The deubiquitinase OTUB1 augments NF-κB-dependent immune responses in dendritic cells in infection and inflammation by stabilizing UBC13

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INTRODUCTION

Dendritic cells (DCs) are key sentinel cells and professional antigen-presenting cells (APCs) of the immune system. They bridge innate and adaptive immune responses and play indispensable roles in host defense against invading pathogens, including viruses, bacteria, and parasites. Individual DC populations are adapted to their anatomical niche and particular function. Type 1 conventional DCs (cDC1s) and cDC2s develop in BATF3/IRF8- and IRF4-dependent manners, respectively, from a common DC precursor, whereas plasmacytoid DCs (pDCs) may arise from common DCs and common lymphoid progenitors.1,2

The detection of pathogens by DCs is primarily mediated by pattern recognition receptors (PRRs), which are essential for DC activation and subsequent immune responses. Among PRRs are the Toll-like receptors (TLRs), which consist of 10 human and 12 murine members. Interestingly, TLR11 and TLR12, which are expressed in only mice, sense very few pathogen-associated molecular pattern molecules (PAMPs), including Toxoplasma (T.) gondii profilin (TgPFN), which activates the MyD88/nuclear factor-κB (NF-κB) pathway, leading to protective interleukin-12 (IL-12) production by CD8+ cDC1s within a few hours after infection.3-5 In contrast, TLR4 is expressed by many cell types in mice and humans, including cDC1s, cDC2s, and pDCs, and induces activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways upon engagement by Gram-negative bacterial lipopolysaccharides (LPS). Notably, exaggerated stimulation of TLR4 by LPS may lead to severe immunopathology, as observed in sepsis.6

Dendritic cells (DCs) are indispensable for defense against pathogens but may also contribute to immunopathology. Activation of DCs upon the sensing of pathogens by Toll-like receptors (TLRs) is largely mediated by pattern recognition receptor/nuclear factor-κB (NF-κB) signaling and depends on the appropriate ubiquitination of the respective signaling molecules. However, the ubiquitinating and deubiquitinating enzymes involved and their interactions are only incompletely understood. Here, we reveal that the deubiquitinase OTU domain, ubiquitin aldehyde binding 1 (OTUB1) is upregulated in DCs upon murine Toxoplasma gondii infection and lipopolysaccharide challenge. Stimulation of DCs with the TLR11/12 ligand T. gondii profilin and the TLR4 ligand lipopolysaccharide induced an increase in NF-κB activation in OTUB1-competent cells, resulting in elevated interleukin-6 (IL-6), IL-12, and tumor necrosis factor (TNF) production, which was also observed upon the specific stimulation of TLR2, TLR3, TLR7, and TLR9. Mechanistically, OTUB1 promoted NF-κB activity in DCs by K48-linked deubiquitination and stabilization of the E2-conjugating enzyme UBC13, resulting in increased K63-linked ubiquitination of IRAK1 (IL-1 receptor-associated kinase 1) and TRAF6 (TNF receptor-associated factor 6). Consequently, DC-specific deletion of OTUB1 impaired the production of cytokines, in particular IL-12, by DCs over the first 2 days of T. gondii infection, resulting in the diminished production of protective interferon-γ (IFN-γ) by natural killer cells, impaired control of parasite replication, and, finally, death from chronic T. encephalitis, all of which could be prevented by low-dose IL-12 treatment in the first 3 days of infection. In contrast, impaired OTUB1-deficient DC activation and cytokine production by OTUB1-deficient DCs protected mice from lipopolysaccharide-induced immunopathology. Collectively, these findings identify OTUB1 as a potent novel regulator of DCs during infectious and inflammatory diseases.

Keywords: OTUB1; dendritic cell; signal transduction; ubiquitination; innate immunity

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The NF-κB pathway is tightly modulated by post-translational modifications (PTMs), including phosphorylation and ubiquitination. These PTMs are highly dynamic and reversible and thereby enable a swift and economical adaptation to environmental changes. Ubiquitination is a process involving the covalent fusion of one or more monomers of ubiquitin, an 8.5 kDa regulatory protein, to target proteins. Ubiquitin molecules can be linked via Met 1 (M1) or more monomers of ubiquitin, an 8.5 kDa regulatory protein, to form distinct polyubiquitin chains. Depending on the type of ubiquitination, proteins may be degraded or functionally altered, particularly during signal transduction. Ubiquitination of target proteins requires the concerted actions of E2-conjugating enzymes, which determine the type of ubiquitination, and E3 ubiquitin ligases, which provide substrate specificity. Activation of the NF-κB pathway is critically regulated by several E2-conjugating enzymes and E3 ligases, including UBC13, Pellino, and TRAF6 (TNF receptor-associated factor 6). UBC13, which binds TLR4, for the indicated time and analyzed by western blot, resulted in the equivalent nuclear accumulation of IRF8 in BMDCs in contrast to NF-κB reporter activity, we cloned the NF-κB reporter construct into a retroviral vector and transduced BMDCs with virus containing the construct. The nuclear accumulation of IRF8 was determined by luciferase reporter assay. The results showed that OTUB1 positively regulates proinflammatory NF-κB signaling in DCs.

RESULTS

OTUB1 is upregulated in DCs during T. gondii infection and LPS challenge

Since OTUB1 has been reported to interact with molecules critical for activation of the immune system and DCs are key immune cells that protect the host from various infectious diseases, including toxoplasmosis, but also contribute to immunopathology in sepsis, we asked whether DC-specific OTUB1 is regulated during murine toxoplasmosis and LPS-induced sepsis. The OTUB1 protein was constitutively expressed in splenic CD11c+ cells, which comprise all major populations of DCs, but its levels were significantly increased during T. gondii infection and LPS challenge (Fig. 1a, b). To substantiate these in vivo findings, in vitro-expanded bone marrow-derived DCs (BMDCs) were stimulated with T. gondii, which activates TRIF, TLR3, and TLR1/2. To investigate whether OTUB1 also acts as a feedback regulator of this signaling pathway, we crossed CD11c-Cre mice with OTUB1fl/fl mice. OTUB1 protein levels in OTUB1-deficient BMDCs were significantly decreased compared to WT BMDCs, suggesting that OTUB1 positively regulates proinflammatory NF-κB signaling in DCs.

To determine the functional role of OTUB1 in DCs, we crossed OTUB1fl/fl mice with OTUB1-deficient BMDCs produced significantly less IL-12, TNF, and IL-6 after stimulation with T. gondii, T. gondii lysate antigen (TLA), and IL-6 after stimulation with T. gondii, T. gondii lysate antigen (TLA), and LPS. The reduced cytokine production in OTUB1-deficient BMDCs was also confirmed by the mRNA expression of IL-12α, IL-12β, TNF, and IL-6 after stimulation with T. gondii and LPS-induced stimulation by quantitative PCR. In addition, engagement of TLR2, TLR7, and TLR9, which all signal via MyD88, resulted in significantly reduced IL-12, TNF, and IL-6 production in OTUB1-deficient BMDCs (Supplementary Fig. 1f, g), suggesting that OTUB1 positively regulates proinflammatory NF-κB signaling in DCs.

