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E3 Ubiquitin Ligases Pellinos as Regulators of Pattern Recognition Receptor Signaling and Immune responses

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SUMMARY

Pellinos are a family of E3 ubiquitin ligases discovered for their role in catalyzing K63-linked polyubiquitination of Pelle, an IL-1 receptor-associated kinase homologue in the *Drosophila* Toll pathway. Subsequent studies have revealed the central and non-redundant roles of mammalian Pellino-1, Pellino-2 and Pellino-3 in signaling pathways emanating from IL-1 receptors, Toll-like receptors, NOD-like receptors, T- and B-cell receptors. While Pellinos ability to interact with many signaling intermediates suggested their scaffolding roles, recent findings in mice expressing ligase-inactive Pellinos demonstrated the importance of Pellino ubiquitin ligase activity. Cell-specific functions of Pellinos have emerged, *e.g.*, Pellino-1 being a negative regulator in T-lymphocytes and a positive regulator in myeloid cells, and details of molecular regulation of receptor signaling by various members of the Pellino family have been revealed. In this review, we have summarized current information about Pellino-mediated regulation of signaling by pattern recognition receptors, T-cell and B-cell receptors and TNF receptors, and discuss Pellino's role in sepsis and infectious diseases, as well as in autoimmune, inflammatory and allergic disorders. We also provide our perspective on the potential of targeting Pellinos with peptide- or small molecule-based drug compounds as a new therapeutic approach for septic shock and autoimmune pathologies.

Keywords

pattern recognition receptors; signal transduction; ubiquitin; inflammation; innate immunity

1. Pattern Recognition Receptors

Macrophages, dendritic cells, neutrophils, epithelial and endothelial cells utilize germ-line encoded pattern recognition receptors (PRRs) to detect conserved microbial structures of invading pathogens or endogenous “alarmins” released during infection and sterile inflammation (1-3). PRRs include membrane-associated Toll-like receptors (TLRs), C-type lectin-like receptors and cytosolic sensors comprised of nucleotide binding and

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oligomerization domain containing leucine-rich repeats receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors, and DNA sensors, e.g. absent in melanoma (AIM)-2 (3). This review focuses on TLR and NLR signaling, and the readers are referred to excellent reviews elsewhere on the subject of C type lectin-like and RIG-I-like receptors, as well as AIM-2-like receptors (4-9).

TLRs share a common structural organization, expressing an ectodomain with multiple leucine-rich repeats through which they interact with agonists and co-receptor molecules, a transmembrane region, and an intracellular tail containing a Toll-IL-1R resistance (TIR) signaling domain (1, 2). They are expressed on the cell surface, e.g. TLR1, TLR5, TLR6, or in intracellular endosomes, e.g. TLR7, TLR8, TLR9 and TLR11, while certain TLRs, such as TLR2 and TLR4, could be expressed in both compartments (1). TLR2 cooperates with TLR1 or TLR6 to detect tri-acylated or di-acylated lipoproteins from Gram positive bacteria, mycobacteria, mycoplasma, and fungi (10, 11), while TLR3 recognizes viral dsRNA (12). TLR4 is the main membrane-associated PRR for Gram negative bacterial lipopolysaccharide (LPS) (13, 14), but it can also recognize structurally unrelated molecules, e.g. fusion protein of respiratory syncytial virus (RSV) (15), chlamydia heat shock proteins (16), taxol (17), and oxidized lipoproteins (18, 19). TLR5 detects extracellular bacterial flagellin (20, 21), TLR7 and TLR8 recognize viral ssRNA (22, 23), while TLR9 senses unmethylated CpG motifs present in bacterial, viral and fungal DNA (24-26). Controversial results have been reported regarding TLR10 functions. TLR10 was shown to cooperate with TLR2 to sense bacterial lipopeptides (27, 28) and regulate production of pro-inflammatory cytokines in human peripheral blood monocytes in response to RNA-protein complexes during influenza virus infection (29). In contrast, another group reported TLR10 to down-regulate cytokine production elicited by TLR2 and to induce anti-inflammatory cytokines, such as IL-1R antagonist (30). Murine TLR11 is critical for protection against infection with uropathogenic *E. coli* (31) and, in cooperation with TLR12, detects *Toxoplasma gondii* profilin-like protein (32, 33), while murine TLR13 senses bacterial 23S ribosomal RNA (34).

Ligand sensing initiates TLR dimerization that brings together intracellular TIR domains, creating docking platforms to enable recruitment of adapter proteins. All TLRs except TLR3 signal via a common myeloid differentiation primary response protein (MyD) 88 pathway. TLR4 activates the MyD88-dependent pathway from the cell surface and translocate to endosomes to trigger the pathway utilizing TIR domain containing adapter inducing IFN- β (TRIF), while TLR3 exclusively employs TRIF for signal transduction (reviewed in (1, 35). Recruited MyD88 associates with TLRs via homotypic TIR domain interactions, forming a scaffold to recruit IL-1R-associated kinases (IRAK) 4, IRAK1, IRAK2 and IRAK-M via death domain-death domain interactions (36). Clusterization of IRAK4 leads to its trans-autophosphorylation (37), activating IRAK4 kinase activity and resulting in IRAK4-mediated phosphorylation and activation of IRAK1. IRAK1 interplay with Pellinos (discussed in the next sections) promotes engagement of downstream adapter-kinase signaling modules comprised of TNFR-associated factor (TRAF) 6 and transforming growth factor (TGF)- β -activated kinase (TAK) 1, leading to up-regulation of TAK1 kinase activity (reviewed in (1, 38, 39). Activated TAK1 triggers activation of mitogen-activated protein kinases (MAPKs) and inhibitor of nuclear factor- κ B (NF- κ B) kinase (IKK) complex,

