe YL, Skanland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, van Wijk SJ, Goswami P, Nagy V, Terzic J *et al.* (2011) SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis. *Nature* **471**, 637–641.
- 5 Emmerich CH, Ordureau A, Strickson S, Arthur JS, Pedrioli PG, Komander D & Cohen P (2013) Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. *Proc Natl Acad Sci USA* **110**, 15247–15252.
- 6 Tokunaga F, Sakata S, Saeki Y, Satomi Y, Kirisako T, Kamei K, Nakagawa T, Kato M, Murata S, Yamaoka S *et al.* (2009) Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. *Nat Cell Biol* **11**, 123–132.
- 7 Cohen P & Strickson S (2017) The role of hybrid ubiquitin chains in the MyD88 and other innate

- immune signalling pathways. *Cell Death Differ* **24**, 1153–1159.
- 8 Lopez-Pelaez M, Lamont DJ, Pegg M, Shpiro N, Gray NS & Cohen P (2014) Protein kinase IKK β -catalyzed phosphorylation of IRF5 at Ser462 induces its dimerization and nuclear translocation in myeloid cells. *Proc Natl Acad Sci USA* **111**, 17432–17437.
 - 9 Ren J, Chen X & Chen ZJ (2014) IKK β is an IRF5 kinase that instigates inflammation. *Proc Natl Acad Sci USA* **111**, 17438–17443.
 - 10 Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, Kano S, Honda K, Ohba Y, Mak TW *et al.* (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* **434**, 243–249.
 - 11 Blackwell TS & Christman JW (1997) The role of nuclear factor-kappa B in cytokine gene regulation. *Am J Respir Cell Mol Biol* **17**, 3–9.
 - 12 Motshwene PG, Moncrieffe MC, Grossmann JG, Kao C, Ayaluru M, Sandercock AM, Robinson CV, Latz E & Gay NJ (2009) An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4. *J Biol Chem* **284**, 25404–25411.
 - 13 Lin SC, Lo YC & Wu H (2010) Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* **465**, 885–890.
 - 14 Ye H, Arron JR, Lamothe B, Cirilli M, Kobayashi T, Shevde NK, Segal D, Dziveno OK, Vologodskaja M, Yim M *et al.* (2002) Distinct molecular mechanism for initiating TRAF6 signalling. *Nature* **418**, 443–447.
 - 15 Ordureau A, Smith H, Windheim M, Pegg M, Carrick E, Morrice N & Cohen P (2008) The IRAK-catalysed activation of the E3 ligase function of Pellino isoforms induces the Lys63-linked polyubiquitination of IRAK1. *Biochem J* **409**, 43–52.
 - 16 Smith H, Pegg M, Campbell DG, Vandermoere F, Carrick E & Cohen P (2009) Identification of the phosphorylation sites on the E3 ubiquitin ligase Pellino that are critical for activation by IRAK1 and IRAK4. *Proc Natl Acad Sci USA* **106**, 4584–4590.
 - 17 Schaubliege R, Janssens S & Beyaert R (2006) Pellino proteins are more than scaffold proteins in TLR/IL-1R signalling: a role as novel RING E3-ubiquitin-ligases. *FEBS Lett* **580**, 4697–4702.
 - 18 Strickson S, Emmerich CH, Goh ETH, Zhang J, Kelsall IR, Macartney T, Hastie CJ, Knebel A, Pegg M, Marchesi F *et al.* (2017) Roles of the TRAF6 and Pellino E3 ligases in MyD88 and RANKL signaling. *Proc Natl Acad Sci USA* **114**, E3481–E3489.
 - 19 Wang C, Deng L, Hong M, Akkaraju GR, Inoue J & Chen ZJ (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**, 346–351.
 - 20 Rahighi S, Ikeda F, Kawasaki M, Akutsu M, Suzuki N, Kato R, Kensche T, Uejima T, Bloor S, Komander D *et al.* (2009) Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. *Cell* **136**, 1098–1109.
 - 21 Lo YC, Lin SC, Rospigliosi CC, Conze DB, Wu CJ, Ashwell JD, Eliezer D & Wu H (2009) Structural basis for recognition of diubiquitins by NEMO. *Mol Cell* **33**, 602–615.
 - 22 Hauenstein AV, Xu G, Kabaleeswaran V & Wu H (2017) Evidence for M1-linked polyubiquitin-mediated conformational change in NEMO. *J Mol Biol* **429**, 3793–3800.