To understand how OTUB1 promotes cytokine production in DCs, we stimulated OTUB1-deficient BMDCs with TLR ligands. As shown in Fig. 2a, b, OTUB1-deficient BMDCs produced significantly less IL-12, TNF, and IL-6 after stimulation with T. gondii, T. gondii lysate antigen (TLA), and LPS. The reduced cytokine production in OTUB1-deficient BMDCs was also confirmed by the mRNA expression of IL-12α, IL-12β, TNF, and IL-6 after stimulation with T. gondii and LPS-induced stimulation by quantitative PCR. In addition, engagement of TLR2, TLR7, and TLR9, which all signal via MyD88, resulted in significantly reduced IL-12, TNF, and IL-6 production in OTUB1-deficient BMDCs (Supplementary Fig. 1f, g), suggesting the stimulatory role of OTUB1 in proinflammatory NF-κB signaling.

In vitro-stimulated, granulocyte–macrophage colony-stimulating factor (GM-CSF)- and FMS-like tyrosine kinase 3 ligand (FLT3L)-expanded BMDCs (Fig. 1c–h). The upregulation of OTUB1 was a consequence of increased gene transcription, as indicated by increased OTUB1 messenger RNA (mRNA) levels after TgP FN- and LPS-induced stimulation (Supplementary Fig. 1a, b). The finding that OTUB1 is upregulated in T. gondii-infected, TgP FN- and LPS-stimulated DCs raises the question of whether OTUB1 is induced only upon stimulation of TLR/MyD88/NF-κB signaling or whether OTUB1 also acts as a feedback regulator of this signaling pathway.

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Fig. 1  OTUB1 expression is upregulated in DCs by *T. gondii* infection and LPS stimulation. **a, b** Mice were infected i.p. with five *T. gondii* cysts (a) or challenged with LPS (b) for the indicated time periods. CD11c⁺ cells were isolated from spleens by magnetic sorting, and OTUB1 expression was measured by WB analysis (upper panels). The lower panels show the relative expression of OTUB1 (*n* = 3 for each group). **c–h** GM-CSF-expanded (c, e, and g) and FLT3L-expanded (d, f, and h) BMDCs generated from C57BL/6 mice were stimulated in vitro with tachyzoites at an MOI of 3 (c, d), 1 μg/ml TgPFN (e, f), or 500 ng/ml LPS (g, h) for the indicated time periods. OTUB1 expression in BMDCs was analyzed by WB analysis (upper panels). The lower panels show the relative expression of OTUB1 (*n* = 3 for each group). Data are displayed as the mean ± SD. *p* < 0.05, **p** < 0.01, and ***p*** < 0.001.
Abolishment of NF-κB activity by the inhibitor dramatically reduced IL-12 production by both OTUB1-sufficient and OTUB1-deficient BMDCs and blunted the difference in IL-12 production between the two cell populations (Fig. 2h).

Since the phosphorylation of TAK1 is a crucial step for activation of the NF-κB pathway in TLR-initiated signaling pathways, we assessed the phosphorylation of TAK1 following the stimulation of TLR2, TLR3, TLR4, TLR7, and TLR9 with their specific ligands. In good agreement with the increased TAK1 phosphorylation in OTUB1-competent BMDCs upon TLR11/12 stimulation (Fig. 2g), the phosphorylation of TAK1 was also strongly increased in OTUB1-competent BMDCs upon the engagement of TLR2, TLR4, TLR7, TLR9, and TLR3 (Fig. 2i).
Stimulation of TLR3 activates NF-κB by RIP1-mediated TAK1 phosphorylation.36 Therefore, we further explored whether increased MyD88-independent TLR3/TRIF-mediated activation of TAK1 in OTUB1-competent BMDCs also resulted in the enhanced activation of NF-κB. In fact, stimulation with polyinosinic-polycytidylic acid (poly I:C) augmented kBα and p65 phosphorylation in OTUB1-competent DCs compared to OTUB1-deficient DCs (Supplementary Fig. 2d). Likewise, stimulation with TNF, which also induces RIP1-dependent TAK1 activation,35 or IL-β, which activates TAK1 via MyD88 and TRAF6,39 increased the phosphorylation of kBα and p65 in OTUB1-competent BMDCs (Supplementary Fig. 2e, f, respectively), further illustrating that OTUB1 supports NF-κB activation in DCs upon their stimulation with both MyD88-dependent and MyD88-independent proinflammatory stimuli.

In addition to the activation of NF-κB, the engagement of TLR4 and TLR11/12 resulted in activation of the MAPKs p38, extracellular signal-regulated kinase 1/2 (ERK1/2), and JNK phosphorylation compared to that in OTUB1-decient BMDCs (Supplementary Fig. 2e, f). In addition, expression of MAPKs p38, ERK1/2, and JNK phosphorylation was increased in the presence of OTUB1 (Fig.3h, i), which is consistent with sequential K63- and K48-linked polyubiquitination of IRAK1 in TLR-induced MyD88-dependent NF-κB activation.43,52

To directly verify that the reduced production of proinflammatory cytokines in OTUB1-decient BMDCs was a consequence of the rapid degradation of UBC13, we overexpressed UBC13 with lentivirus (Supplementary Fig. 3h–k). In contrast, K48-linked polyubiquitination of TRAF6 was only weakly induced by UBC13 overexpression in OTUB1-competent BMDCs (Supplementary Fig. 3g). Interestingly, K48-linked polyubiquitination of IRAK1 was also increased in OTUB1-competent BMDCs (Fig. 3k), which is consistent with sequential K63- and K48-linked polyubiquitination of IRAK1 in TLR-induced MyD88-dependent NF-κB activation.43,52