resulting in activation and nuclear translocation of transcription factors NF- κ B, activator protein (AP)-1 and ATF-2 that drive transcription of inflammatory cytokine genes and genes encoding co-stimulatory, MHC and adhesion molecules (39). Endosomal-associated TLR7, TLR8 and TLR9 utilize the MyD88-dependent pathway activating the module IRAK1-IKK- α -IRF7 to trigger expression of type I IFN (Figure 1) (39, 40). TLR3 and TLR4 associate with TRIF in the endosomal compartment to recruit TRAF3 and activate TANK-binding kinase (TBK) 1 and IKK- ϵ that phosphorylate and activate IRF-3 leading to its nuclear translocation and induction of type I IFN and type I IFN-dependent genes (1, 40-42). The TRIF pathways also results in delayed activation of MAPKs, NF- κ B and pro-inflammatory cytokines via engaging receptor-interacting protein (RIP) 1-TAK1 module (Figure 1) (40). TLRs mediate antimicrobial responses by inducing expression of pro-inflammatory cytokines, chemokines and interferons (IFNs), up-regulating expression of co-stimulatory, adhesion, and MHC molecules, providing signal 1 (up-regulation of pro-IL-1 β and pro-IL-18 levels) for inflammasome activation, and activating autophagy responses (3, 40).

The family of cytosolic NLRs includes 24 members, with known functions associated with only a few receptors. NOD1 and NOD2 recognize peptidoglycan components meso-diaminopimelic acid and muramyl dipeptide, respectively, and activate expression of inflammatory cytokines and antimicrobial peptides via adapter-kinase modules involving RIP-2-TAK1-MAPKs/NF- κ B (reviewed in (42)). NOD2 has also been shown to recognize RNA genomes of a few viruses, *e.g.* RSV and vesicular stomatitis virus (43), and engage Atg16L1 protein to induce autophagy responses (44). NLRs with pyrin domain, *e.g.* NLRP1 detecting anthrax lethal toxin (45), NLRP3 recognizing bacterial RNA, toxins and crystalline substances (46-50), and NLR with caspase activation and recruitment domain (CARD) 4 (NLRC4) detecting cytoplasmic flagellin and rod proteins of type 3 secretion system (51), assemble to form multiprotein complexes termed inflammasomes. These complexes contain oligomerized NLRs, an adapter apoptosis-associated speck-like protein containing CARD (ASC), and multiple molecules of procaspase-1 (52). Oligomerization of pro-caspase-1 molecules leads to their auto-cleavage by a close proximity mode, generating active caspases-1 that cleaves pro-IL-1 β and pro-IL-18, generating mature cytokines that undergo secretion. Another outcome of inflammasome activation is induction of specialized form of programmed inflammatory cell death termed pyroptosis (52). Caspase-11 is a critical mediator of pyroptosis in response to cytoplasmic LPS and caspase-11 deficiency protected mice from endotoxin shock (53, 54). How caspase-11 becomes activated is poorly understood, with possible mechanisms involving the existence of a hypothetical “non-canonical” inflammasome sensors activating caspase-11, possibly, via guanylate-binding proteins (55), or direct sensing of cytoplasmic LPS by caspase-11 (56).

2. The ubiquitin system

Ubiquitination, a covalent attachment of ubiquitin (Ub) moieties (a 76-amino acid peptide) to protein substrates, is an important post-translational modification that regulates signal transduction by virtually all receptor systems. It involves a E1 Ub-activating enzyme that activates Ub by forming a thioester bond between the C-terminus of Ub and an E1 active-site cysteine in an ATP-dependent manner, Ub is then transferred to the active site cysteine of a E2 Ub-conjugating enzyme. Finally, an E3 Ub ligase associates with the Ub-loaded E2

complex and binds to the target protein, which catalyzes the ligation of the C-terminal residue of Ub to a lysine residue on the target protein, resulting in the formation of Ub polymers (Figure 2) (57). Lysine residues K6, K11, K27, K29, K33, K48 and K63 of Ub can potentially form seven different types of linkages in branched polyUb chains, and Ub molecules can also be attached to each other via linear linkages (57, 58).

Monoubiquitination promotes endocytic trafficking and DNA repair (59), K48-linked Ub moieties mark proteins for degradation via the proteasomal system, while K63-linked and linearly-linked Ub chains provide a docking site to recruit downstream intermediates and generally promote protein-protein interactions and signaling (Figure 2) (58).

