Optineurin Insufficiency Impairs IRF3 but Not NF-κB Activation in Immune Cells

Ivana Munitic, Maria Letizia Giardino Torchia, Netra Pal Meena, Guozhi Zhu, Caiyi C. Li and Jonathan D. Ashwell

*J Immunol* published online 15 November 2013
http://www.jimmunol.org/content/early/2013/11/15/jimmunol.1301696
Optineurin Insufficiency Impairs IRF3 but Not NF-κB Activation in Immune Cells

Ivana Munitic,1 Maria Letizia Giardino Torchia,1 Netra Pal Meena, Guozhi Zhu, Caiyi C. Li, and Jonathan D. Ashwell

Optineurin is a widely expressed polyubiquitin-binding protein that has been implicated in regulating cell signaling via its NF-κB essential modulator–homologous C-terminal ubiquitin (Ub)-binding region. Its functions are controversial, with in vitro studies finding that optineurin suppressed TNF-mediated NF-κB activation and virus-induced activation of IFN regulatory factor 3 (IRF3), whereas bone marrow–derived macrophages (BMDMs) from mice carrying an optineurin Ub-binding point mutation had normal TLR-mediated NF-κB activation and diminished IRF3 activation. We have generated a mouse model in which the entire Ub-binding C-terminal region is deleted (Optn470T). Akin to C-terminal optineurin mutations found in patients with certain neurodegenerative diseases, Optn470T was expressed at substantially lower levels than the native protein, allowing assessment not only of the lack of Ub binding, but also of protein insufficiency. Embryonic lethality with incomplete penetrance was observed for 129 × C57BL/6 Optn470T mice, but after further backcrossing to C57BL/6, offspring viability was restored. Moreover, the mice that survived activation but necessary for optimal IRF3 activation in immune cells.

Activation of the transcription factor NF-κB is essential in signaling induced by pathogen- or damage-associated molecular patterns, as well as cellular stresses such as DNA damage, hypoxia, and excitotoxicity (1, 2). More than 400 NF-κB–responsive genes have been implicated in regulating stimulus- and cell-type–specific responses (3). Cells of the innate immune system such as macrophages and dendritic cells (DC) express a variety of NF-κB–coupled receptors (e.g., TLRs, Rig-like receptors, and Nod-like receptors) that have a major role in orchestrating immune responses and resolving tissue damage. The importance of NF-κB in immune responses is underscored by the fact that subsequent lines of defense, adaptive T and B cell responses, also require NF-κB for signaling via Ag, costimulatory, and cytokine receptors (4). Another major pathway in immune responses is the activation of the inhibitor of κB kinase (IKK)–related kinase, TANK binding kinase 1 (TBK1), which although occurring simultaneously with NF-κB activation, leads to a fundamentally different outcome. TBK1 is the major kinase that phosphorylates the transcription factor IFN regulatory factor 3 (IRF3), causing its dimerization, nuclear localization, and initiation of type I IFN production (5, 6).

Ubiquitination is a major mechanism of regulation of both NF-κB and IRF3 pathways. The canonical pathway leading to activation of NF-κB is initiated when the IKK, comprising the kinases IKKα and IKKβ and the regulatory subunit NF-κB essential modulator (NEMO), causes phosphorylation and subsequent lysine 48 (K48)–linked polyubiquitination and proteasomal degradation of the inhibitor of κB (IκB) (7). In addition, nondegradatory ubiquitination with polyubiquitin (polyUb) chains linked via lysine 63 (K63) or linear chains in which the N-terminal methionine of one ubiquitin (Ub) is linked to the C-terminal glycine of another (M1) is required for assembly of multimeric signaling complexes. K63 and/or M1 ubiquitination of various receptor-associated adaptor molecules such as RIP1, IRAK1, and Bcl10 allows the recruitment of NEMO and IKK activation (8–11). Similarly, TBK1 activation requires recruitment of NEMO to signaling complexes containing polyUb chains (12–14).

NEMO has two Ub-binding domains in its C-terminal half, an ∼30-aa region termed Ub-binding domain of ABIN proteins and NEMO (UBAN) and a more distal zinc finger (ZF) (15, 16). The presence of both domains confers high-affinity binding to K63- and M1-linked Ub chains. Four other proteins contain a UBAN, optineurin, and three A20 interacting proteins (ABIN-1, -2 and -3), whereas only optineurin and ABIN-2 have the ZF domain as well. Notably, replacing NEMO’s UBAN and ZF with the C terminus of optineurin or ABIN-2 restored Ub binding and NF-κB activation in response to a variety of stimuli (16, 17).