To directly verify that the reduced production of proinflammatory cytokines in OTUB1-deficient BMDCs was a consequence of the rapid degradation of UBC13, we overexpressed UBC13 with lentivirus (Supplementary Fig. 3h). As shown in Fig. 3i, untransduced and mock-transduced OTUB1-deficient BMDCs produced significantly lower amounts of IL-12, TNF, and IL-6 than OTUB1-deficient BMDCs, whereas OTUB1-deficient BMDCs transduced with UBC13 produced amounts of proinflammatory cytokines similar to those in UBC13-transduced OTUB1-deficient BMDCs (Fig. 3i). This demonstrates that the restoration of UBC13 corrected the defect in cytokine production in OTUB1-deficient cells. Taken together, these results indicate that OTUB1 promotes...
OTUB1 stabilizes UBC13 by reducing its K48-linked polyubiquitination. a–e Cytoplasmic proteins from unstimulated, TgPFN-stimulated (a, b, e) and LPS-stimulated (c, d) BMDCs were immunoprecipitated with the indicated antibodies. Immunoprecipitates and input were analyzed by WB analysis with the indicated antibodies. f OTUB1-sufficient and OTUB1-deficient FLT3L-expanded BMDCs were pretreated with TgPFN (1 μg/ml) for 1 h or left unstimulated. Then, cycloheximide (CHX, 100 μg/ml) was added for the indicated time period. Protein levels of UBC13 in whole-cell lysates were analyzed by WB analysis (upper panel). The lower panel shows the relative levels of UBC13 normalized to βACT levels (n = 3). g NIH 3T3 cells were transfected with OTUB1 siRNA for 36 h. Then, the cells were transfected with GFP, GFP-OTUB1, GFP-OTUB1 ΔN, or GFP-OTUB1 C91S plasmids. After 24 h, the cells were treated with CHX + LPS for 0, 3, and 6 h. Whole-cell lysates were then isolated and analyzed with the indicated antibodies. h–k GM-CSF-expanded BMDCs were left unstimulated or stimulated with TgPFN in the presence of MG132. Cytoplasmic proteins were isolated and immunoprecipitated with anti-TRAF6 (h, i) and anti-IRAK1 (j, k) antibodies. Immunoprecipitates and input were analyzed with the indicated antibodies. l FLT3L-expanded BMDCs were left untreated or transduced with UBC13-expressing lentivirus or vector lentivirus for 72 h, followed by stimulation with TgPFN for 24 h. Cytokines in the supernatants of cell cultures were measured by flow cytometry (n = 4). Data are displayed as the mean ± SD (d) or mean ± SD (g). *p < 0.05, **p < 0.01, and ***p < 0.001; n.s. not significant.
T. gondii-induced NF-kB activation and cytokine production in DCs by directly deubiquitinating and stabilizing the E2 enzyme UBC13. Ubiquitinated TRAF6 induces the activation of its downstream kinase TAK1, which is essential for NF-kB and MAPK activity. In addition to TRAF6, TAK1 can be directly activated by unanchored ubiquitin chains. Unanchored ubiquitin chains can originate from the DUB-dependent release of ubiquitin from substrates during proteasomal degradation or can be synthesized de novo using monomeric ubiquitin as a substrate. Since OTUB1 is a DUB and UBC13 in cooperation with Uev1a and TRAF6 mediates the formation of unanchored ubiquitin,53,54,55,56 we hypothesized that unanchored ubiquitin would be increased in TgPFN-activated OTUB1-competent DCs. We performed a free ubiquitin assay57 and discovered that 30 min after TgPFN stimulation, the accumulation of unanchored ubiquitin monomers, dimers, and, in polyubiquitin chains was strongly increased in OTUB1-sufficient BMDCs compared to OTUB1-deficient BMDCs (Supplementary Fig. 3i). Thus, in OTUB1-competent BMDCs, the increased amount of free ubiquitin may further contribute to the activation of TAK1 and increased NF-kB activation.

OTUB1 is required for high levels of cytokine production by CD11c+ cells and subsequent IFN-γ responses in early toxoplasmosis
To determine the functional role of OTUB1 in DCs in vivo, we induced toxoplasmosis in CD11c-Cre OTUB1fl/fl and control mice. CD8- cDC1s are the main source of IL-12 within a few hours after infection,26,30,38 and shortly thereafter, interferon-γ (IFN-γ)-primed CD11b+ cDC2s and pDCs additionally contribute to IL-12 production.34 Therefore, we explored the impact of OTUB1 on DC-mediated IL-12 production during T. gondii infection. CD11c-Cre OTUB1fl/fl and OTUB1+/fl control mice were i.p. infected with 50,000 tachyzoites expressing GFP (PTG-GFP strain, type II strain), and cells in the peritoneal cavity were collected at 12 and 24 h postinfection (p.i.) for further analysis. In good agreement with the OTUB1-dependent cytokine production observed in vitro (Fig. 2a), the CD8- cDC1s of CD11c-Cre OTUB1fl/fl mice showed a significantly reduced percentage and absolute number of IL-12-producing DCs in comparison to OTUB1+/fl mice at 12 and 48 h p.i. (Fig. 4a). Additionally, the CD11b+ cDC2s of CD11c-Cre OTUB1fl/fl mice exhibited reduced IL-12 production 48 h, but not 12 h p.i. (Fig. 4a). In contrast, IL-12 production by F4/80+ macrophages was identical in both mouse strains (Supplementary Fig. 4a). Additionally, the production of TNF and IL-6 was significantly reduced in all DC subsets of CD11c-Cre OTUB1fl/fl mice at 72 h p.i. (Supplementary Fig. 4b, c). In good agreement with the UBC13-stabilizing function of OTUB1 in TgPFN-stimulated BMDCs (Fig. 3f), we detected the increased expression of UBC13 in CD8- cDC1s, PDCA1+ DCs, and CD11b+ cDC2s isolated from OTUB1+/fl mice after infection with T. gondii (Fig. 4b, first top panel), confirming the critical role of OTUB1 in stabilizing UBC13 in DCs in vivo. In addition to the induction of cytokines, NF-kB activity drives the expression of immunologically important cell surface molecules, such as MHC (major histocompatibility complex) class II, CD40, CD80, and CD86, which regulate the interaction of DCs with T cells.59 Here, we observed significantly reduced expression of MHC II, CD80, and CD86 in the CD8- cDC1s of CD11c-Cre OTUB1fl/fl mice 48 h p.i. (Fig. 4b), substantiating the role of OTUB1 in NF-kB activation, particularly that in this DC subtype.

Given that CD8- cDC1-derived IL-12 is indispensable for the initiation of a potent IFN-γ response in NK cells upon T. gondii infection,60-62 the reduction in early IL-12 production in the CD8- cDC1s of OTUB1+/fl mice was accompanied by a reduced IFN-γ response in NK cells (Fig. 4c), but not in CD4+ and CD8+ T cells (Supplementary Fig. 4d). The strong OTUB1-dependent reduction in IL-12 production by CD11b+ cDC2s at 48 h p.i. is consistent with the observation that IFN-γ-mediated priming of these DC populations is required for their TLR11/12-dependent production of IL-12.5,63 The impaired immune response of OTUB1-deficient DCs resulted in a reduction in serum IL-12 and IL-6, and, to a lesser extent, TNF at 48 h p.i. (Supplementary Fig. 4e).