 - 23 Zhang J, Clark K, Lawrence T, Pegg MW & Cohen P (2014) An unexpected twist to the activation of IKK β : TAK1 primes IKK β for activation by autophosphorylation. *Biochem J* **461**, 531–537.
 - 24 Zhang J, Macartney T, Pegg M & Cohen P (2017) Interleukin-1 and TRAF6-dependent activation of TAK1 in the absence of TAB2 and TAB3. *Biochem J* **474**, 2235–2248.
 - 25 Nanda SK, Venigalla RK, Ordureau A, Patterson-Kane JC, Powell DW, Toth R, Arthur JS & Cohen P (2011) Polyubiquitin binding to ABIN1 is required to prevent autoimmunity. *J Exp Med* **208**, 1215–1228.
 - 26 Skaug B, Chen J, Du F, He J, Ma A & Chen ZJ (2011) Direct, noncatalytic mechanism of IKK inhibition by A20. *Mol Cell* **44**, 559–571.
 - 27 Bosanac I, Wertz IE, Pan B, Yu C, Kusam S, Lam C, Phu L, Phung Q, Maurer B, Arnott D *et al.* (2010) Ubiquitin binding to A20 ZnF4 is required for modulation of NF-kappaB signaling. *Mol Cell* **40**, 548–557.
 - 28 Tokunaga F, Nishimasu H, Ishitani R, Goto E, Noguchi T, Mio K, Kamei K, Ma A, Iwai K & Nureki O (2012) Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF-kappaB regulation. *Embo J* **31**, 3856–3870.
 - 29 Heyninck K, Kreike MM & Beyaert R (2003) Structure-function analysis of the A20-binding inhibitor of NF-kappa B activation, ABIN-1. *FEBS Lett* **536**, 135–140.
 - 30 Kelsall IR, Zhang J, Knebel A, Arthur JSC & Cohen P (2019) The E3 ligase HOIL-1 catalyses ester bond formation between ubiquitin and components of the Myddosome in mammalian cells. *Proc Natl Acad Sci USA* **116**, 13293–13298.
 - 31 Fujita H, Tokunaga A, Shimizu S, Whiting AL, Aguilar-Alonso F, Takagi K, Walinda E, Sasaki Y, Shimokawa T, Mizushima T *et al.* (2018) Cooperative domain formation by homologous motifs in HOIL-1L and SHARPIN plays a crucial role in LUBAC stabilization. *Cell Rep* **23**, 1192–1204.
 - 32 Peltzer N, Darding M, Montinaro A, Draber P, Draberova H, Kupka S, Rieser E, Fisher A, Hutchinson C, Taraborrelli L *et al.* (2018) LUBAC is essential for embryogenesis by preventing cell death and enabling haematopoiesis. *Nature* **557**, 112–117.

- 33 Peltzer N, Rieser E, Taraborrelli L, Draber P, Darding M, Pernaute B, Shimizu Y, Sarr A, Draberova H, Montinaro A *et al.* (2014) HOIP deficiency causes embryonic lethality by aberrant TNFR1-mediated endothelial cell death. *Cell Rep* **9**, 153–165.
- 34 Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K & Akira S (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**, 143–150.
- 35 Jorgensen I, Lopez JP, Laufer SA & Miao EA (2016) IL-1beta, IL-18, and eicosanoids promote neutrophil recruitment to pore-induced intracellular traps following pyroptosis. *Eur J Immunol* **46**, 2761–2766.
- 36 Yasuda K, Nakanishi K & Tsutsui H (2019) Interleukin-18 in health and disease. *Int J Mol Sci* **20**, 649.
- 37 Clark K, Pegg M, Plater L, Sorcek RJ, Young ER, Madwed JB, Hough J, McIver EG & Cohen P (2011) Novel cross-talk within the IKK family controls innate immunity. *Biochem J* **434**, 93–104.
- 38 Regan J, Breitfelder S, Cirillo P, Gilmore T, Graham AG, Hickey E, Klaus B, Madwed J, Moriaki M, Moss N *et al.* (2002) Pyrazole urea-based inhibitors of p38 MAP kinase: from lead compound to clinical candidate. *J Med Chem* **45**, 2994–3008.
- 39 Kuma Y, Sabio G, Bain J, Shpiro N, Marquez R & Cuenda A (2005) BIRB796 inhibits all p38 MAPK isoforms *in vitro* and *in vivo*. *J Biol Chem* **280**, 19472–19479.