Despite their high level of homology, optineurin was not found in the same TNF signaling complex as IKKβ and NEMO, and its
expression could not complement NEMO deficiency in a TNF-signaled pre-B cell line (17). To the contrary, several studies have directly implicated optineurin in negative regulation of NF-κB signaling. In one, overexpressed optineurin inhibited TNF-induced NF-κB activation by competing with NEMO for ubiquitinated RIP1 (18). In addition, optineurin recruited the deubiquitinating CYLD to the TNF signaling complex, where CYLD removed polyUb from RIP1 (19). It is notable that like NEMO, optineurin binds TBK1 (20). It has been reported that this interaction is constitutive and occurs via the optineurin N terminus (20, 21). However, another report has suggested that efficient binding of optineurin to TBK1 requires an intact UBAN (22). As with NF-κB, there is evidence that optineurin is a negative regulator of the IRF3 pathway because overexpressed optineurin inhibited, and silencing enhanced, IRF3 activation in virus-infected cells (22). In contrast, bone marrow–derived macrophages (BMDMs) from mice with a U-binding disruptive point mutation in the UBAN (Optn

expression) had diminished TLR-mediated activation of TBK1 and IFN-β secretion, suggesting that U binding by optineurin positively regulates the IRF3 pathway (21). At the same time, LPS-mediated activation of NF-κB and secretion of the proinflammatory cytokines IL-6 and IL-12 was unperturbed. Possible note is that there was a small amount of residual U-binding activity in Optn

expression) cells, and that the expression of the mutant protein was substantially higher than that of the wild type (WT).

Because of the existence of these contradictory data on the function of optineurin, we generated a mouse in which both the optineurin UBAN and ZF U-binding domains were deleted (Optn

expression)). As often happens upon introduction of a premature termination codon, the truncated protein was expressed at substantially lower levels than the WT, allowing us to study the effects of defective U binding, as well as the repercussions of optineurin insufficiency. This is particularly relevant because loss-of-function optineurin mutations due to C-terminal truncations and/or reduced protein levels have been associated with human neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and primary open-angle glaucoma (23–29).

Although understanding of the pathogenesis of both of these diseases is incomplete, it is clear that a nonautonomous neuronal death is a major contributor to disease, with immune cells such as microglia (resident macrophages of the CNS), peripheral cells of the monocyte/macrophage lineage, and T cells having a prominent part in lesion progression (30, 31). In this report, we have analyzed all of the major immune cell subsets and an in vivo response in a relevant model of optineurin insufficiency.

Materials and Methods

Mice

β-ACTin Cre transgenic mice were obtained from the Mouse Cancer Genetics Program in National Cancer Institute (NCI)-Frederick. C57BL/6 (B6) mice were obtained from the Frederick Cancer Research Facility (Frederick, MD). Mice harboring a conditional optineurin C-terminal deletion were generated by gene recombining (32) (http://frederick.cancer.gov). The 2.9-kB upstream and 3.9-kB downstream recombination arms surrounding exon 12 were subcloned from the BAC clone RP24-255E22 (BACPAC Resource Center) and placed in the thymidine kinase-containing targeting vector pLMJ235. To introduce the upstream loxP site, we placed a loxP-neoR-loxP cassette upstream of exon 12 and subsequently excised it by Cre induction in EL350 E cell lines. The FRT-loxP- targeted cassette was inserted downstream of exon 12. The vector was linearized with Nod digestion and electroporated into B6 × 129 hybrid mouse embryonic stem cells (C57BL/6 × 129/SvJae). The chimeric mice were screened by Southern blotting and PCR analysis. Southern blotting of HindIII-restricted WT DNA detected a 13-kB fragment, whereas introduction of an additional HindIII site by the FRT-loxP-FRT-loxP cassette led to the generation of a 10-kB fragment in chimeras. Whole-body deletion of the loxP-flanked segment was achieved by directly crossing mice that carried the recombinant allele with β-actin Cre. These mice are designated as Optn

expression). Optn

expression) mice were subsequently back-crossed onto the B6 background to generate F3 and F5 offspring, which were used in these studies. The homozygous Optn

expression) were always compared with WT mice of the same generation. All animal care and Use Committees were approved by the NCI Animal Care and Use Committee.

Reagents

Abs to the C terminus of optineurin were purchased from Cayman (#100000), to the central region (ab101592) from Abcam, and to the N terminus (C-2) from Santa Cruz. Abs to phospho-S473 Akt, p-ERK, phospho-S172 TBK1, TBK1, IRF3, and phospho-S396 IRF3 were from Cell Signaling, to IFN-β, and to IFN-β ELISA (7F-D5) from Abcam, and to IκBα from Santa Cruz. Secondary Abs conjugated with HRP were purchased from GE Healthcare, and Abs conjugated to IR670 or IR800 were purchased from Rockland. Nicotinamide membranes were from Bio-Rad. Protease and phosphatase inhibitor cocktails and SuperScript First-Strand Synthesis System were obtained from Roche, and enhanced chemiluminescence reagent from Pierce. 2-ME and Abs to β-actin and Flag, and LPS were from Sigma. Purified anti-mouse CD3 (145–2C11), anti-mouse CD28, anti-mouse CD4, and flow cytometry Abs to CD3e, CD4, CD8, CD25, CD44, CD42L, CD96, TCRβ2, B220, CD21/35, CD23, CD11b, NK1.1, CD19, CD11b, and 2.4G2 (Fc block) were from BD Pharmingen. Abs against CD8, CD11c, IFN-γ, and MHC class II, ELISA kits for IL-12p70, TNF, and IL-6, TMB solution, and FlowCytomix Simplex kits for the cytokines IL-12p70, TNF, IL-1β, IFN-γ, IL-6, and CXCL1/CXCL were purchased from eBioscience. Power SYBR green and luciferase/β-galactosidase reagents for reporter assays were obtained from Applied Biosystems. IFN-β, IFN-γ, and polyinosinic:polycytidylic acid (poly[I:C]) and CpGA were from InvivoGen. Fixable Dead Cell Stain, NuPAGESDS-PAGE, CFSE, and Lipofectamine 2000 were from Invitrogen/Life Technologies, and luciferase reagents were from Applied Biosystems. IFN, IFN-γ, and secondary rabbit polyclonal anti–IFN-β Abs for ELISA were purchased from R&D Systems, and rGM-CSF and rIL-12 were obtained from Prospec. GP33–41 (GP33) and NP396–404 H-2D b tetramers (NP396) were obtained from the National Institutes of Health Tetramer facility at Emory University (Atlanta, GA). GP33–41 peptide was purchased from Peptide 2.0.