Together, these data suggest a pivotal role for OTUB1 in driving NF-kB-dependent immune reactions in DCs, including IL-12 production, which is critical for a potent NK cell-dependent IFN-γ response in toxoplasmosis.62

CD11c-Cre OTUB1fl/fl mice fail to control expansion of T. gondii
To investigate whether the diminished immune responses of OTUB1-deficient DCs affected parasite control, we i.p. infected the mice with 50,000 GFP-expressing tachyzoites and determined the composition of leukocytes and parasite load in the peritoneal cavity at 2 and 4 days p.i. The percentages of T. gondii-infected cDC2s, pDCs, and, to a lesser extent, CD8- cDC1s were increased in CD11c-Cre OTUB1fl/fl mice at 4 days p.i. (Fig. 5a). The number of leukocytes present at the site of infection was comparable between the two mouse strains (Supplementary Fig. 5a), but the intracellular parasite loads of the total CD45+ leukocytes in the peritoneal cavity (Fig. 5b) and the individual leukocyte subpopulations NK1.1+ NK cells, CD11b+ Ly6Chigh inflammatory monocytes, CD11b+ F4/80+ macrophages, and CD11b+ Ly6G+ neutrophils (Fig. 5c) were significantly increased in CD11c-Cre OTUB1fl/fl mice. This suggests that the early defect in immune activation of OTUB1-deficient DCs resulted in insufficient control of the parasite at the early stage of infection. The massive release of T. gondii from infected cells may augment parasite dissemination to adjacent cells, and, subsequently, to other organs. Indeed, quantitative analysis of T. gondii genomic DNA revealed that multiple organs of CD11c-Cre OTUB1fl/fl mice harbored significantly higher parasite loads than those of OTUB1+/fl control mice at day 10 p.i. (Fig. 5d).

Interestingly, in vitro infection with T. gondii resulted in similar parasite burdens in OTUB1-sufficient and OTUB1-deficient BMDCs with or without IFN-γ prestimulation, suggesting that OTUB1 does not interfere with the cell-intrinsic parasite killing mechanism in DCs (Supplementary Fig. 5b). Consistently, the production of the antiparasitic effector molecules inducible nitric oxide synthase (iNOS), IFN-γ-induced GTPase (IGPT), and guanylate-binding proteins (GBP) were comparable between OTUB1-sufficient and OTUB1-deficient BMDCs (Supplementary Fig. 5c). Toxoplasma gondii promotes its systemic dissemination by hijacking the migratory properties of parasitized DCs.66 To address whether OTUB1 has an impact on Toxoplasma-induced hypermigration of DCs, we performed motility analyses. Upon challenge with T. gondii, parasitized OTUB1-sufficient and OTUB1-deficient BMDCs exhibited similar elevated velocities, and unchallenged DCs exhibited a nonsignificant difference in baseline velocity (Supplementary Fig. 5d). Thus, T. gondii-induced DC hypermigration was OTUB1 independent. Collectively, these data suggest that OTUB1 has no impact on DC-intrinsic parasite control or DC-mediated T. gondii dissemination.

Hou et al.61 demonstrated that impaired IL-12 production by CD8- cDC1s in acute toxoplasmosis resulted in a more severe course of chronic Toxoplasma encephalitis (TE) with an increased intracerebral parasite load. To address whether DC-specific OTUB1 regulates the course of chronic TE, we infected CD11c-Cre OTUB1fl/fl mice and control mice with three brain cysts of the type II DX strain. At day 30 p.i., the intracerebral parasite load in CD11c-Cre OTUB1fl/fl mice was significantly higher than that in control mice (Fig. 5e). In addition, the mortality of CD11c-Cre OTUB1fl/fl mice during chronic TE was significantly increased (Fig. 5f). Notably, the intracerebral immune response of CD11c-Cre OTUB1fl/fl mice was not impaired, as illustrated by the normal number of the intracerebral leukocyte populations DCs and T cells at day 30 p.i. (Supplementary Fig. 5e–g). Additionally, IL-12 production by DCs and IFN-γ production by CD4+ and CD8-
OTUB1 deletion in DC impairs the cytokine response during *T. gondii* infection. a–c OTUB1<sup>fl/fl</sup> and CD11c-Cre OTUB1<sup>fl/fl</sup> mice were infected i.p. with 50,000 tachyzoites for 12 and 48 h, respectively. Peritoneal cells were collected by lavage of the peritoneal cavity and analyzed by flow cytometry to detect IL-12 production in CD11c<sup>+</sup>CD8<sup>a</sup><sup>+</sup>, CD11c<sup>+</sup>PDCA-1<sup>+</sup>, and CD11c<sup>+</sup>CD11b<sup>+</sup> DCs (a); UBC13, MHC II, CD40, CD86 and CD80 expression in CD11c<sup>+</sup>CD8<sup>a</sup><sup>+</sup>, CD11c<sup>+</sup>PDCA-1<sup>+</sup>, and CD11c<sup>+</sup>CD11b<sup>+</sup> DCs (b); and IFN-γ production in NK cells (c). Representative flow cytometry plots (left panels) and statistics (right panels) are shown (n = 8). Data are shown as the mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001; n.s. not significant.
T cells were equal in mice of both genotypes (Supplementary Fig. 5h, i, respectively). In parallel to the increased intracerebral load, even the production of antiparasitic IGTP and iNOS as well as IL-1β and IL-18 was increased in CD11c-Cre OTUB1<sup>fl/fl</sup> mice (Supplementary Fig. 5j), further indicating that the aggravated course of TE in CD11c-Cre OTUB1<sup>fl/fl</sup> mice was caused by an impaired DC immune response early after infection but not by an insufficient intracerebral immune response.

