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Report

Intracellular Trafficking of Interleukin-1 Receptor I Requires Tollip

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Summary

Interleukin-1 receptor (IL-1RI) is a master regulator of inflammation and innate immunity [1]. When triggered by IL-1β, IL-1RI aggregates with IL-1R-associated protein (IL-1RAcP) and forms a membrane proximal signalosome that potently activates downstream signaling cascades. IL-1β also rapidly triggers endocytosis of IL-1RI [2, 3]. Although internalization of IL-1RI significantly impacts signaling [4], very little is known about trafficking of IL-1RI and therefore about precisely how endocytosis modulates the overall cellular response to IL-1β. Upon internalization, activated receptors are often sorted through endosomes and delivered to lysosomes for degradation. This is a highly regulated process that requires ubiquitination of cargo proteins as well as protein-sorting complexes that specifically recognize ubiquitinated cargo [5]. Here, we show that IL-1β induces ubiquitination of IL-1RI and that via these attached ubiquitin groups, IL-1RI interacts with the ubiquitin-binding protein Tollip [6]. By using an assay to follow trafficking of IL-1RI from the cell surface to late endosomes and lysosomes, we demonstrate that Tollip is required for sorting of IL-1RI at late endosomes. In Tollip-deficient cells and cells expressing only mutated Tollip (incapable of binding IL-1RI and ubiquitin), IL-1RI accumulates on late endosomes and is not efficiently degraded. Furthermore, we show that IL-1RI interacts with Tom1, an ubiquitin-, clathrin-, and Tollip-binding protein [7, 8], and that Tom1 knockdown also results in the accumulation of IL-1RI at late endosomes. Our findings suggest that Tollip functions as an endosomal adaptor linking IL-1RI, via Tom1, to the endosomal degradation machinery.

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Results and Discussion

IL-1RI Is Ubiquitinated

IL-1RI is rapidly internalized, in an IL-1β-dependent manner [4, 9], and slowly degraded [3, 10] (see also Figure S1 in the Supplemental Data available online), suggesting that it, like other receptors delivered to the lysosomes for degradation, may be ubiquitinated. This was addressed by immunoprecipitating IL-1RI from a cell line stably expressing Flag-tagged IL-1RI (293-Flag IL-1RI) before and after IL-1β stimulation with conditions that exclude association of IL-1R-interacting proteins (Figure 1). IL-1β treatment rapidly induced IL-1RI ubiquitination, which remained so, albeit with diminishing intensity, over the duration of the time course (4 hr) (Figure 1). The ladder-like pattern of ubiquitin species conjugated to IL-1R were immunoreactive with a ubiquitin antibody (P4D1) that recognizes monoubiquitin as efficiently as polyubiquitin but not with a second antibody (FK1) that is specific for polyubiquitin [11] (Figures S2A and S2B). This suggests that IL-1RI may be conjugated by multiple monoubiquitin moieties, as is the case for EGFR [11].

Tollip Is An Ub-Receptor for IL-1Rs

Ub-receptors are components of trafficking complexes that have one or more ubiquitin-binding domains (UBD) [12]. Tollip has a C-terminal UBD called the CUE (coupling of ubiquitin to endoplasmic reticulum degradation) domain [6, 13–15] that is crucial for its interaction with IL-1Rs [14]. To determine whether this is because Tollip acts as an Ub-receptor for ubiquitinated IL-1RI, we transfected 293T cells with VSV-Tollip and Flag-IL-1Rs (inducing both the expression of ubiquitinated and nonubiquitinated forms) and then immunoprecipitated VSV-Tollip. The VSV-Tollip immunoprecipitate was probed with Flag antibodies, revealing that Tollip only interacts with a higher-molecular-weight form of the Flag-tagged receptor (Figure 2A, left panel). That this was an ubiquitinated form of Flag-IL-1RI was confirmed in a second experiment in which it was dissociated from Tollip (by boiling in SDS), immunoprecipitated with Flag antibodies, and subsequently immunoblotted with anti-ubiquitin or Flag antibodies as indicated (Figure 2A, right panels). Tollip's preference for ubiquitinated IL-1RI correspondingly implied that Tollip's capacity to bind ubiquitin was required for its interaction with IL-1RI. To confirm this, we generated a form of Tollip that was mutated at the MFP motif (residues 240–242 in the CUE domain), a high-affinity ubiquitin-binding site common to several different CUE domains