Cell lines and plasmids

Human embryonic kidney 293 cells (293) were obtained from American Type Culture Collection and 293 cells expressing human TLR3 (hTLR3-293) from Invivogen. Cell lines were maintained in DMEM and primary cells in RPMI 1640, supplemented with 10% FCS, 5 mM glutamine, 5 μM 2-ME, and 100 μg/ml gentamicin. Human full-length optineurin (WT), the C-terminal truncation (1-467), and the previously described D474N mutation (18) were placed in N-terminal Flag-tagged mammalian expression vector Gateway pDEST26 (Invitrogen). The p-55D1BLuc construct that expresses luciferase under the control of repeated positive regulatory domain I recognized by IRF3 was kindly provided by Dr. Takashi Fujita (33). pcDNA3.1/HisLacZ containing the gene encoding β-galactosidase was obtained from Invitrogen, and pNF-κB luc encoding luciferase was from Clontech.

Cell transfection and luciferase reporter assays

Plasmids encoding luciferase under the control of the NF-κB or IRF3 promoters, β-galactosidase, and optineurin variants or control vector were cotransfected in 293 cells using Lipofectamine 2000, and were left unstimulated or stimulated with 20 ng/ml TNF or 10 μg/ml poly(I:C) 24 h later. Cells were lysed 5 h after stimulation, and luciferase activity was measured using a 96-well luminometer and Venus software and was normalized to β-galactosidase.

Cell preparation and purification

Mouse embryonic fibroblasts were prepared from embryonic day 11.5 (E11.5) and E13.5 embryos as described previously (34). BMDMs and bone marrow–derived DCs (BMDCs) were generated from bone marrow cultured like other primary cells but with 30% L929 supernatant for 5 d (BMDM) or rGM-CSF for 8 d (BMDC). BMDMs and BMDCs were stimulated with 100–500 ng/ml LPS or 10–20 ng/ml TNF. T and B cells were purified from lymph nodes or spleen, respectively, using the specific Easy Sep enrichment kit (StemCell Technologies) following the manufacturer’s protocol, and the number of live cells was assessed by trypan blue exclusion. Purity was determined by flow cytometry and for all experiments was >95%.

T and B cell proliferation assay

Proliferation assays were performed in 96-well flat-bottom plates in a final volume of 200 μl. For T cells, wells were coated with anti-CD3 and anti-CD28 overnight at 4°C in PBS. Cells were cultured for 48 h, pulsed with 1 μCi [3H]thymidine, and harvested 18 h later. [3H]thymidine uptake was determined with a Wallac 1450 MicroBeta Liquid Scintillation Counter.
All experimental points were performed in triplicate and the error bars represent the SEM. CFSE was used at a concentration of 500 nM, and cells were stained following the manufacturer’s instructions.

**Flow cytometry**

After Fc blockade, cells were stained with the indicated Abs and flow cytometry was performed with a BD LSRFortessa cytometer using BD FACSDiva software (BD Biosciences). The same cytometer was used to analyze Flowcytomix beads after cytokine capture. All flow cytometry data analysis was performed with FlowJo software (Tree Star).

** Immunoblotting**

Cells were normalized by number and lysed either in IP lysis Buffer (Pierce) or in sample buffer (50 mM Tris pH 6.8, 10% glycerol, 2% SDS, 2% 2-ME, and 0.04% bromophenol blue), resolved with NuPAGE SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with the indicated Abs. Samples were lysed in RIPA buffer (Sigma-Aldrich) and normalized to protein concentration to detect p-Akt and p-IRF3. Immunoblots were developed with either HRP-conjugated secondary Abs and enhanced chemiluminescence or fluorescently labeled secondary Abs, and blots were developed with either HRP-conjugated secondary Abs and enhanced chemiluminescence or fluorescently labeled secondary Abs and an Odyssey imaging system (Li-COR Biosciences). Densitometry was performed using ImageJ software.

**Immunodetection assays**

ELISA for IL-12, TNF, and IL-6 were done according to manufacturer’s instructions (eBioscience). IFN-β ELISA was performed as described previously (35). In brief, rat monoclonal anti-mouse IFN-β was used for plate coating, rabbit polyclonal anti-mouse IFN-β in solution, and HRP-conjugated goat anti-rabbit IgG was used for detection. Flowcytomix was used according to manufacturer’s protocol.