Fig. 5 OTUB1 deletion in DCs leads to impaired parasite control in toxoplasmosis. a–c OTUB1<sup>fl/fl</sup> and CD11c-Cre OTUB1<sup>fl/fl</sup> mice were infected i.p. with 50,000 tachyzoites. Peritoneal cells were isolated from the peritoneal cavities and analyzed by flow cytometry at 2 and 4 days p.i. a Percentages of infected CD11c<sup>+</sup> CD8α<sup>+</sup>, CD11c<sup>+</sup> PDCA-1<sup>+</sup>, and CD11c<sup>+</sup> CD11b<sup>+</sup> DCs. b Percentages of infected peritoneal CD45<sup>+</sup> cells in OTUB1<sup>fl/fl</sup> and CD11c-Cre OTUB1<sup>fl/fl</sup> mice. c Percentages of infected peritoneal leukocyte subpopulations in OTUB1<sup>fl/fl</sup> and CD11c-Cre OTUB1<sup>fl/fl</sup> mice. Representative FACS plots (upper panel) and statistics (lower panel) are shown (n = 10 for each group). d Mice were infected i.p. with 50,000 tachyzoites for 10 days. Parasite loads in different organs were determined by semiquantitative PCR of tissue DNA (n = 5 for each group). e, f Mice were infected i.p. with three cysts. Parasite load in the brain 30 days p.i. was determined by semiquantitative PCR of tissue DNA (n = 4 for each group) (e). Survival was monitored for 70 days p.i. (f) (n = 10 for each group). Data are expressed as the mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001; n.s. not significant.
Supplementation with IL-12 restored the ability of CD11c-Cre OTUB1−/− mice to control T. gondii. Mice selectively lacking CD8+ cDC1s are extremely susceptible to toxoplasmosis due to impaired IL-12 and subsequent IFN-γ production. Treatment of these Batf3−/− mice with 0.5 μg of IL-12 at days 0, 1, 2, 3 and 4 after infection restored resistance, illustrating that IL-12 production by CD8+ DCs is of central importance and independent of all other protective immune reactions in this cell population. To study whether the increased parasite expansion and dissemination in CD11c-Cre OTUB1−/− mice were caused by the early defect in IL-12 production by DCs, we administered 150 ng of IL-12 i.p. to CD11c-Cre OTUB1−/− and control mice at days 0, 1, 2, and 3 after infection, as shown in Fig. 6a. Here, although the defect in the production of cytokines by DCs was not limited to only IL-12 but also extended to TNF and IL-6, administration of IL-12 alone significantly reduced the T. gondii load in both CD11c-Cre OTUB1−/− and control mice and abolished the difference in parasite load between mice of the two genotypes at 4 days p.i. (Fig. 6b–e), indicating that the early defect in IL-12 production by DCs is responsible for the uncontrolled parasite expansion in acute infection. Interestingly, IL-12 administration in acute toxoplasmosis was also able to dramatically reduce the intracerebral parasite load in the brains of chronically infected CD11c-Cre OTUB1−/− and OTUB1+/+ mice, and importantly, the intracerebral parasite load did not differ between mice of the two genotypes (Fig. 6f). Histopathology confirmed that IL-12 treatment strongly reduced parasite numbers and abolished the difference between the two mouse strains (Fig. 6g). Consistent with the finding that a higher brain parasite load is associated with increased mortality (Fig. 5e, f), supplementation with IL-12 restored the ability of CD11c-Cre OTUB1−/− mice to control and mediate DC-dependent protection in infectious disease, but augments immunopathology upon LPS challenge.

Mechanistically, we identified that UBC13 interacts with OTUB1 upon LPS stimulation, extending the results of a previous study showing that, in the cell nucleus, OTUB1 binds UBC13 in response to DNA double-strand breaks. With respect to TLR signaling, UBC13 is a key molecule that bolsters MyD88-mediated NF-κB activity by cooperating with the E3 ligases Pellino and TRAF6. First, engagement of TLR11/12 and TR24 induces recruitment and interaction with MyD88, which is followed by the recruitment of IRAK4 to MyD88. This oligomeric Mydosome acts as a platform for the binding of IRAK1, IRAK2, and IRAK3. Activated IRAK1 catalyzes the phosphorylation of Pellino, which is a prerequisite for its activity as an E3 ubiquitin ligase. In collaboration with the cognate E2 ubiquitin-conjugating enzyme UBC13, active Pellino acts back against IRAK1 by enabling its K63-specific ubiquitination and subsequent activation of NEMO. Second, even without K63-linked ubiquitination, IRAK1 can also activate the IKK complex via inducing the association and activation of TRAF6. As an E3 ligase, TRAF6 adds K63-linked polyubiquitin chains to itself, with UBC13 acting as the E2 conjugase. The bifunctional nature of UBC13-mediated ubiquitination regulation makes UBC13 a pivotal molecule that bolsters MyD88-mediated NF-κB activity. In good agreement with this dual function of UBC13 in MyD88/NF-κB signaling, K63-linked ubiquitination of both IRAK1 and TRAF6 was augmented in TgPFN-stimulated OTUB1−/− BMDCs. Additionally, OTUB1 increased the amount of free ubiquitin in MyD88-activated DCs, which may further contribute to augmented NF-κB activation, since free ubiquitin can directly stimulate TAK1. The stimulatory effect of OTUB1 on TAK1/NF-κB activation upon TNF and TR23 engagement may also be mediated by increased activation of TAK1 by unanchored ubiquitin chains and UBC13-mediated K63-linked polyubiquitination of RIP1, which also contributes to TAK1 activation.

Interestingly, UBC13 is also a target of ubiquitination, and its protein stability is compromised by the addition of K48-linked polyubiquitin chains by A20. Here, we identified that K48-linked ubiquitination of UBC13 is counteracted by OTUB1. Since K48-linked ubiquitination is associated with protein degradation, OTUB1 increases the stability of UBC13. In this way, OTUB1 preserves the E2 activity of UBC13 and thereby enhances K63-linked ubiquitination of IRAK1 and TRAF6, leading to increased NF-κB activation. We found that the UBC13 protein is not stable and can be degraded even in the presence of OTUB1 (Fig. 3f, g and Supplementary Fig. 3). However, OTUB1 can significantly delay the degradation of UBC13, showing that OTUB1 serves as a mechanistic agent by which protein ubiquitination and degradation are finely tuned. Interestingly, transfection with OTUB1 mutants revealed that the prevention of UBC13 degradation requires both the N terminus and catalytic domain of OTUB1, given that only wild-type but not the OTUB1-C91S-GFP and the OTUB1-ΔN-GFP mutants rescued UBC13 from degradation (Fig. 3g). Interestingly, consistent with our findings, both the C91 levels of IL-12, TNF, and IL-6 (Supplementary Fig. 6g) and the viral load in the liver, spleen, lung, and lymph nodes (Supplementary Fig. 6h) were equal in both infected mouse strains.

**DISCUSSION**

The swift sensing of invading pathogens by DCs plays a critical role in defense against infections. In this process, TLR-mediated activation of NF-κB is essential for the rapid induction of immune responses. However, hyperactivation of NF-κB signaling may lead to immunopathology and diseases, as observed in LPS-mediated TR24 activation during Gram-negative sepsis. The results presented in the present study provide evidence that the DUB activity of OTUB1 critically supports canonical NF-κB activation in DCs and mediates DC-dependent protection in infectious disease, but augments immunopathology upon LPS challenge.
residue and the N terminus of OTUB1 are required for its effect on SMAD3.20

Both the canonical NF-κB pathway, which is induced by the engagement of TLRs and dependent on degradation of the NF-κB inhibitor IκBα, and the noncanonical NF-κB pathway, which is dependent on the degradation of the IκB-like inhibitor p100, play fundamental roles in immune responses. Due to the central importance of NF-κB pathways, several DUBs, including TNFAIP3.