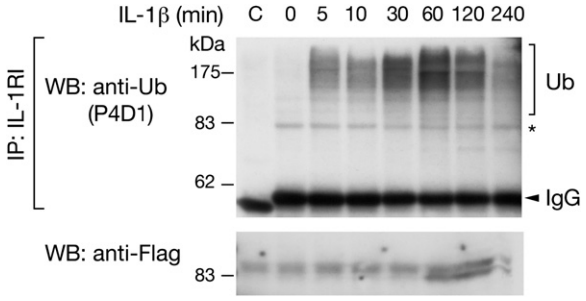

Figure 1. IL-1β Induces Ubiquitination of IL-1Rs

IL-1β rapidly induces ubiquitination of IL-1RI. 293-Flag-IL-1RI cells were stimulated with IL-1β (20 ng/ml) for the indicated times and then lysed in RIPA buffer. The Flag immunoprecipitates were probed with antibodies against ubiquitin (Ub), P4D1, or Flag. The asterisk denotes a band that is probably nonspecific in nature. As a control for the stringency of immunoprecipitation, recruitment of Tollip, IRAK, and MyD88 to the IL-1RI was examined and not detected.

[6]. This mutation, subsequently referred to as Tollip*MF/AA*, rendered Tollip incapable of binding to endogenously ubiquitinated proteins (Figure S3A). As expected, coimmunoprecipitation of Tollip*MF/AA* and IL-1Rs was severely attenuated (Figures 2A and 2B); however, interestingly, coimmunoprecipitation of Tollip*MF/AA* and IRAK (a protein that is also ubiquitinated after IL-1β stimulation and interacts with Tollip's CUE domain) was not affected (Figure 2C). In summary, these results indicate that Tollip has the capacity to act as an Ub-receptor for ubiquitinated IL-1RI.

Tollip Is Ubiquitinated via Its CUE Domain

Several Ub-receptors are themselves ubiquitinated, a predictable consequence of their ability to bind directly to the activated intermediate of the ubiquitination process [16]. It was thus not surprising to detect minor forms (approximately 15 kDa higher) of Tollip but not of Tollip*MF/AA* (See Figure 2A). His-tagged Tollip was immunoprecipitated under denaturing conditions (6 M guanidine-HCL), used to exclude noncovalent association of ubiquitin, and analyzed by 2D gel electrophoresis revealing that Tollip (at least when overexpresssed) was modified, in a CUE-domain-dependent manner, by conjugation of multiple ubiquitin moietes (Figures S4A and S4B). The functional role of Tollip ubiquitination is presently unknown. However, it is tempting to speculate, based on a recent report [17], that ubiquitination of Tollip induces intramolecular interaction with its CUE domain and thereby triggers release of Tollip from IL-1R. Because ubiquitinated Tollip is enriched by immunoprecipitation of IL-1R complex (see Figure 2A), it may also promote the assembly of ubiquitin-linked protein networks important for sorting of IL-1RI [18].

Tollip Is Necessary for Sorting of IL-1RI at Late Endosomes

IL-1RI is found at very low levels on the cell surface of primary cell types precluding its detection. Therefore, to follow IL-1RI trafficking and analyze Tollip's role in this process, we developed an assay by using wild-type and Tollip-deficient mouse embryonic fibroblasts (MEFs) [19] transfected with IL-1RI and IL-1RAcP. Transfected IL-1RI localized to the plasma membrane in both cell lines as revealed by immunostaining with IL-1RI specific antibodies. Further endocytosis of cell-surface IL-1RI was dependent on the addition of IL-1β and thereby mimicked signal-dependent internalization of endogenous receptors [2, 3] (Figure S5). Internalization of IL-1RI occurred rapidly in both wild-type and Tollip-deficient MEFs, indicating that Tollip is not crucial for internalization per se (Figure 3A). To analyze whether Tollip is subsequently required for trafficking of IL-1RI, we monitored the localization of internalized IL-1RI after different intervals of IL-1β stimulation by costaining it with either early endosome antigen 1 (EEA1) or