3. Pellinos: Discovery, Structural Organization and Regulation of Activity by Post-Translational Modifications

Pellino was discovered as an interacting partner of the *Drosophila* Pelle, an ortholog of IRAK4, which mediates induction of Rel-related transcription factor Dorsal (60), and is required for immune responses against Gram positive bacteria (61). The mammalian family of Pellino proteins contains three members: Pellino-1, Pellino-2 and Pellino-3, which undergoes alternative splicing in humans, giving rise to splice variants Pellino-3a and Pellino-3b (reviewed in (38, 62)). Structural analyses and bioinformatic studies revealed the presence of an N-terminal forkhead-associated domain and “wing” or appendage structure in Pellinos that promotes their association with phosphothreonine residues presented by interacting proteins, such as phospho-IRAK1 (63). At the carboxyl termini, Pellinos express a conserved pattern of cysteine and histidine residues resembling a RING motif in, which is a characteristic feature of the RING class of E3 Ub ligases (Figure 3) (38, 62). Consistent with this, Pellinos have been reported to act as E3 Ub ligases: *in vitro* Ub assays indicated that Pellinos are capable of mediating K11-, K48-, and K63-linked conjugation of Ub (64), while *in vivo* analyses in cells demonstrated primarily K63-linked ubiquitination of IRAK1, RIP-1, and RIP-2 mediated by Pellinos (65-68), with only one example of Pellino-1-mediated K48-linked ubiquitination of c-Rel (69). Because all three Pellinos interact with multiple intermediates, including IRAK4, IRAK1, TAK1, and TRAF6 (70-76), Pellinos were initially suggested to function as scaffolding proteins in IL1R/TLR signaling (72). However, subsequent studies demonstrated specificity in interactions between different members of the Pellino family: *e.g.* Pellino-1, but not Pellino-2 or Pellino-3, has been reported to interact with MyD88 (77, 78), and TBK-1 (76); and Pellino-3, but not Pellino-1 or Pellino-2, associates with NF- κ B-inducing kinase (75, 79). Pellino 1 and Pellino-2 bind IRAK1, become phosphorylated, and mediate subsequent IRAK1 ubiquitination (79), and mice expressing ligase-inactive Pellino-1 had inhibited expression of IL-1- or TLR-inducible cytokine genes (66), supporting a critical role for E3 Ub ligase activity in mediating Pellino functions.

Ubiquitination assays *in vitro* demonstrated the ability of IRAK1 and IRAK4 to phosphorylate all Pellino proteins (65, 73, 79), leading to Pellino autoubiquitination on several lysine residues and inducing enhanced E3 Ub ligase activity of Pellinos (65, 80). Pellino 1 expresses multiple phosphorylation sites that could be phosphorylated by IRAK4/1 kinases (postulated to function in IL-1R signaling), TBK1/IKK- ϵ kinases (postulated to act

in TLR signaling) or both (Figure 3), with several phosphorylation sites required for full activation. It is likely that dephosphorylation of critical sites is required for termination of Pellino activity (80), but the respective protein phosphatases remain unknown. Interestingly, co-expression of active IRAK1 decreases levels of Pellino1 (64), while cells expressing catalytically inactive IRAK1 or lacking IRAK1 did not show decreased expression of Pellino 1 (65, 76, 81), suggesting that Pellino ubiquitination may serve as a degradation signal. Pellino-1 also undergoes sumoylation upon overexpression by small-ubiquitin-related modifier-1 (SUMO-1) and E2 ubiquitin-conjugating enzyme Ubc9 (81). The functional significance of the sumoylation of Pellino 1 is unknown, and the ability of other members of the Pellino family to undergo sumoylation has not been studied. Due to high evolutionary conservation of phosphorylation and sumoylation sites within the Pellino family members, it is tempting to speculate that coordinate ubiquitination and sumylation events represent molecular framework that regulates fine-tuning of IL1R/TLR signaling by Pellinos. Further studies are required to address this hypothesis.