**Fig. 6** IL-12 supplementation rescues the defect in parasite control in CD11c-Cre OTUB1fl/fl mice. a Experimental design scheme of IL-12 administration and analysis. Mice were i.p. injected with either PBS or 150 ng IL-12 daily from 0 to 3 days p.i. b The parasite load in peritoneal cells was determined by semiquantitative PCR of tissue DNA after infection with 50,000 tachyzoites for 4 days (n = 8 for each group). c Percentages of infected peritoneal CD45+ cells were analyzed by flow cytometry at day 4 after infection with 50,000 tachyzoites. Representative flow cytometry plots (left panel) and statistics (right panel) are shown (n = 8 for each group). d, e Percentages of infected peritoneal leucocyte subpopulations (d) and DC subsets (e) were analyzed by flow cytometry at day 4 after infection with 50,000 tachyzoites (n = 8 for each group). f Mice were i.p. infected with five cysts of the DX strain. At day 30 p.i., the parasite loads in the brains of surviving mice were determined by semiquantitative PCR. Two independent experiments with comparable results were performed. One representative experiment is shown (n = 4 for each group). g Intracerebral parasites in the brains of T. gondii-infected OTUB1fl/fl and CD11c-Cre OTUB1fl/fl mice at 30 days p.i. A PBS-treated OTUB1fl/fl mouse (top left) shows a single focus consisting of a few T. gondii cysts and some tachyzoites in the white matter of the frontal lobe. The brain of a PBS-treated CD11c-Cre OTUB1fl/fl mouse (top right) shows an increased number of parasitic foci (arrows) with T. gondii cysts and tachyzoites in the white matter of the frontal lobe. In both an OTUB1fl/fl and a CD11c-Cre OTUB1fl/fl mouse, IL-12 application reduced the intracerebral parasitic load, with only single cysts scattered throughout the frontal lobe (arrows). Immunohistochemistry with polyclonal rabbit anti-T. gondii (BioGenex, Fremont, CA, USA) and slight counterstaining with hemalum; original magnification ×200; scale bar (a-d): 50 µm. The photomicrographs shown are representative of three mice per experimental group. Similar results were obtained in a second independent experiment. h The survival of the mice was monitored daily up to 30 days after infection with cysts of strain 5 DX (n = 8 for each group). Data are displayed as the mean ± SD. *p < 0.05, and ***p < 0.001; n.s. not significant.
MCMV actively manipulates IRF3 and NF-κB resulting in activation, both of which induce MyD88-independent signaling, and production of the cytokines IL-12, TNF, and IL-6 upon TLR3,68 TLR7,69 TLR9,70,71 RLR,72 and cGAS/STING,72 and these underlying pathogen determines the importance of OTUB1 in pathogen control under MCMV infection illustrate that the protective DC functions in MCMV infection.71,72,77 Additionally, receptors can compensate for each other with respect to their activity against chemokines and other cytokines.61 IL-12 production by CD8+ T cells mediates activation of TLR11/12 in DCs. Engagement of TLR11/12 promotes LPS-induced TLR4/MyD88-dependent NF-κB activation and inflammatory stimuli, therefore contributing to the proinflammatory responses in a wide range of infections and inflammatory disorders. However, our data on OTUB1-independent cytokine production by DCs and pathogen control under MCMV infection illustrate that the underlying pathogen determines the importance of OTUB1 in DC activation. MCMV activates several PRRs in DCs, including TLR3,68 TLR7,69 TLR9,70,71 RLR,72 and cGAS/STING,72 and these receptors can compensate for each other with respect to their protective DC functions in MCMV infection.71,72,77 Additionally, MCMV actively manipulates IRF3 and NF-κB activation to promote viral spread.76-80 These data extend those of a previous in vitro study on the inhibition of Sendai virus-induced IFN regulatory factor 3 (IRF3) and NF-κB signaling by OTUB128 and further indicate the interplay between the DC-specific function of OTUB1 with pathogen-mediated modulation of host cell signaling.

To address the importance of OTUB1-mediated NF-κB activation in vivo, we studied a murine model of toxoplasmosis because Toxoplasma gondii infection and in vivo LPS challenge. Mice were infected i.p. with five freshly prepared tissue cysts of the type II DX strain, which was maintained by the chronic infection of NMRI mice and have been prepared from the brain as described before.82 Alternatively, mice were infected i.p. with 20,000 or 50,000 GFP-expressing tachyzoites of the type II PTG-GFP strain, which were prepared from the brains of type II infected NMRI mice according to methods described before.83 In this study, we identified OTUB1 as a novel DUB that regulates TLR-induced DC activation via deubiquitinating and stabilizing UBC13, thereby providing a potential target for the treatment of infectious and inflammatory diseases.

**MATERIALS AND METHODS**

**Mice**

CS7BL/6 OTUB1fl/fl mice24 were crossed with CS7BL/6 1211c-Cre mice32 to obtain CD11c-Cre OTUB1fl/fl transgenic mice. Genotyping of newborn mice was performed by PCR of tail DNA with primers targeting CD11c-Cre and OTUB1fl/fl. Animals were kept under specific pathogen-free conditions in the animal facility at Magdeburg University Hospital (Magdeburg, Germany). All mice were used at 8–12 weeks of age and were sex and age matched. Animal care and experiments were performed according to the European Animal Protection Law and approved by local authorities (Landesverwaltungsamt Halle, Germany; license number 2-1175). The regional Animal Research Ethical Board (Stockholm, Sweden) approved procedures and protocols involving the extraction of cells from mice (N135/15, N78/16), following procedures described in EU legislation (Council Directive 2010/63/EU).

**Toxoplasma gondii** infection and in vivo LPS challenge

Mice were infected i.p. with five freshly prepared tissue cysts of the type II DX strain, which was maintained by the chronic infection of NMRI mice and have been prepared from the brain as described before.82 Alternatively, mice were infected i.p. with 20,000 or 50,000 GFP-expressing tachyzoites of the type II PTG-GFP strain, which were prepared from the brains of type II infected NMRI mice according to methods described before.83 In this study, we identified OTUB1 as a novel DUB that regulates TLR-induced DC activation via deubiquitinating and stabilizing UBC13, thereby providing a potential target for the treatment of infectious and inflammatory diseases.
upon ATCC (Manassas, VA, USA) and maintained by in vitro passage in Vero cultures. Upon 60–80% lysis of Vero cell monolayers, cell cultures were mixed by pipetting and subsequently centrifuged at 50 × g for 5 min to pellet cellular debris. Thereafter, the supernatant containing tachyzoites was transferred to a new tube and centrifuged at 2000 × g for 10 min to spin down the tachyzoites. The number of tachyzoites was determined with a hemocytometer. LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as per the manufacturer’s recommended concentrations. Recombinant murine IL-1β and TNF were purchased from PeproTech (Rocky Hill, USA) and used at working concentrations of 10 and 20 ng/ml, respectively.

Flow cytometry

Leukocytes were isolated from the peritoneal cavities of mice by lavage with PBS. Intracerebral leukocytes were isolated from brains by a Percoll (GE Healthcare Life Sciences, Marlborough, USA) gradient according to previously published protocols.