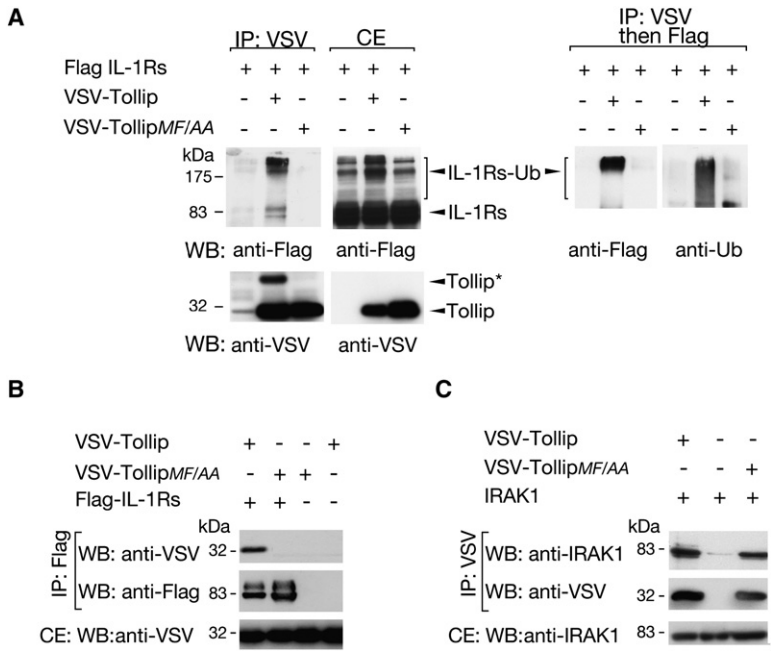

Figure 2. The Tollip-IL-1Rs Interaction Requires Ubiquitin

(A) Tollip binds the ubiquitinated form of IL-1Rs. 293T cells were cotransfected with Flag-IL-1Rs (IL-1RI + IL-1RAcP) and VSV-Tollip or VSV-TollipMF240/241 (Tollip*MF/AA*). Tollip/IL-1Rs complexes were immunoprecipitated with VSV antibody and either analyzed directly by western blotting with anti-Flag and anti-VSV (left panel) or dissociated by boiling in SDS and then subjected to a second immunoprecipitation with Flag antibody (right panel). The Flag immunoprecipitate was then analyzed by western blotting with anti-Ub and anti-Flag as indicated. The asterisk (*) indicates ubiquitinated Tollip. CE denotes cellular extract.

(B and C) Tollip*MF/AA* binds IRAK but not IL-1Rs. 293T cells were cotransfected with Flag IL-1Rs, IRAK-1 D340N and VSV-Tollip, or VSV-TollipMF240/241 (Tollip*MF/AA*). The Flag (B) or VSV (C) immunoprecipitates were revealed with antibodies against IRAK-1, Flag, or VSV as indicated.