**PCR and quantitative RT-PCR**

The following primers were used to generate the Southern blot probe: forward, 5’-TATTCGATCATTCTCCTGGCCC-3’; reverse, 5’-ACAGATTTCTCTCCTCTGAGACGC-3’. The primers used to distinguish fixed and WT optineurin alleles were: forward, 5’-GTCACATGCTAGGCCAGAGTTTC-3’; reverse, 5’-GGCTCTAGGGATGCTAATGCT-3’. The Optn470T allele was detected with the following primers: forward, 5’-GCAACACAGACCC-TGAACACAGCG-3’; reverse, 5’-ACTCCACCATAAGTCATCAGAAAGC-3’. Real-time PCR was performed with SYBR green using a 7500 Real Time PCR System by Applied Bioscience. Housekeeping ribosomal 18S RNA was amplified to normalize RNA content of the lysate and to obtain a value for the difference in threshold cycles (ΔCT). The primers used were: Optn-forward, 5’-GCTCCGAATCTCAAGTGAGGAC-3’; reverse, 5’-GCAGAGTGCTAACTCCCGAGC-3’; Ifnb-forward, 5’-CTGGCTGCTCAGTTGGAAGA-3’; reverse, 5’-AGAGGCTGTGGGAGAGA-3’; 18S-forward, 5’-AAATCAGTTATGGCTCCTTGG-3’; reverse, 5’-GCTCTGAATTTACACGATCCCA-3’.

**In vivo studies**

LPS (3 mg/kg) was injected i.p. and blood was drawn 6 h later. For lymphocytic choriomeningitis virus (LCMV) infection, Armstrong strain 53b was grown in baby hamster kidney cells, and viral titers were determined as reported previously (36). WT and Optn470T mice were injected i.p. with 2 × 10⁶ PFU virus, and spleens were collected 8 d postinfection. For intracellular cytokine detection, 3 × 10⁶ splenocytes were stimulated with either medium alone or 0.3 μg/ml GP33–41 for 4 h in the presence of BD GolgiStop. Cells were stained with Abs to surface markers and fixed. The 2.4G2 Ab was added to the permeabilization buffer to block FcR binding before the addition of anti-cytokine Abs.

**Statistical analysis**

Statistical analysis was done using Student t test with GraphPad Prism software.

**Results**

**In vitro suppression of NF-κB and IRF3 by overexpressed optineurin requires the C-terminal region**

The importance of the C-terminal Ub-binding region of optineurin to NF-κB- and IRF3-dependent transcription was assessed in 293 cells transiently expressing WT or mutated human optineurin (Fig. 1). Stimulation with TNF highly induced NF-κB reporter activity, which was substantially inhibited by overexpression of full-length optineurin, but not the Ub-binding mutant optineurinD474N, as described previously (18). Importantly, the C-terminal deletion, Optn467T (corresponding to mouse Optn470T), was incapable of mitigating NF-κB activation (Fig. 1B). Stimulation with the TLR3 agonist poly I:C increased IRF3-reporter activity, which was reduced by cotransfection of full length but not Optn467T, as reported previously (22), or Optn467T (Fig. 1C). Thus, overexpression of optineurin inhibits both NF-κB and IRF3 pathways in a manner dependent on its Ub-binding domain.

---

**FIGURE 1.** WT but not Optn470T expression inhibits activation of NF-κB and IRF3. Human optineurin constructs, homologous to murine WT (1-584), the Ub-binding point-mutant D477N, and the C-terminal truncation (1-470) are depicted (A). 293 cells were transiently transfected with the indicated constructs and stimulated or not with TNF. Reporter luciferase activity was normalized to β-galactosidase activity (B, left panel), and anti-Flag blotting for Flag-optineurin variants is shown (B, right panel). hTLR3-293 cells, which stably express TLR3, were transiently transfected as indicated and stimulated or not with poly(I:C). Luciferase activity (C, left panel) and anti-Flag blotting (C, right panel) were performed as in (B). An average of duplicate experiments of one representative experiment is shown.
**Generation of Optn\(^{470T}\) knock-in mice**

Exon 12 was flanked with loxP recombination sites to generate a mouse with a conditional optineurin C-terminal deletion (Fig. 2A). Upon the predicted Cre-mediated deletion, a stop codon would be exposed immediately at the beginning of exon 13, resulting in translation of a truncated protein of 470 aa. Mice carrying the recombinant floxed allele were verified by Southern blot (Fig. 2B) and PCR (Fig. 2C), and were crossed to mice expressing Cre-recombinase under the control of the β-actin promoter to achieve exon 12 deletion. The excision of exon 12 was verified by PCR of tail DNA (Fig. 2D), and the lysates of BMDMs from homozygous WT and Optn\(^{470T}\) mice were blotted for optineurin. An Ab that binds the C terminus detected the WT 75-kDa protein, but no band was found in homozygous Optn\(^{470T}\) cells (Fig. 2E). Abs against central (Fig. 2F) and N-terminal regions of optineurin (Fig. 2G) showed the expected smaller band of ∼55 kDa in Optn\(^{470T}\) cells. Notably, the level of the truncated protein was ∼7-fold lower than that of the WT. Attempts to rescue protein expression with inhibitors of common proteases (aprotinin, leupeptin, pepstatin), caspases (zVAD), lysosomal proteases (chloroquine), and the proteasome (MG-132) had no effect, suggesting that the lower levels were not the result of proteolysis (M.L. Giardino Torchia, I. Munitic, and J.D. Ashwell, unpublished observations). In contrast, as often happens after introduction of a premature termination signal, Optn\(^{470T}\) mRNA expression was decreased (∼3-fold) in Optn\(^{470T}\) mice (Fig. 2H). The likely cause was a conserved eukaryotic quality-control mechanism known as nonsense-mediated mRNA decay (NMD), which is often initiated when a premature stop codon is detected (37, 38). In our case, the introduced stop signal was located ∼80 nt downstream of the exon 13/14 boundary, which is a greater distance than the minimum 50- to 55-nt separation from an exon–exon boundary that activates NMD. Thus, the substantial reduction of Optn\(^{470T}\) protein (Fig. 2F, 2G) could be largely accounted for by mRNA instability and translational repression associated with NMD (37).