Cell cultures and transfection

Vero cells were cultured in RPMI medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS) and 1% (vol/vol) pen/strep solution in a cell incubator at 37 °C with 5% CO2. NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FCS and 1% pen/strep in an incubator at 37 °C with 5% CO2. Control siRNA and OTUB1 siRNA (Thermo Fisher Scientific) were transfected into NIH 3T3 cells with Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. pCMV6-GFP, pCMV6-GFP-OTUB1 (Origene), pCMV6-GFP-ΔN, and pCMV6-GFP-OTUB1-C915 expression plasmids (generated with a Q5 Site-Directed Mutagenesis Kit, NEB) were transfected into NIH 3T3 cells with Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s protocols.

Bone marrow-derived DCs and bone marrow-derived macrophages

BMDCs were obtained and cultured as described previously. Cells were differentiated with 35 ng/ml GM-CSF (Peprotech Tebu-Bio, Offenbach, Germany) or 400 ng/ml FLT3L (Peprotech Tebu-Bio) for 8–10 days. The purity of the BMDC cultures was >90%, as determined by flow cytometry to detect CD11c. To obtain BMDMs, bone marrow was isolated from the femurs of CD11c-Cre OTUB1fl/fl and OTUB1fl/fl mice and then incubated for 8 days with 35 ng/ml M-CSF (Peprotech Tebu-bio). The purity of the BMDM cultures was >90%, as determined by flow cytometry to detect CD11b and F4/80.

In vitro stimulation

After in vitro expansion, BMDCs were washed once with PBS and plated at the desired concentration with appropriate medium.

After 4 h of incubation at 37 °C, the cells were stimulated as indicated. Samples for IP were always stimulated in the presence of the proteasome inhibitor MG132 (Sigma-Aldrich). For the preparation of TLA, freshly released tachyzoites were isolated as described before and then freeze-thawed in liquid nitrogen five times. The TLA concentration was quantified with a spectrophotometer. TgPFN was produced in the Protein Science core facility of the Karolinska Institute (Stockholm, Sweden). The purity of TgPFN was controlled by mass spectrometry, and the protein concentration was determined by Bradford assay. LPS (Sigma, St. Louis, MO, USA) and poly I:C were purchased from Invivogen (San Diego, CA, USA) and used as per the manufacturer’s recommended concentrations. Recombinant murine IL-1β and TNF were purchased from PeproTech (Rocky Hill, USA) and used at working concentrations of 10 and 20 ng/ml, respectively.

Quantitative and semiquantitative PCR

DNA and RNA were isolated from mouse tissues or BMDCs. Mouse organs were first mechanically homogenized with the QIAshredder Kit (Qiagen, Hilden, Germany) before the isolation of DNA and RNA using DNeasy and RNeasy Kits (Qiagen), respectively. RNA was then reverse transcribed into complementary DNA with the SuperScript Reverse Transcriptase Kit (Thermo Fisher Scientific). Quantitative and semiquantitative PCR was performed on a LightCycler 480 System (Roche, Ludwigshafen, Germany). The following primers for quantitative PCR of T. gondii sequences were custom produced by Eurofins MWG Operon (Ebersberg, Germany): primer sequences 5′→3′: sense GGAACGTATCCGTTCATGAG;
antisense TCTTAAAGCGTTCGTC. The following primers for semiquantitative PCR were purchased from Applied Biosystems (Darmstadt, Germany): OTUB1 (Mm00506597_m1, lot 1189970), UBC13 (Mm00779119_s1, lot 1388107), HPRT (Mm01545399_m1, lot P181210-001), INOS (Mm00440485_m1, lot 1799604), iGTP (Mm00497611_m1, lot 1467539), GBP3 (Mm00497606_m1, lot 1162514), GBP5 (Mm00463729_m1, lot P160617-002), IL-1β (Mm00434228_m1, lot 1276462), IL-12α (Mm00801778_m1, lot 1272494), TNF (Mn00443258_m1, lot 1139366), IL-12β (Mm99999067_m1, lot P160325-006), IFN-γ (Mm00801778_m1, lot 1272494), TNF (Mm00443258_m1, lot P180926-011), and IL-6 (Mm00446190_m1, lot P140131-000).

WB analysis

Cells were stimulated as indicated and lysed on ice with complete RIPA lysis buffer (Cell Signaling Technologies, Denver, CO, USA). Nuclear and cytoplasmic fractions were isolated with the Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific). Protein samples were diluted and heated in Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific) at 95 °C for 5 min. Equal amounts of samples were separated on 8–15% sodium dodecyl sulfate-polyacrylamide gels and subsequently transferred to polyvinylidene difluoride membranes, which were blocked with 5% BSA, followed by incubation with specific antibodies as indicated. Mouse monoclonal anti-HDAC-1 (cat. #5c-S81598), mouse monoclonal anti-UBC13 (cat. #5c-S58452), mouse monoclonal anti-TRAF6 (cat. #5c-8409), rabbit polyclonal anti-TRAF6 (cat. #5c-7221), mouse monoclonal anti-Pellino1/2 (cat. #5c-271065), and mouse monoclonal anti-Uev1a (cat. #5c-390047) antibodies were purchased from Santa Cruz Biotechnology (Dallas, USA). Rabbit polyclonal anti-IRAK1 (cat. #10478-2-AP) and rabbit polyclonal anti-UBC13 (cat. #10243-1-AP) antibodies were purchased from Proteintech Group (Manchester, UK). Rabbit polyclonal anti-OTUB1 antibody (cat. #NPB1-49934) was purchased from Novus Biologicals (Centennial, USA). Rabbit polyclonal anti-phospho-IRAK1 (Thr209) antibody (cat. #SA4505246) was purchased from Sigma-Aldrich. Rabbit polyclonal anti-cIAP1 (cat. #ab2399) was purchased from Abcam (Cambridge, UK). Rabbit monoclonal anti-lysine 48-specific polyubiquitin chains (cat. #05-1307) were purchased from Millipore (Burlington, MA, USA). The following remaining primary antibodies were purchased from Cell Signaling Technologies: rabbit monoclonal anti-GAPDH (cat. #2118), rabbit monoclonal anti-β-actin (cat. #8457), rabbit polyclonal anti-p65 (cat. #3034), mouse monoclonal βTUB (cat. #86289), rabbit monoclonal anti-IRF8 (cat. #56285), rabbit polyclonal anti-MyD88 (cat. #3699), rabbit polyclonal anti-IRAK4 (cat. #4363), rabbit polyclonal anti-phospho-IRAK4 (cat. #76535), rabbit polyclonal anti-TAK1 (cat. #4505), rabbit polyclonal anti-phospho-TAK1 (Ser412) (cat. #9339), rabbit monoclonal anti-IκBα (cat. #4812), rabbit monoclonal anti-phospho-IκBα (Ser32) (cat. #2859), rabbit monoclonal anti-phospho-p65 (Ser536) (cat. #4887), rabbit polyclonal anti-A20 (cat. #46255), mouse monoclonal anti-Tubulin (cat. #3936), rabbit monoclonal anti-K48 linkage-specific polyubiquitin (cat. #4289), rabbit polyclonal anti-p38 (cat. #92145), rabbit monoclonal anti-phospho-p38 (Thr180/Tyr182) (cat. #92155), rabbit polyclonal anti-ERK (cat. #9102), rabbit polyclonal anti-phospho-ERK (Thr202/Tyr204) (cat. #91015), rabbit polyclonal anti-SAPK/JNK (cat. #9252), and rabbit monoclonal anti-phospho-SAPK/JNK (Thr183/Tyr185) (cat. #4668). The following secondary antibodies were purchased from Dako (Glostrup, Denmark): swine polyclonal anti-rabbit (cat. #P0217) and rabbit polyclonal antimouse (cat. #P0260). WB images were developed with an ECL Plus system (Intas, Göttingen, Germany).