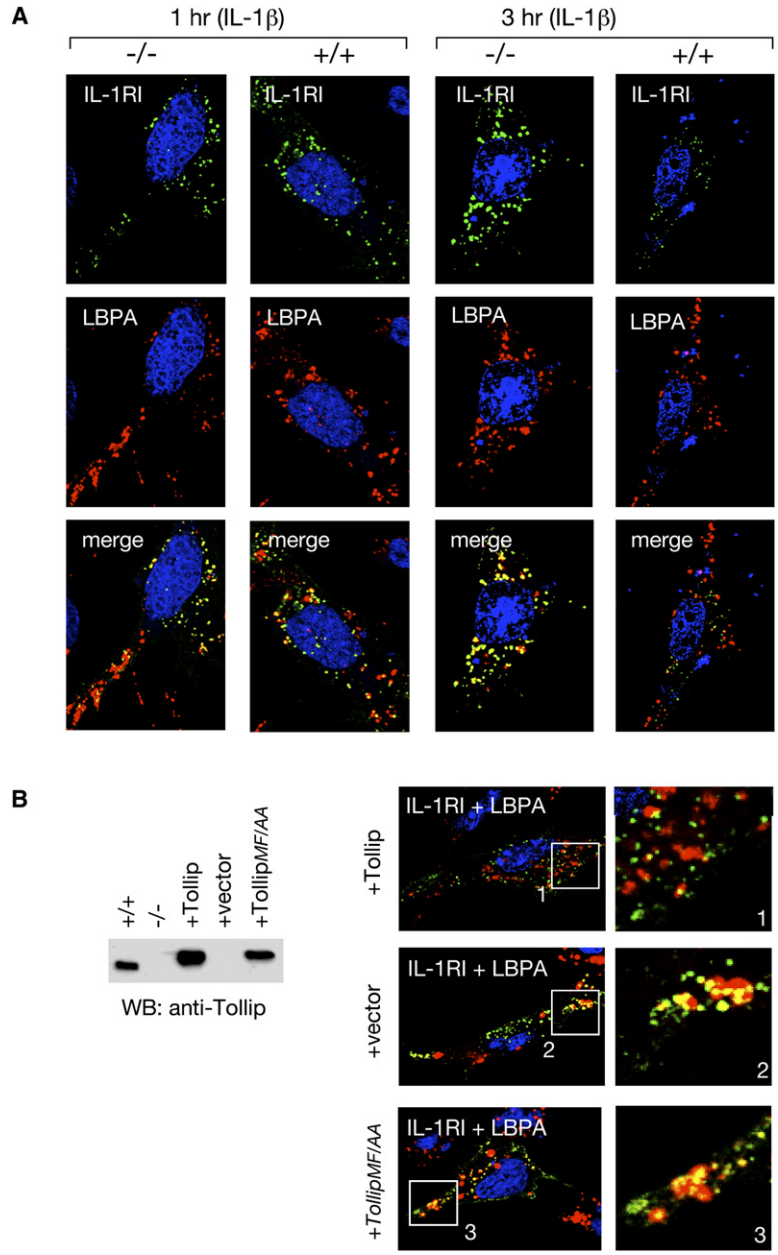

Figure 3. Sorting of IL-1RI at Late Endosomes Requires Tollip's CUE Domain

(A) IL-1RI accumulates on late endosomes in the absence of Tollip. −/− (Tollip-deficient) or +/+ (wild-type) MEFs were transfected with IL-1RI and IL-1RAcP then 24 hr later were incubated with anti-IL-1RI (6B5) and IL-1β (50 ng/ml) for 1 or 3 hr at 37°C. Cells were then permeabilized, incubated with secondary antibody against IL-1RI, and immunostained for lysobisphosphatidic acid (LBPA). Central sections imaged by confocal microscopy are shown. IL-1RI is shown in green; LBPA is shown in red; and Draq 5 nuclear staining is shown in blue.

(B) Tollip's CUE domain mediates sorting of IL-1RI at late endosomes. The left panel shows cellular extracts that were prepared from the following MEF cell lines: +/+ (wild-type), −/− (Tollip deficient), +Tollip, +vector, or +Tollip*MF/AA* (Tollip-deficient MEFs stably reconstituted with the indicated vectors). Equal amounts of protein were analyzed by immunoblotting with Tollip antibodies. The right panel shows trafficking of IL-1RI was followed after 3 hr, as in (A) above. IL-1RI is shown in green; Draq 5 nuclear staining is shown in blue; and LBPA is shown in red. Colocalization of IL-1RI and LBPA is shown in the enlarged images.

lysobisphosphatidic acid (LBPA), markers specific for early and late endosomes, respectively (data not shown and Figure 3A). Shortly after IL-1β stimulation (i.e., 30–60 min), the distribution of internalized IL-1RI was similar in both wild-type and Tollip-deficient cells. Trafficking of IL-1RI from early to late endosomes was also detected in both cell types. However, after IL-1β stimulation for longer periods (i.e., 3–6 hr) striking differences in the levels of the IL-1RI were increasingly apparent in −/− and +/+ cells. In wild-type cells, IL-1RI became progressively more difficult to detect, whereas in Tollip-deficient cells, significant levels of IL-1RI remained, the majority of which colocalized with LBPA (Figure 3A). IL-1RI was therefore accumulating at late endosomes in Tollip-deficient MEFs. This accumulation of IL-1RI could be reversed by stable reconstitution of Tollip-deficient cells with Tollip (+Tollip MEFs) but not with empty vector (+vector MEFs) or Tollip*MF/AA* mutant (+Tollip*MF/AA* MEFs), indicating that Tollip and specifically its capacity to act as an Ub-receptor for IL-1RI was required for efficient sorting of IL-1RI at late endosomes (Figure 3B).