4. Pellino-1 functions

4.1. Role of Pellino-1 in IL-1R Signaling

IL-1 is an important pro-inflammatory cytokine initially identified as an “endogenous pyrogen” (82) that plays a critical role in the regulation of hematopoiesis, recruitment and activation of neutrophils, macrophages, T- and B-lymphocytes and mediates the acute phase response during inflammation (83-87). IL-1 β secretion requires two signals, TLR-mediated up-regulation of expression of precursor pro-IL-1 β , and NLR-induced activation of caspase-1 that cleaves pro-IL-1 β to yield mature IL-1 β form to be secreted (50). IL-1 acts via IL-1R and IL-1R-accessory protein to engage the MyD88-dependent signaling cascade involving IRAK4, IRAK1 and IRAK2 kinases, TRAF6 and TAK1 to activate MAP kinases and NF- κ B (88). Jiang et al. (71) first identified mammalian Pellino-1 as a critical participant of the IL-1R signaling cascade via promoting IL-1-dependent interactions within the IRAK4-IRAK1-TRAF6 complex and showed that knockdown of Pellino-1 in HEK293 cells inhibits IL-1-mediated activation of NF- κ B and induction of NF- κ B-dependent IL-8. Likewise, Pellino-1 knockdown in the BEAS-2B epithelial cell line was shown to reduce IL-1 β -induced expression of pro-inflammatory cytokines (89). TGF- β was reported to inhibit IL-1R signaling via the induction of SMAD6 and SMAD7 proteins that bind Pellino-1 via their MAD homology 2 domains and disable Pellino-1 association with IRAK4, IRAK1 and TRAF6, blocking activation of NF- κ B and induction of pro-inflammatory cytokines (78, 90). These results support a critical role for Pellino-1 in IL-1R-driven NF- κ B activation and pro-inflammatory cytokine expression. In contrast, a few publications question this conclusion. For example, mouse embryonic fibroblasts from WT and Pellino-1 KO mice were published to have comparable IL-1-induced NF- κ B activity and expression of pro-inflammatory cytokines TNF- α , IL-6 and CXCL10 (66), and Pellino-1 knockdown in primary human bronchial epithelial cells did not affect IL-1-driven pro-inflammatory cytokine production (89). Furthermore, cells expressing kinase-inactive IRAK1 have significantly impaired IL-1-inducible E3 UB ligase activity of Pellino-1, yet showing only a very modest effect on MAPK and NF- κ B activation (76). Thus, more studies on Pellino-1 ablation in cell lines and experiments with Pellino-1KO mice and E3 UB ligase-inactive

Pellino-1 knockin mice are required to definitively elucidate the role of Pellino-1 in IL-1R signaling.

4.2. Role of Pellino-1 in TLR Signaling

Generation of Pellino-1 KO mice led to the discovery of a critical role for Pellino-1 in regulating TLR3- and TLR4-mediated septic shock and inflammatory cytokine expression. These mice exhibit normal viability, growth and survival and are protected from septic shock induced by administration of poly (I:C) or LPS, associated with deficient TLR3- or TLR4-mediated activation of NF- κ B and induction of pro-inflammatory cytokines TNF- α , IL-6 and IL-12 (66). Other tested TLR agonists utilizing the MyD88 pathway, *e.g.* Pam3Cys (TLR2-TLR1), MALP-2 (TLR2-TLR6), R837 (TLR7/8), and CpG oligonucleotides (TLR9) showed comparable activities in WT and Pellino-1-deficient mice, suggesting that Pellino-1 targets the TRIF-dependent signaling cascades (66). Recent studies showed that IKK- ϵ and TBK1 mediate the activation of Pellino 1's E3 ligase activity, as well as inducing the transcription of its gene and protein expression in response to TLR3 and TLR4 agonists (91). TBK1 and IKK- ϵ -mediated-phosphorylation and activation of Pellino-1 leads to Pellino-1-mediated ubiquitination of RIP-1 (76) that provides molecular platforms to recruit and activate TAK1-binding proteins-TAK1 and IKK complexes, inducing NF- κ B (92). The TRIF pathway also activates expression of type I IFNs and type I IFNs-dependent genes via TBK1/IKK- ϵ -mediated phosphorylation and activation of IRF3 (1). Pellino-1 ablation was found to specifically impair RIP-1 ubiquitination, activation of NF- κ B and induction of pro-inflammatory cytokines while not affecting TLR3- or TLR4-mediated activation of IKK- ϵ , and induction of type I IFNs and Cxcl10 (66) (Figure 4). Similarly, Pellino-1 knockdown in human primary bronchial epithelial cells was reported to attenuate poly (I:C)-mediated expression of pro-inflammatory cytokines IL-6 and Cxcl8, while leaving intact the induction of type I IFN (89). These results suggest that Pellino-1 promotes TLR3- and TLR4-driven ubiquitination of RIP-1, activation of NF- κ B and pro-inflammatory cytokines but does not influence TRIF-driven induction of IKK- ϵ and type I IFNs.

In contrast to this conclusion, other studies showed a significant reduction of poly (I:C)- and LPS-mediated expression of type I IFNs in mice and cells expressing E3 Ub ligase-inactive version of Pellino-1 and normal induction of pro-inflammatory cytokines (93). Macrophages and DCs from ligase-deficient Pellino-1 knock-in mice had normal TLR3- or TLR4-induced phosphorylation of TBK-1 and IRF3 but deficient IRF3 recruitment to the IFN- β promoter, and normal poly (I:C)-mediated ubiquitination of RIP-1, induction of NF- κ B, MAPKs and expression of pro-inflammatory cytokine genes (93). These findings suggest that Pellino-1 promotes recruitment of IRF3 to the IFN- β promoter, possibly, via the transcription factor deformed epidermal autoregulatory factor 1 homologue that interacts with Pellino-1 and also associates with IRF3 and the IFN- β promoter to drive type I IFN expression (94). Thus, there are conflicting data in the literature using Pellino-1^{-/-} mice vs Pellino-1 E3 Ub ligase-inactive knock-in mice with respect to the role for Pellino-1 in regulating TLR signaling cascades that drive expression of pro-inflammatory cytokines and type I IFNs. Our unpublished observations showed that Pellino-1 knockdown in THP-1 cells inhibits LPS-mediated induction of pro-inflammatory cytokine genes TNF- α , IL-6, pro-IL-1 β and induction of RANTES and IFN- β mRNA (Murphy M, Xiong Y, Medvedev AE. Submitted),

suggesting that both MyD88- and TRIF-dependent branches of the TLR4 signaling pathway are affected by Pellino-1. Further experiments are required to delineate functional roles of Pellino-1 in regulating TLR-driven MyD88- and TRIF-dependent signaling cascades in human and mouse macrophages.