- 40 Zhang T, Inesta-Vaquera F, Niepel M, Zhang J, Ficarro SB, Machleidt T, Xie T, Marto JA, Kim N, Sim T *et al.* (2012) Discovery of potent and selective covalent inhibitors of JNK. *Chem Biol* **19**, 140–154.
- 41 Waterfield M, Jin W, Reiley W, Zhang M & Sun SC (2004) IkappaB kinase is an essential component of the Tpl2 signaling pathway. *Mol Cell Biol* **24**, 6040–6048.
- 42 Beinke S, Robinson MJ, Hugunin M & Ley SC (2004) Lipopolysaccharide activation of the TPL-2/MEK/extracellular signal-regulated kinase mitogen-activated protein kinase cascade is regulated by IkappaB kinase-induced proteolysis of NF-kappaB1 p105. *Mol Cell Biol* **24**, 9658–9667.
- 43 Keusekotten K, Elliott PR, Glockner L, Fiil BK, Damgaard RB, Kulathu Y, Wauer T, Hospenthal MK, Gyrd-Hansen M, Krappmann D *et al.* (2013) OTULIN antagonizes LUBAC signaling by specifically hydrolyzing Met1-linked polyubiquitin. *Cell* **153**, 1312–1326.
- 44 Rivkin E, Almeida SM, Ceccarelli DF, Juang YC, MacLean TA, Srikumar T, Huang H, Dunham WH, Fukumura R, Xie G *et al.* (2013) The linear ubiquitin-specific deubiquitinase gumby regulates angiogenesis. *Nature* **498**, 318–324.
- 45 Kawagoe T, Sato S, Matsushita K, Kato H, Matsui K, Kumagai Y, Saitoh T, Kawai T, Takeuchi O & Akira S (2008) Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. *Nat Immunol* **9**, 684–691.
- 46 Pauls E, Nanda SK, Smith H, Toth R, Arthur JS & Cohen P (2013) Two phases of inflammatory mediator production defined by the study of IRAK2 and IRAK1 knock-in mice. *J Immunol* **191**, 2717–2730.
- 47 Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW *et al.* (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372**, 739–746.
- 48 Rousseau S, Papoutsopoulou M, Symons A, Cook D, Lucocq JM, Prescott AR, O'Garra A, Ley SC & Cohen P (2008) TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages. *J Cell Sci* **121**, 149–154.
- 49 Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C & Chen ZJ (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**, 351–361.
- 50 Carvajal AR, Gomez Diaz C, Vogel A, Sonn-Segev A, Schodl K, Deszcz L, Orban-Nemeth Z, Sakamoto S, Mechtler K, Kukura P *et al.* (2020) The linear ubiquitin chain assembly complex LUBAC generates heterotypic ubiquitin chains. *BioRxiv*. [PREPRINT]
- 51 Phadke R, Hedberg-Oldfors C, Scalco RS, Lowe DM, Ashworth M, Novelli M, Vara R, Merwick A, Amer H, Sofat R *et al.* (2020) RBCK1-related disease: A rare multisystem disorder with polyglucosan storage, auto-inflammation, recurrent infections, skeletal, and cardiac myopathy-Four additional patients and a review of the current literature. *J Inherit Metab Dis* **43**, 1002–1013.
- 52 MacDuff DA, Reese TA, Kimmey JM, Weiss LA, Song C, Zhang X, Kambal A, Duan E, Carrero JA, Boisson B *et al.* (2015) Phenotypic complementation of genetic immunodeficiency by chronic herpesvirus infection. *Elife* **4**, e04494.
- 53 Emmerich CH, Bakshi S, Kelsall IR, Ortiz-Guerrero J, Shpiro N & Cohen P (2016) Lys63/Met1-hybrid ubiquitin chains are commonly formed during the activation of innate immune signalling. *Biochem Biophys Res Commun* **474**, 452–461.
- 54 Windheim M, Stafford M, Pegg M & Cohen P (2008) Interleukin-1 (IL-1) induces the Lys63-linked polyubiquitination of IL-1 receptor-associated kinase 1 to facilitate NEMO binding and the activation of IkappaBalpha kinase. *Mol Cell Biol* **28**, 1783–1791.
- 55 Cohen ES, Scott IC, Majithiya JB, Rapley L, Kemp BP, England E, Rees DG, Overed-Sayer CL, Woods J, Bond NJ *et al.* (2015) Oxidation of the alarmin IL-33 regulates ST2-dependent inflammation. *Nat Commun* **6**, 8327.