A Pool of Tollip Localizes to LBPA- and LAMP1-Positive Vesicles

Consistent with a role in sorting IL-1RI at late endosomes, a significant pool of endogenous Tollip colocalized with LBPA- and LAMP1- (late endosome and lysosome marker) positive vesicles (see Figures S6A–S6G and S6Q–S6S). Because a small pool of Tollip was also found on early endosomes (Figures S6H–S6M) and a significant fraction was found in the cytosol (supported by cellular fractionation), it is likely that the localization of Tollip to late endosomes is a dynamic and highly regulated process, controlled, at least in part, through

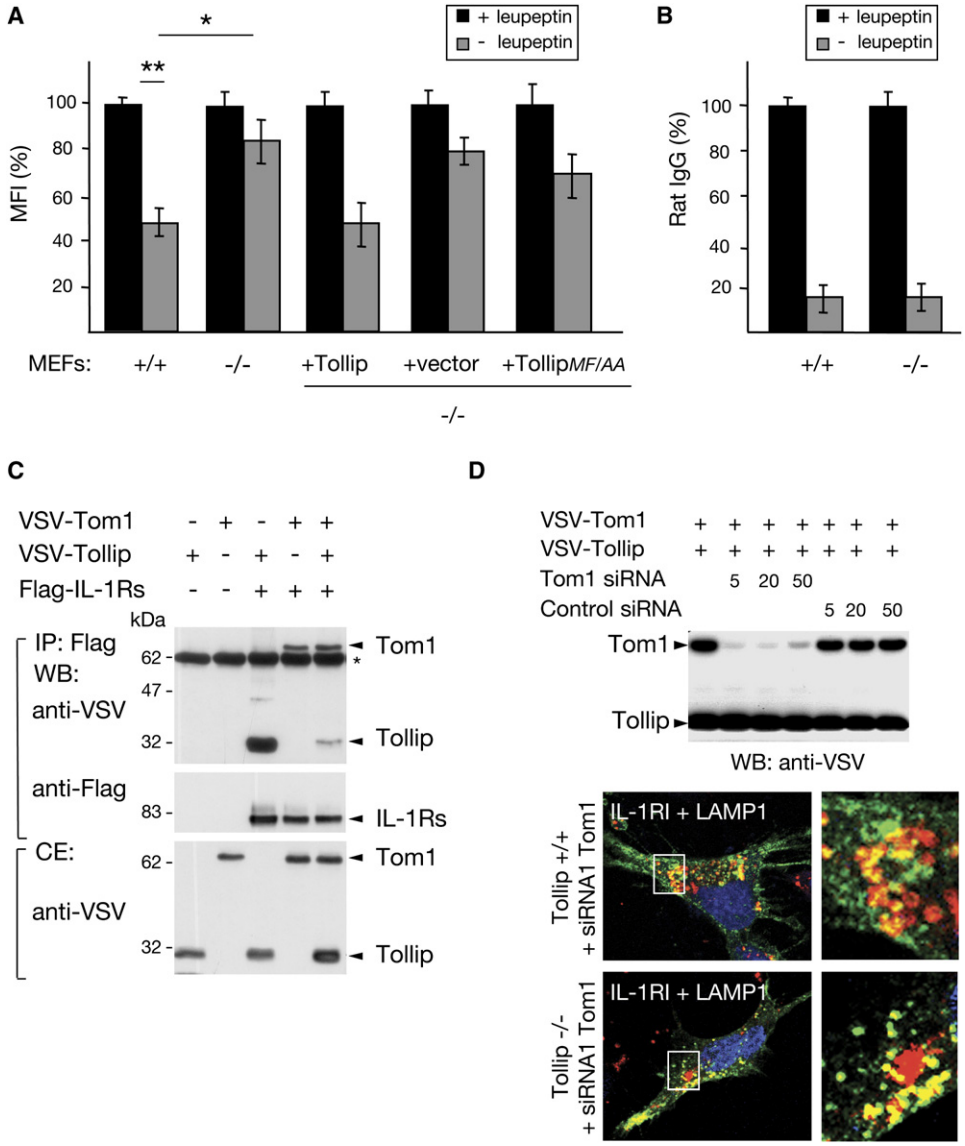

Figure 4. Tollip Facilitates Lysosomal Targeting of IL-1RI

(A) Degradation of IL-1RI is attenuated in the absence of Tollip. The indicated cell lines were transfected with IL-1RI and IL-1RAcP then 24 hr later were incubated with IL-1RI (6B5) and IL-1β (50 ng/ml) for 6 hr at 37°C in the presence or absence of leupeptin. Internalized IL-1RI was revealed as described in Figure 3A. The mean fluorescent intensity (MFI) of IL-1RI-positive cells was calculated on >350 cells per condition in two separate experiments. The data are represented as a percentage of the MFI in leupeptin-treated cells (100%) (+SE) for facilitation of direct comparison. The asterisk (*) indicates a significant difference between +/+ and −/− cells, in the absence of leupeptin (*$p < 0.005$). A double asterisk (**) signifies a significant difference in MFI between wild-type cells with or without leupeptin (**$p < 0.0001$), indicating efficient IL-1RI degradation; however, this is not so in the case of −/− cells ($p = 0.2$).