4.3. Pellino-1 and Neuroinflammation

Recent findings have highlighted an important role of Pellino-1 as a mediator of neuroinflammation during development of multiple sclerosis. Post-mortem brain samples from a patient with multiple sclerosis showed significantly increased levels of Pellino-1 compared to healthy volunteers, and Pellino-1^{-/-} mice were reported to exhibit a drastically reduced severity of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (95). Microglia cells express very high levels of Pellino-1 that plays an important role in eliciting expression of pro-inflammatory cytokines and chemokines and activating recruitment of autoreactive T cells into the central nervous system (Figure 4) (95). Pellino-1 was shown to critically regulate MyD88-dependent signaling in microglia cells, as evidenced by ablated induction of pro-inflammatory cytokines in Pellino-1^{-/-} microglia cells stimulated with TLR1-TLR2, TLR7 or TLR9 agonists utilizing the MyD88-dependent pathway. (95). At the molecular levels, loss of Pellino-1 led to impaired MYD88-induced activation of the ERK, JNK and p38 MAP kinases without affecting NF-κB activation, due to attenuated TRAF6-induced ubiquitination of IAP2, resulting in blocked TRAF3 degradation, reducing MAPK activation and decreasing pro-inflammatory gene expression (95). The indispensable role of Pellino-1 in eliciting MyD88-dependent MAPK activation observed in microglial cells could be accounted for by the highest expression of Pellino-1 compared to other Pellinos (95), reducing functional compensation by Pellino-2/3. Overall, these data highlight a highly specialized role for Pellino 1 as a mediator of neuroinflammation in microglial cells.

5. Pellino-2 Functions in IL-1R/PRR Signaling

Pellino-2 has been recognized as an important regulator of IL-1R/TLR signaling and interacts with IRAK4, IRAK1, TRAF6 and TAK1 (73, 74). Studies in cell lines have produced mixed results: overexpression of Pellino-2 induces activation of ERK and JNK MAP kinases, leading to the induction of transcription factors AP-1 and Elk-1 (74), and Pellino-2 knockdown in 293/IL1R cells and primary bone marrow-derived macrophages reduces IL-1β- and LPS-mediated induction of ERK and JNK (68). The ability of Pellino-2 to activate the MAPK pathways could account for retained MAPK signaling in myeloid cells from Pellino-1 KO mice, setting an example of functional compensation between Pellino family members. In contrast, Strelow et al. (73) failed to detect activation of ERK and JNK in cells overexpressing Pellino-2. Kim et al (68) uncovered a critical role for Pellino-2 in IL-1R/TLR-mediated NF-κB activation and posttranscriptional control of cytokine expression. Pellino-2 knockdown in 293/IL-1R cells and primary BMDMs led to inhibition of TAK1-dependent activation of NF-κB in response to LPS and IL-1 and reduced stabilization of mRNA encoding pro-inflammatory cytokines (68). Structurally, Pellino-2 contains a forkhead-associated (FHA) domain which is a well-characterized phosphothreonine-binding motif. This domain in Pellino-2 drives the interaction with

phosphorylated IRAK1 (64). Mechanistically, Pellino-2 ablation blocked IL-1- and LPS-induced polyubiquitination of IRAK1 (68), suggesting suppression of IRAK1 as a mechanism responsible for deficient downstream activation of the MAPK and NF- κ B pathways in Pellino-2 knockdown cells. Pellino-2-B cell lymphoma (Bcl10) complex represents another important molecular hub that critically regulates TLR4 signal transduction. Bcl10 is the signaling adapter activated following LPS engagement of TLR4-MD2 that subsequently interacts with Pellino-2 to activate NF- κ B (96). Suppressor of cytokine signaling (SOCS)-3, a negative regulator of TLR4 signaling, was reported to attenuate TLR4-mediated NF- κ B activation and iNOS expression by disrupting Pellino-2-Bcl10 interaction (96). While these reports provide valuable information regarding functions of Pellino-2 in IL-1R/TLR4 signaling, no information is available regarding possible involvement of Pellino-2 in TRIF-dependent signaling cascades and whether it regulates functions of other TLRs, and how Pellino-2 functions *in vivo*. Future studies on the generation of Pellino-2-deficient mice, mice expressing E3 Ub ligase-inactive Pellino-2 and identification of human patients lacking Pellino-2 or expressing Pellino-2 polymorphisms will clarify the physiological roles and functional activities of Pellino-2.