**Homogeneous Optn\(^{470T}\) knock-in is embryonic lethal with incomplete penetrance**

Interbreeding of heterozygous (het) WT/Optn\(^{470T}\) F3 mice resulted in few homozygous Optn\(^{470T}\) offspring. Of the first >100 mice from such het × het breedings, instead of the expected 25% homozygous Optn\(^{470T}\) mice, only 3% were found (Table I). Similarly, only 1 of 20 embryos taken between E11.5 and E13.5 was homozygous Optn\(^{470T}\). Embryonic death occurred early, as the inspection of embryos on E11.5 found that ∼25% of the placental sacs had completely resorbed embryos, arguing that the critical period when optineurin is required for development occurs well before E11.5. The few live homozygous Optn\(^{470T}\) mice were physically indistinguishable from WT littermates and were fertile when intercrossed, indicating incomplete phenotypic penetration.

---

**FIGURE 2.** Generation of Optn\(^{470T}\) mice. (A) To generate mice with C-terminal truncation of optineurin (Optn\(^{470T}\)), a targeting construct with loxP sites flanking exon 12 was inserted into the endogenous locus by homologous recombination. Upon Cre-mediated deletion, a stop codon was exposed at the beginning of exon 13, resulting in a truncated protein of 470 aa. Neo cassette, Southern probe, and recombination sites are indicated. (B) Southern blot distinguishing WT and chimeric mice (WT/Fl) is shown. The introduced HindIII site in the Neo cassette leads to generation 10-kb band in the Fl allele. The PCR of the indicated mice distinguishing WT and floxed (C) and WT and deleted alleles is shown (D). Blotting BMDMs from the indicated mice with optineurin Abs raised against C-terminal (E), central (F), and N-terminal epitopes (G). Optineurin mRNA was detected in BMDMs by quantitative RT-PCR. ΔCt of Optn\(^{470T}\) was designated as 1, and the difference between ΔCt of WT and Optn\(^{470T}\) is depicted as mean ± SEM for three independent experiments (H).
The initial embryonic stem cells used to create the knock-ins were 129 × B6. Further breeding of het Optn470T mice onto the B6 background for the total of five backcrosses resulted in viable Optn470T offspring that were born at the expected Mendelian ratios (Table II). The mice studied in this report are from a colony generated from the survivor animals of independent het breeding couples. In conclusion, our results suggest that embryos of the 129 background are particularly prone to death when there are suboptimal levels of optineurin and/or optineurin lacks the C terminus.

The C terminus of optineurin is dispensable for immune cell development and T and B cell activation

Because many molecules in the NF-κB signaling pathway influence the development of cells of the immune system (39, 40), we analyzed the peripheral lymphoid tissues of homozygous Optn470T animals. Homozygous Optn470T F3 survivors and F5 mice were indistinguishable, so the data from all homozygous mice were pooled. Splenocyte numbers were comparable (data not shown), and all major innate and adaptive immune subsets were present in normal frequencies and numbers (Fig. 3A, 3B). Moreover, no signs of excessive activation were detected in the T cell compartment, as the number of regulatory CD4 T cells (Fig. 3C) and ratios of naive to memory cell subsets within both CD4 (Fig. 3C) and CD8 T cell (Fig. 3D) compartments were comparable between WT and Optn470T mice.

TCR-mediated signaling in Optn470T cells was analyzed. Anti-CD3/CD28 induced rapid degradation of IκBα and phosphorylation of ERK1/2 and Akt, with no obvious differences between WT and Optn470T T cells (Fig. 3E, 3F). Flow cytometric analysis of the activation markers CD25 and CD69 also found no difference between WT and Optn470T CD4 and CD8 T cells at rest or over the course of 2 d of in vitro activation (Fig. 3G and data not shown). T cell proliferation, analyzed by CFSE dilution and [3H]thymidine incorporation, was similar between WT and Optn470T CD4 and CD8 T cells (Fig. 3H, 3I). Optn470T mice were infected with the LCMV Armstrong strain to test in vivo responses. LCMV infection causes extensive CD8 T cell proliferation that is crucial in clearing the virus (41). Infection of Optn470T mice with LCMV Armstrong induced expansion of GP33+(Fig. 3J), NP396+, and GP276+ (data not shown) Ag-specific CD8 T cells, and IFN-γ production upon restimulation with peptide comparable with WT mice. Analysis of the viral load at day 8 showed clearance of LCMV by both WT and Optn470T mice (data not shown). Therefore, optineurin insufficiency has no apparent effect on T cell activation and function.