(B) +/+ and −/− cells were incubated overnight with 40 μg/ml rat IgGs in the presence or absence of 50 μg/ml leupeptin. Endocytosed IgGs were revealed with rat antibodies, and cells were analyzed by confocal microscopy. The total number of labeled vesicles (containing IgGs) was counted for each condition in 15 cells in three different experiments. Numbers were similar whether Tollip was present and are expressed as a percentage of the total number of vesicles in leupeptin-treated cells (+SE) for facilitation of direct comparison.

(C) Recruitment of Tom1 to IL-1R abolishes Tollip interaction. 293T cells were cotransfected with Flag-IL-1Rs and VSV-Tom1 in the presence or absence of VSV-Tollip. The Flag immunoprecipitates were revealed with antibodies against Flag or VSV as indicated. The asterisk (*) denotes the IgG heavy chain.

(D) Tom1 knockdown results in accumulation of IL-1RI in LAMP1-positive vesicles. VSV-Tom1 (mouse) and VSV-Tollip were transfected in the presence of increasing concentration of Tom1 siRNA or control siRNA. Lysates were prepared 24 hr later, and western-blot analysis was carried out with a VSV antibody (upper panel). +/+ or −/− MEFs were transfected with Tom1 siRNA (50 nM) and after 24 hr, with IL-1RI and IL-1RAcP. The cells were incubated with IL-1RI (6B5) antibody 24 hr later and with 50 ng/ml IL-1β for 6 hr at 37°C. Cells were then permeabilized, incubated with secondary antibody against the IL-1RI, and immunostained for LAMP1. Central sections imaged by confocal microscopy are shown. IL-1RI is shown in green; LAMP1 is shown in red; and Draq 5 nuclear staining is shown in blue.

Tollip's internal C2 domain (a region that binds phosphatidylinositol-3-phosphate and phosphatidylinositol-3,4,5-phosphate, phospholipids abundant on endocytic vesicles) [20].