6. Pellino-3 Functions

6.1. Role of Pellino-3 in IL-1R/TLR Signaling

Initial overexpression studies demonstrated that in contrast to Pellino-1, Pellino-3 is unable to activate NF- κ B but drives phosphorylation and activation of MAP kinases ERK, JNK and p38 (72). Our results ((97) and Murphy M, Xiong Y, Medvedev A. Submitted) uncovered the ability of Pellino-3b, a splice variant of Pellino-3, to act as a dominant-negative inhibitor of IL-1R/TLR4-mediated NF- κ B activation, as judged by overexpression and knockdown studies, and showed enhanced IL-1R/TLR4-mediated K63-linked ubiquitination of IRAK1. We hypothesize that Pellino3-mediated K63 ubiquitination of IRAK1 stabilizes its assembly with MyD88-IL-1R/TLR4 complex, preventing IRAK1 degradation and release of downstream signaling components TRAF6-TAK1 in the cytoplasm, thereby attenuating signaling. This hypothesis somewhat contradicts a well-accepted model according to which K63-linked ubiquitination promotes downstream signaling, and we cannot exclude alternative explanations, such as modulation of linear ubiquitination and formation of unanchored Ub chains. Notably, TLR-mediated activation of IKK kinases involves initial conjugation of K63-linked polyUb moieties to IRAK1, followed by addition linear Ub chains (98). These hybrid Ub chains have been suggested to recruit and activate the TAK1 and IKK complexes, resulting in activation of IKKs (98). Another possible mechanism to be explored could represent Pellino-3-assisted recruitment of negative inhibitory molecules in the receptor complexes, e.g. A20 or SHIP-1, which could subsequently disable downstream signaling.

Pellino-3 KO mice do not show abnormalities in viability and growth, but exhibit increased levels of type I IFNs in response to TLR3 stimulation with poly (I:C), have enhanced protection against EMCV due to increased expression of type I IFNs and augmented viral clearance (99). In contrast, Pellino-3^{-/-} cells and mice did not show deficiencies in pro-inflammatory cytokines upon poly (I:C) stimulation or EMCV infection (99). TLR3

signaling via the TRIF–TBK1–IRF3 axis increases expression and activity of Pellino-3 and initiates its assembly with TRAF6 and TRAF6 polyubiquitylation. This event suppresses TRAF6–IRF7 interactions and blocks IRF7 polyubiquitylation (99) required for its activation (100), leading to impaired translocation of IRF7 and inhibited expression of IFN- β (99). Thus, Pellino-3 plays an important physiological role by negatively regulating TLR3 signaling via suppression of TRAF6-mediated polyubiquitination of IRF7, blocking its activity and expression of IFN- β .

Pellino-3 has been implicated as an important regulatory molecule in atherosclerosis, due to its ability to suppress TLR4-mediated expression of IFN β (101). Macrophages localized to atherosclerotic lesions ingest oxidized low-density lipoprotein (oxLDL) to yield lipid-laden foam cells that exhibit increased pro-inflammatory gene expression but reduced IFN β induction in response to LPS (102). OxLDL delivered via the scavenger receptor A1 binds and activates Pellino-3, promoting mono-ubiquitination of the adaptor TRAF-family-member-associated NF- κ B activator (TANK) (101), leading to impaired recruitment of TBK1 to TRAF3 and inhibition of IFN β expression (101). It remains unknown however whether pellino-3 directly ubiquitinates TANK or acts indirectly via activation of another E3 Ub ligase.

6.2. Pellino-3 and NOD2 signaling

Very little is known about the role of Pellino proteins in NLR signaling. Pellino-3 has been described as a critical regulator of NOD2 (67), whose loss-of-function mutations are linked to prevalence of Crohn's disease in human patients and in mouse models (103). Upon sensing MDP, NOD2 triggers Pellino-3-mediated K63-linked ubiquitination of RIP2 (Figure 6) that facilitates the recruitment of TAK1 and IKK kinase complexes, leading to activation of NF- κ B, antimicrobial peptides and inflammatory cytokines (67). Pellino 3^{-/-} BMDMs were found to be defective in NOD2-driven RIP2 poly-ubiquitylation and induction of pro-inflammatory cytokines and chemokines, and Pellino-3 KO mice exhibited drastically reduced serum levels of MDP-induced pro-inflammatory cytokines and reduced colonic inflammation in models of chemically-induced colitis (67). Notably, Crohn's disease patients have decreased Pellino-3 levels in intestinal cells, and WT mice, but not NOD2^{-/-} or Pellino-3^{-/-} mice could be rescued from colitis-associated morbidity by administration of MDP (67). Thus, Pellino 3 is an important regulator of NOD2-induced K63-linked ubiquitination of RIP2, which is followed by its linear ubiquitylation by the linear ubiquitin chain assembly complex (LUBAC) (104) and the induction of downstream cytokines (Figure 6), and plays a critical role in the homeostatic control of intestinal inflammation.

It is currently unknown whether Pellino proteins control signaling by other members of the NLR family and if they could regulate inflammasome activation, IL-1 β /IL-18 secretion and pyroptosis initiated by NLRP1, NLRP3, NLRC4 or AIM-2. Based on the ability of Pellinos to modulate expression of cytokines and type I IFNs, it is possible that they could regulate inflammasomal NLRs via modulation of transcription of pro-IL-1 β and pro-IL18, and, via modulation of activity of IRAK4/1 kinases, Pellinos could affect rapid NLRP3-mediated inflammasome activation. Future studies will shed a light into whether Pellinos fine-tune signaling via inflammasomal NLR receptors or other members of the NLR family.