Dysregulation of NF-κB signaling is associated with impaired generation of B cells with a marginal zone (MZ) phenotype (42, 43). Notably, the percentage of CD21hiCD23lo splenic MZ B cells was indistinguishable between WT and Optn470T mice (Fig. 3K). Intact NF-κB signaling is also required for optimal B cell function (40). Engagement of TLR4 and CD40 induced IκBα degradation with comparable kinetics in WT and Optn470T B cells (Fig. 3L, 3M), and triggered proliferation to a similar extent (Fig. 3N, 3O). Therefore, the Optn470T mutation does not perturb B cell NF-κB signaling or activation.

Optineurin does not influence the NF-κB activation in innate immune cells

Experiments in cell lines have suggested a role for optineurin in the negative regulation of TNF-induced NF-κB (Fig. 1) (18, 19). To assess TNF-induced NF-κB activation in primary cells, we generated BMDMs from bone marrows cultured with L929 supernatants containing M-CSF. Of note, BMDM differentiation was unperturbed in Optn470T mice (data not shown). Comparable kinetics of IκBα degradation and resynthesis in TNF-treated WT and Optn470T cells were observed (Fig. 4A). Because optineurin is itself upregulated by various cytokines, including TNF and IFN-β (17), it is possible that it regulates later phases of NF-κB activation. To test this, we pretreated BMDMs with IFN-β for 16 h and then treated them with TNF (Fig. 4B). Both WT and Optn470T protein were upregulated by IFN-β. However, WT and Optn470T BMDMs showed similar patterns of TNF-induced IκBα degradation, arguing against a role for optineurin in late NF-κB activation. TNF treatment also activates MAPKs such as ERK1/2. The degree of ERK1/2 activation, as measured by phosphorylation of its activation loop, was indistinguishable between WT and Optn470T BMDMs (Fig. 4C). Analysis of IκBα degradation in response to the TLR4 ligand LPS also showed no difference between WT and Optn470T BMDMs (Fig. 4D). Similar results were obtained in another innate immune cell type, BMDCs (data not shown). Thus, no obvious function of optineurin was found in innate immune cells activated via TNFR or TLR4.

LPS induces a large number of signaling events, and it is possible that pathways that were not examined are regulated by optineurin. To examine more global readouts of activation with LPS, we

### Table I. Optn470T mice on the 129 × B6 genetic background die embryonically

<table>
<thead>
<tr>
<th></th>
<th>WT/WT/470T × WT/WT/470T</th>
<th>WT/WT/470T × WT/WT/470T</th>
<th>WT/WT/470T × WT/WT/470T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected frequency</td>
<td>25%</td>
<td>50%</td>
<td>25%</td>
</tr>
<tr>
<td>Adult mice (n)</td>
<td>48</td>
<td>83</td>
<td>4</td>
</tr>
<tr>
<td>Observed frequency of adult mice</td>
<td>35%</td>
<td>61%</td>
<td>3%</td>
</tr>
<tr>
<td>Developed embryos (n)</td>
<td>5</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Observed frequency of developed embryos</td>
<td>25%</td>
<td>70%</td>
<td>5%</td>
</tr>
</tbody>
</table>

Het OptnWT/WT/470T F3 mice were intercrossed and the genotype of the offspring was analyzed by PCR from tail DNA. The expected and observed frequencies from 135 adult mice and 20 embryos are shown.

### Table II. Optn470T mice further backcrossed to B6 background were born at the expected Mendelian ratios

<table>
<thead>
<tr>
<th></th>
<th>WT/WT/470T × WT/WT/470T</th>
<th>WT/WT/470T × WT/WT/470T</th>
<th>WT/WT/470T × WT/WT/470T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected frequency</td>
<td>25%</td>
<td>50%</td>
<td>25%</td>
</tr>
<tr>
<td>Adult mice (n)</td>
<td>14</td>
<td>38</td>
<td>15</td>
</tr>
<tr>
<td>Observed frequency of adult mice</td>
<td>21%</td>
<td>57%</td>
<td>22%</td>
</tr>
</tbody>
</table>