Tollip Is Required for Efficient Degradation of IL-1RI

Taken together, the above results suggested that Tollip might be required for sorting of IL-1RI to lysosomes and thereby facilitation of IL-1RI degradation. However, because of the low levels of IL-1RI (even in transfected MEFs), it was not possible to obtain direct evidence that this is the case by western-blot analysis. Therefore, the content of internalized and immunolabeled IL-1RI in transfected MEFs was measured by quantitative image analysis. Cells treated with leupeptin to block degradation served to calibrate total receptor content (Figure 4A). Strikingly, 6 hr after IL-1β stimulation, the IL-1RI signal was significantly diminished in +/+ or +Tollip MEFs but remained virtually unchanged in −/− as well as in +vector and + Tollip*MF/AA* MEFs (Figure 4A). The observed differences in the levels of immunolabeled IL-1RI were not the result of defective immunoglobulin degradation, which occurred with similar efficiency in −/− and +/+ cells (Figure 4B). Tollip deficiency also had no effect on transport of fluorescent dextran, a soluble marker of bulk transport, to LBPA- or LAMP1-positive vesicles (Figure S7A). Moreover, EGFR (an ubiquitinated receptor sorted to lysosomes) was degraded after EGF treatment with similar kinetics in −/− and +/+ MEFs (Figures S7B and S7C). Therefore, Tollip and specifically its CUE domain were required for efficient degradation of IL-1RI. Further, Tollip is not a general Ub-receptor but has specificity for endocytic cargo, which includes IL-1RI and conceivably other ubiquitinated TIR-family receptors (i.e., TLR2, 4) with which it interacts [13, 21]. Interestingly, Tollip interacts, via its N terminus, with Tom1 and the related proteins, Tom1L1 and L2, which are ubiquitin- and clathrin-binding proteins likely to be involved in the delivery of both biosynthetic and endosomal cargo to lysosomes [8, 16, 22]. Recruitment of Tom1 to the IL-1Rs complex was tested by coimmunoprecipitation revealing that Tom1 also binds IL-1Rs and therefore may have a role in IL-1RI degradation (Figure 4C). For confirmation of this, the cellular levels of Tom1 were knocked down with siRNA against Tom1 (Figure 4D, upper panel) and the distribution of internalized IL-1RI was monitored after different intervals of IL-1β stimulation. In both wild-type and Tollip-deficient cells, IL-1RI was found to accumulate on LAMP1-positive vesicles, even after prolonged intervals of IL-1β stimulation, suggesting that Tom1 is involved in IL-1RI degradation and that it acts downstream of Tollip (Figure 4D, lower panels). Collectively, our findings support a model whereby IL-1RI is shuttled from Tollip to Tom1, which in turn recruits components of the endosomal-degradation machinery, such as Hrs (suggested by coimmunoprecipitation of Tom1 and Hrs; see Figure S8A) and or TSG101 [22], crucial for multivesicular-body formation and ultimately lysosomal degradation [23, 24]. Such a model is consistent with the observation that Tom1 abrogated Tollip's interaction with the IL-1Rs (Figure 4C), with the report that ubiquitin and Tollip bind in a mutually exclusive manner to an overlapping region in the GAT domain of Tom1 [25, 26], and that a Tom1 mutant (Tom1 ΔGAT) only binds weakly to IL-1R (Figure S8B). Because Tom1 is a cytoplasmic protein that is recruited to endosomes [25, 27] (See Figure S8C, showing partial localization to late endosomes), regulated recruitment of Tom1 to Tollip and IL-1R complexes may have an important role in controlling lysosomal targeting of IL-1RI. In summary, the effects of Tollip depletion reported here most plausibly reflect a function of this protein in endosomes. Nonetheless, because Tollip is also rapidly recruited to IL-1RI complex after IL-1β stimulation [14], it cannot be excluded that the observed effects on IL-1R degradation are partially a consequence of this earlier event.

Growing evidence indicates that the endocytic and signaling machinery of a receptor are intimately linked such that signaling events modify the endocytic machinery, which in turn modulates signaling (reviewed in [28, 29]). Canonical endocytic adapters (defined as proteins with domains for protein–protein or protein–lipid interaction and sites, lacking catalytic activity, for inducible post-translational modifications) have been described as the junction ring between signaling and endocytosis [30]. On one side, they are involved in the assembly and perhaps specificity of signalosomes, and on the other side, they are necessary to select and sort receptor cargo. It is therefore important to mention that Tollip-deficient mice produce less TNFα and IL-6 after IL-1β administration, despite the absence of obvious defects in signaling pathways (i.e., IκB degradation, JNK, p38, and ERK phosphorylation) in Tollip −/− MEFs [19]. Thus, it is tempting to speculate that the magnitude of the IL-1β response is attenuated in Tollip-deficient mice because of Tollip's role as an endocytic adaptor. Given the important interplay that exists between endocytosis and signaling and the pivotal position of endocytic adapters, it will now be pertinent to understand how Tollip regulates this exchange, the modulation of which may provide an effective means of regulating IL-1β-triggered inflammation.

Supplemental Data

Supplemental Data include Experimental Procedures and eight figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/22/2265/DC1/.

Acknowledgments

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