Immunoprecipitation

Whole-cell lysates of stimulated and unstimulated BMDCs were preincubated by incubation with Sepharose G Beads (GE Healthcare) with gentle shaking at 4 °C for 1 h. After removal of the beads by centrifugation, equal amounts of lysates were then incubated with specific antibodies with gentle shaking at 4 °C overnight. The next day, Sepharose G beads were added to the lysates and incubated at 4 °C for 2 h with gentle shaking. To capture the immunocomplex, samples were centrifuged, and beads were washed five times with PBS by pulse centrifugation. The beads were resuspended in 2× Lane Marker Reducing Sample Buffer and boiled at 95 °C for 5 min. Then, the samples were centrifuged at 14,000 × g at 4 °C for 1 min, and the supernatants were harvested for WB analysis.

Free ubiquitin assay

To test unanchored ubiquitin chains in FLT3L-expanded BMDCs, we followed the protocol published by Gilda et al.77 One milligram of purified proteins from unstimulated and stimulated samples was depleted of substrate-conjugated ubiquitin chains using TUBE1s (UM402, LifeSensors, PA, USA). TUBE1s have been shown to be highly efficient at removing polyubiquitinated proteins from lysates. Two incubation steps with TUBE probes were carried out, and significantly more bait (TUBE1s) than required was used to ensure that the lysate had been depleted of ubiquitinated proteins. For every milligram of total protein, 25 μl of resin was utilized and incubated for 1 h at 4 °C with slow movement. TUBE agarose was collected by low speed centrifugation (1000 × g, 4 °C) for 2 min. The beads were washed with Tris-buffered saline containing 0.05% Tween-20 and collected by low-speed centrifugation. The supernatant was collected and subsequently analyzed by WB. After pretreatment with 0.5% glutaraldehyde, we used anti-ubiquitin VU-101 antibody (LifeSensors) to detect unanchored ubiquitin chains.

Transduction of BMDCs

The pLenti-C-Myc-DDK and pLenti-C-Myc-DDK-UB2EN plasmids were purchased from OriGene (Rockville, USA). Lentivirus overexpressing UBC13 and control lentivirus were produced with lentiviral packaging kits (OriGene). At 24 and 48 h after transfection, supernatants containing lentivirus were collected, filtered, aliquoted, and stored at −80 °C. FLT3L-expanded BMDCs were transduced with lentivirus in the presence of 4 μg/ml polybrene (Sigma-Aldrich). The transduction mixture was centrifuged at 1000 × g at 32 °C for 90 min and further incubated in the cell incubator for 5 h. Thereafter, the transduced cells were incubated in normal BMDC culture medium for 3 days.

Motility assays

Motility assays were performed as previously described. Briefly, DCs were cultured in chamber slides (Lab-Tek®, Nalge Nunc International) with complete medium + freshly egressed GFP-expressing T. gondii tachyzoites (type II PTG-GFP, MOI = 3, 4 h incubation). Bovine collagen I (1 mg/ml, Life Technologies) was added, and live-cell imaging was performed for 1 h at 1 frame/min and ×10 magnification (21 Observer with Zen 2 Blue v. 4.0.3, Zeiss, Oberkochen, Germany). Time-lapse images were consolidated into stacks, and motility data were obtained from 30 cells/condition (Manual Tracking, ImageJ), yielding mean velocities (Chemotaxis and Migration Tool v. 2.0). GFP+ cells harboring tachyzoites were defined as infected cells.

MCMV infection

Seven- to ten-week-old CD11c-Cre OTUb1/fl and OTUb1/fl control mice were intravenously infected with 10^6 MCMV 3DR. After 36 h, the mice were perfused with PBS; the lymph nodes, lungs, spleens, and livers were removed; and organ homogenates were plated in serial log 10 dilutions on primary murine embryonic fibroblasts. Centrifugal enhancement was performed (2000 r.p.m., 15 min), and after 2 h of incubation at 37 °C and 5% CO2, the cells were overlaid with 1% methylcellulose. Plaques were counted after 4 days of culture under a light microscope (Zeiss). In addition, leukocytes were isolated from the spleen and liver and analyzed by flow cytometry.
Intracellular staining for IL-12 was performed after 4 h of incubation at 37 °C with GolgiPlug™ (BD Bioscience). Sera were collected from infected animals and used to measure cytokines.

Histology
At 30 day p.i. with *T. gondii*, anesthetized mice were intracardially perfused with 0.9% NaCl, following which their brains were dissected, snap frozen in isopentane (Flika, Neu-Ulm, Germany), precooled on dry ice, and stored at −80 °C until the preparation of 10-µm-thick frozen sections. Immunohistochemistry was performed with polyclonal rabbit anti-*T. gondii* (BioGenex, Fremont, USA) using an ABC protocol with 3,3'-diaminobenzidine (Merck, Darmstadt, Germany) and H2O2 as cosubstrates.

Quantification and statistical analysis
Quantification of WB data was performed using the NIH ImageJ software. Statistical analysis and graphic design were performed using GraphPad Prism 6. Differences for which &\( P < 0.001 \) by Student’s t test were considered statistically significant; *P* indicates a &\( P < 0.05 \), **P** indicates a &\( P < 0.01 \), and ***P*** indicates a &\( P < 0.001 \). All experiments were performed at least twice.

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AUTHOR CONTRIBUTIONS
F.M. designed and conducted experiments, analyzed data, and wrote the manuscript; S.S., G.N., W.T., and F.K.L. designed and conducted experiments and analyzed the data; A.B. performed histology; B.I., A.B., M.N., U.K., and M.D. designed experiments and provided helpful discussion; X.W. and D.S. conceived the project, designed and conducted experiments, analyzed data, and wrote the manuscript.

ADDITIONAL INFORMATION
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Competing interests The authors declare no competing interests.

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