Het OptnWT/WT/470T mice backcrossed onto the B6 background for five generations (F5) were intercrossed and the genotype of the offspring was analyzed by PCR from tail DNA. The expected and observed frequencies from 67 adult mice are shown.
FIGURE 3. T and B cell development and activation are unimpaired in Optn$^{470T}$ mice. T and B (A), conventional DC (cDC), macrophage, and NK cell numbers (B) in spleens of the indicated mice are shown. CD4 (C) and CD8 T cell (D) numbers and their respective compartments are shown. Naive cells were gated as CD44loCD62Lhi, TCM as CD44hiCD62Lhi, and TEM as CD44hiCD62Llo. The data from eight mice are shown with mean ± SEM. T cells freshly purified or stimulated with anti-CD3 (3 μg/ml) and anti-CD28 (2 μg/ml) were lysed at the indicated times, and IκBα degradation and ERK1/2 phosphorylation (E) and Akt phosphorylation (F) were detected by immunoblotting. Expression of the indicated activation markers was monitored on WT and Optn$^{470T}$ purified T cells at the indicated times after stimulation with plate-bound anti-CD3 (3 μg/ml) and anti-CD28 (2 μg/ml) (G). CFSE-labeled T cells were stimulated as in (C), and CFSE dilution was monitored after 60 h (H). Purified T cells were cultured with 2 μg/ml anti-CD28 and the indicated concentrations of anti-CD3 for 48 h, pulsed with [3H]thymidine, and harvested 18 h later (I). WT or Optn$^{470T}$ mice were infected with LCMV Armstrong and the absolute numbers of GP33-tetramer+ CD8 T cells analyzed on the peak of the response (day 8; J, left panel). Splenocytes were restimulated in vitro with GP33 peptide and evaluated for cytokine production by intracellular staining. Absolute numbers of IFN-γ–producing CD8 T cells are shown (J, right panel). Distribution of CD21hiCD23int MZ B cells (gated in B220+TCR-β− population) in splenocytes of WT and Optn$^{470T}$ mice was determined by flow cytometry. Numbers represent the percentage of cells in the gated region, and the bar graph below represents the average of three mice per group ± SEM (K). B cells freshly purified or stimulated with LPS (100 ng/ml) (L) or anti-CD40 (1 μg/ml) (M) were lysed at the indicated times and IκBα was detected by immunoblotting. Purified B cells were cultured with the indicated concentration of LPS (N) or anti-CD40 (O) for 48 h, pulsed with [3H]thymidine, and harvested 18 h later. Each panel is representative of two or three independent experiments.
measured cytokine secretion by BMDMs and BMDCs. No differences were found in LPS-induced TNF and IL-6 production by BMDMs, or TNF and IL-12 production by BMDCs (Fig. 4E, 4F). A broader set of cytokines and chemokines was analyzed upon LPS injection in vivo, a model for septic shock. Notably, CXCL1, IL-6, IFN-γ, TNF, IL-12p70, and IL-1β all had similar levels in the sera of WT and Optn470T mice (Fig. 4G). Together, these results strongly argue that the Ub-binding domain of optineurin is not required for TNF- or LPS-mediated activation of cells of the innate immune system.

**Optineurin positively regulates TBK1 and IRF3**

BMDMs from Optn D474 mice had impaired TBK1 and IRF3 activation and reduced IFN-β secretion in response to TLR3/4 stimulation (21). To determine the effect of deletion of the entire C terminus, as well as very low optineurin expression, we treated BMDMs with LPS and assessed TBK1 activation. Phosphorylated TBK1 is active and required for phosphorylation of IRF3. Whereas stimulation of WT BMDM with LPS induced strong TBK1 phosphorylation that peaked at 30 min, Optn470T BMDM showed much weaker phosphorylation at all time points (Fig. 5A). The subsequent phosphorylation of IRF3 peaked 1–2 h after stimulation in WT BMDMs (Fig. 5B). Although the time course of activation was similar in WT and Optn470T BMDMs, phosphorylation of IRF3 in the latter was diminished by 30–40% at the peak of activation (Fig. 5B, right panel). Consequently, upregulation of mRNA for IFN-β (Fig. 5C) and IFN-β secretion was lower in Optn470T than in WT BMDMs (Fig. 5D). Similar results were found in BMDCs, for which LPS stimulation resulted in lower IFN-β secretion in Optn470T cells (Fig. 5E). WT and Optn470T mice were injected with LPS, and IFN-β levels were measured 6 h later to assess the biological relevance of these findings. Substantially lower levels of IFN-β were detected in the sera of Optn470T mice (Fig. 5F). Until recently, the TIR domain–containing adapter inducing IFN-β (TRIF) was the sole adapter thought to convey TLR3/4-mediated signals that lead to TBK1-mediated type I IFN secretion. However, it was recently shown that BMDM MyD88-coupled TLRs also activate the TBK1 pathway (44). To determine whether optineurin is capable of mediating TBK1 activation without TRIF, we tested assessed IFN-β responses upon BMDM stimulation with the TLR9 ligand CpGA, a potent agent that mimics viral and bacterial (unmethylated) DNA and whose signaling requires MyD88 but is TRIF independent. CpGA stimulation of Optn470T BMDM resulted in substantially lower levels of IFN-β mRNA and protein than in WT cells (Fig. 5G, 5H), providing evidence that optineurin is required for MyD88-mediated TBK1 activation. Together, these results establish optineurin as a positive regulator of IRF3 and type I IFN production.

**Discussion**

Controlled activation of NF-κB is vital to the health of the organism. Whereas suboptimal NF-κB activation can lead to immunodeficiencies, excessive activation is linked to autoimmunity, neurodegeneration, and cancer (45–47). Based upon experiments performed in
cell lines, optineurin has been proposed to be a negative regulator of TNF-induced NF-κB activation by preventing the binding of NEMO and recruitment of CYLD to ubiquitinated RIP1 (18, 19). Another clue that optineurin inhibits excessive NF-κB activation came from studies of two human neurodegenerative diseases, ALS and primary open-angle glaucoma (23, 24), diseases in which microglia-derived TNF is thought to potentiate the activation of glutamate receptors and excitotoxic neuronal death (48). Many of the optineurin mutations associated with neurodegeneration have either large C-terminal deletions or point mutations in the Ub-binding region, suggesting that the lack of Ub-binding is a major pathogenic mechanism. Fittingly, mutations found in ALS patients were incapable of inhibiting TNF-induced NF-κB activation in the NSC-34 neuroblastoma/spinal-cord cell line (24). Our study reproduced some of these findings showing that an intact optineurin Ub-binding domain confers the ability to suppress NF-κB activation in vitro. However, the results obtained with primary immune cells from Optn^{470T} mice, similar to previously published Optn^{D477N} BMDMs, suggest that the in vitro data were an overexpression artifact rather than the demonstration that optineurin is a negative regulator of NF-κB. Off-target activity is not uncommon for Ub-binding proteins (49). Even though NEMO is an essential positive regulator of the NF-κB, its overexpression leads to reduced NF-κB activation because of off-target effects (49).