7. Pellino roles in regulating functions of T- and B-lymphocytes

Very limited information is available regarding the role of Pellinos in T- and B-lymphocytes. In contrast to its functions as a positive regulator of IL1R/TLR signaling in myeloid cells, Pellino-1 acts as a negative regulator in T cells. Pellino-1 KO mice exhibited a loss of tolerance and showed signs of autoimmunity during ageing, characterized by multiorgan inflammation and autoantibody production (69). Subsequent analyses demonstrated increased accumulation of T cell in lymph nodes and the spleen and T cell hyperactivation, judged by increased proliferation and IL-2 and IFN- γ production of both CD4⁺ and CD8⁺ T cells upon stimulation via CD3 and CD28 (69). Pellino-1 deficiency rendered T cells refractory to suppressive effects by Tregs or TGF- β , and at the molecular level, led to increased nuclear accumulation of the c-Rel NF- κ B subunit (playing a pivotal role in T cell activation (105)) due to a loss of Pellino-1-mediated K48-linked ubiquitination of c-Rel that targets it for proteasomal degradation (69) (Fig. 5). It remains unknown whether Pellino-1 directly mediates K48-linked ubiquitination of c-Rel or functions in an indirect fashion. Notably, despite Pellinos generally promote non-degradative K63-linked polyubiquitination of target proteins, in T cells Pellino-1 catalyzes the Lys48-linked polyubiquitylation of c-Rel, suggesting different modes of functions for Pellinos in various pathways and cell types.

Only one report has been published on the role of Pellino-1 in B cells that suggests that up-regulation of Pellino-1 expression could play a role in B cell lymphoma tumorigenesis. Indeed, patients with diffuse large B cell lymphomas showed a close correlation between increased expression of Pellino-1, increased levels of the oncoprotein B cell chronic lymphocytic leukemia (BCL6) and poor prognosis (106). Furthermore, transgenic mice overexpressing human Pellino-1 exhibited a shorter lifespan due to ligand-independent hyperactivation of B cells, with prominent B cell infiltration in multiple organs and the development of a wide range of lymphoid tumors. Mechanistically, Pellino-1 interacted with BCL6, resulting in K63-mediated BCL6 polyubiquitination (106). These data suggest that increased expression of Pellino-1 promotes induction of BCL6, leading to lymphomagenesis, and indicate the potential for therapeutic targeting of this pathway to treat B cell lymphomas. They also indicate that Pellino-1 is an important regulator of not only innate immunity, but also the adaptive immune responses involving T- and B-cells.

8. Pellinos and TNFR Signaling

TNF- α is an important cytokine produced by macrophages, DCs, neutrophils, NK cells, T- and B-lymphocytes that mediates various processes during immunity and inflammation, including proliferation, apoptosis, angiogenesis and cancerogenesis (107). It signals via two TNFRs. TNFR1 elicits apoptosis via engagement of the adapter proteins TNFR-associated death domain, Fas-associated death domain, and caspases 8 and 3, and it can also mediate proliferation and expression of inflammatory mediators by the pathways utilizing TRAF2/ TRAF5-RIP1-TAK1-MAPK/NF- κ B (107). TNFR2 primarily mediates proliferation and induction of inflammatory cytokines via recruitment of TRAF2, RIP-1 and activation of TAK1, subsequently leading to induction of inflammatory cytokines and proliferation (107). Recent studies have identified Pellino-3 as an important regulator of TNFR signaling.

TNFR1 employs E3 Ub ligases TRAF2/5, inhibitor of apoptosis protein (IAP)-1 and IAP-2 to polyubiquitinate RIP1, which initiates recruitment of TAK1 and IKK kinases leading to NF- κ B activation, expression of anti-apoptotic genes, and cell survival (108). Interestingly, deubiquitination of RIP1 inhibits NF- κ B activation via preferential association of unubiquitinated RIP1 with FADD and pro-caspase-8 that promotes the formation of death-inducing signaling complex (DISC) and apoptosis (108). Pellino-3 was found to interact with RIP-1 and block the assembly of the DISC signalosome, activation of caspase-8 and inhibit caspase-8-mediated cleavage of RIP-1, promoting cell survival (109). Functional activities of Pellino-3 in TNFR signaling were found to be dependent of its FHA domain but independent of the RING-like domain, suggesting that Pellino-3 may exert at least some functional effects in the ligase-independent manner.