TNF-mediated NF-κB activation was not assessed in the recently reported Optn^{D477N} mice, although NF-κB activation by TLR ligands resulted in normal IκB degradation and IL-6 and IL-12 secretion in BMDMs (21). Because this particular mutation has not been described in human disease, the possibility remains that it does not completely reproduce findings in patients and/or that the C-terminal region harbors other sites relevant for optineurin function. Moreover, the previously reported Optn^{D477N} mice had levels of optineurin that were higher than in WT mice, whereas many of the optineurin C-terminal truncations found in human disease result in protein insufficiency (24, 25, 27, 28, 50, 51). In this study, we have addressed the role of optineurin in TNFR signaling and other stimulation settings in a genetic mouse model in which WT optineurin was replaced by a mutant lacking both Ub-binding domains (UBAN and ZF) and the linker region between them that harbors a putative binding site for the CYLD deubiquitinase (16). Of note, Optn^{470T} was also expressed at a substantially lower level than WT, allowing us to address the effect of optineurin insufficiency. Although the development of the major immune cell subsets depends on NF-κB (39, 40), no evidence of impaired cellularity or autoimmunity was found in these mice. Moreover, activation of T cells via the TCR, macrophages and DCs via TNFR or TLR4, and B cells via TLR4 or CD40 showed no signs of impaired NF-κB activation, strongly arguing against the role of optineurin in these pathways. Whereas it is
formally possible that low levels of residual OptnT70T, even if devoid of Ub binding, are sufficient for regulating NF-κB, we think this is unlikely because overexpressed OptnT70T was incapable of mitigating NF-κB activation. An alternative possibility is that although expressed in almost all cell types tested, optineurin has cell-specific functions that are perhaps conspicuous only in specific neurons or glial cells. A recent report demonstrated a slight upregulation of TNF-mediated NF-κB activation upon optineurin silencing in a neuroblastoma cell line that resulted in increased cell death (52). It is notable that the phenotype in that cell line was not very prominent, and that further neurologic analysis of the aged OptnT70T mice will allow us to address those issues in primary neuronal cells and in vivo.

A prominent finding in OptnT70T mice with a B6 × 129 background was early embryonic lethality. It is of note that mice with impaired NF-κB activation due to deficiency of either NEMO, IKKβ, or p65 die at a similar or later stage of embryonic development as the OptnT70T mice (53–56). Because crossing all of these strains to either TNF- or TNFR1-deficient animals led to rescue from embryonic lethality, it is thought that the death occurs due to hypersensitivity to TNF caused by inadequate NF-κB activation. Curiously, although TBK1 is not directly implicated in IKK activation and TNF responses in primary cells, embryonic lethality of TBK1-deficient animals was also rescued by disruption of TNFR1 expression (57). We found that simply backcrossing of the OptnT70T mice further onto the B6 background led to normal litters, with IFNAR-deficient cells being almost incapable of expansion (62). However, in vivo CD8 T cell expansion upon LCMV infection was diminished but not absent suggests that either factors other than optineurin contribute to TBK1 activity or that the remaining response depends on the activity of IKKe (63), an inducible TBK1-related kinase expressed in myeloid cells that, unlike TBK1, does not interact with optineurin (17). NF-κB and IRF3 activation were focused on because these pathways have been reported to be regulated by optineurin in a variety of in vitro and in vivo studies. However, it is possible that optineurin has important roles in other cellular events. Indeed, the fact that the OptnT70T mice underwent embryonic death tells us that this protein has vital roles that appear, in this instance, to be strain dependent. Whether this is due to speculated roles in Golgi integrity (64), vesicle trafficking (65, 66), autophagy (67, 68), or cell division (69), remains to be determined.

Acknowledgments

We are grateful to Lino Tesslerollo and Eileen Southon (Mice Cancer Genetics Program, NCI-Frederick, Frederick, MD) for assistance during generation of OptnT70T mice; Mirela Kuka and Paul Mittelstadt for helpful discussions; Bei Dong, Ehydel Castro, Steven Leung, and Neda Savidic for technical assistance; Dr. Takashi Fujita for allowing the use of p-S5-DI-BLuc construct; Dr. Patricia Johnson for the IFN-β ELISA protocol; Dr. Rafi Ahmed for LCMV Armstrong; and the National Institutes of Health Tetrramer facility at Emory University for supply of tetramers.

Disclosures

The authors have no financial interests of conflict.

References

OPTINEURIN IS DISPENSABLE FOR NF-κB BUT NOT IRF3 ACTIVATION