9. Pellinos and Disease

Little is known about functional roles of different members of the Pellino family in the pathogenesis of infectious, allergic, autoimmune and inflammatory diseases and cancer. Given the ability of Pellino proteins to regulate MyD88-IRAK-driven and TRIF-TBK1-activated TLR signaling pathways, altered expression of Pellinos or dysregulations in Pellino functions due to polymorphisms/mutations are likely to affect susceptibility to infectious, allergic, inflammatory diseases and cancer. Studies in mice demonstrated that Pellino-1 gene ablation conferred protection against sepsis initiated by systemic administration of TLR3 and TLR4 agonists (66), while Pellino-3 deficiency is associated with increased production of type I IFNs, viral clearance and protection against infection with EMCV (99). However, there have been no reports in human populations regarding whether single nucleotide polymorphisms (SNPs) in Pellinos could impact susceptibility to infections or prevalence of sepsis. Patients with multiple sclerosis express increased levels of Pellino-1, and Pellino-1 ablation in mice was reported to significantly reduce severity of EAE, a mouse model of multiple sclerosis (95). Increased levels of Pellino-1 and Pellino-2 were found in patients with asthma (110) and kidney transplant recipients experiencing acute rejection showed increased Pellino-2 mRNA levels (111), while Pellino-3 expression is down-regulated in the colon of patients with Crohn's disease (67). Interestingly, Pellino-1 SNPs have been linked to increased risks of coronary artery lesions in children with Kawasaki disease, a rare form of vasculitis (112, 113). Furthermore, patients with diffuse large B cell lymphomas showed a close correlation between increased expression of Pellino-1, increased levels of BCL6 and poor prognosis (106). Pellino-3 was shown as an important molecule regulating OxLDL-mediated inhibition of TLR4-mediated activation of IFN- β in foam cells of atherosclerosis patients (101). These findings indicate that dysregulated expression of Pellino proteins or the presence of SNPs affecting Pellino expression and/or functions could critically underlie pathogenesis of many allergic, infectious, inflammatory diseases and cancer. It is plausible that peptide-based or small molecule inhibitors of Pellino-1 could be a perspective new therapeutic modality for treatment of patients with multiple sclerosis and asthma, and agonist or antagonist drugs targeting other members of the Pellino family may contribute to therapeutic regimens for treatment of patients with sepsis, inflammatory and allergic diseases.

10. Conclusion and Perspectives

Pellino proteins have emerged as important regulators of many receptor systems, including IL-1R, TLRs, NOD2 and TNF receptors (38), and a few published studies have started to shed a light on their role in allergic, inflammatory, infectious diseases and cancer (66, 69, 95, 106, 111). However, conclusions regarding functional activities of Pellinos await their confirmation and clarification in additional research employing Pellino KO and E3 Ub ligase-inactive Pellino knock-in mice. Since conserved signaling modules are utilized by the IL-1R/TLR pathways (1), it is unclear why Pellino-1 affects the MyD88-dependent, IRAK-mediated pathway in the context of IL-1R signaling in macrophages (71) and upon TLR signaling in microglia cells (95), while influencing TRIF-dependent RIP-1 ubiquitination upon TLR3/4 signaling in macrophages (66). The basis for divergent roles of Pellino-3 in TLR signaling (where it acts as a negative regulator of TLR3-mediated, TRAF6-driven ubiquitination of IRF7) and NOD2 signaling (where it positively regulates NOD2-mediated K63-linked ubiquitination of RIP2, promoting interactions with TAK1) needs to be elucidated. It is tempting to speculate that Pellino-3 associates with other E3 Ub ligases in a receptor-specific context and mediates divergent functions depending on the composition of these complexes.

Given that different members of the Pellino family share common structural organization, expressing the FHA domain involved in interactions with substrates and the RING-like domain exerting E3 Ub ligase activity (38), the molecular basis for diversification and non-redundant functions of Pellino-1, Pellino-2 and Pellino-3 remains poorly understood. Discrepancies between phenotypes of Pellino-1-deficient cells and cells expressing E3 Ub ligase-deficient Pellino-1 also need to be confirmed and molecular mechanisms of this phenomenon addressed. Although the RING-like domain endows E3 Ub ligase activity on Pellinos (38), some functions could be mediated by mechanisms independent of E3 Ub ligase activity. It is also possible that specific tissue expression of various Pellinos may promote their specialized roles in specific cells, as demonstrated by the dominant expression of Pellino-1 in T-lymphocytes (69) and microglial cells (95), associated with Pellino-1-mediated regulation of self-tolerance (95) and neuroinflammation (69). It is unclear if ligase-deficient Pellino-1 could still mediate the induction of pro-inflammatory cytokines, *e.g.* via engagement of additional unknown E3 Ub ligases, while acting as an inhibitor of TLR-mediated type I IFN expression, a mechanism lacking in Pellino-1^{-/-} cells.

It will be of importance to delineate whether individual Pellinos could be regulated by specific kinases in cell-dependent and receptor-specific contexts, leading to activation of Pellino functions. While Pellino-1 was reported to be phosphorylated by IRAK1 in IL-1R signaling (65, 71), and by TBK1–IKK-ε in TLR signaling (71), these results need to be confirmed in independent studies, and kinases responsible for activation of Pellino-2 and Pellino-3 await their identification. Furthermore, functional roles for sumoylation and dephosphorylation of Pellinos and the respective enzymes remain unknown. Since Pellino-1 and Pellino-3 have been shown to interact with RIP-1 and of RIP-2, respectively (66, 67), it is possible that RIP kinases could phosphorylate and regulate functions of Pellino proteins. Signals responsible for termination of Pellino functions are also largely unknown. It is likely that one such regulatory negative feedback loop could be protein phosphatases

dephosphorylating critical phosphorylated residues required for activation of Pellino functions. In addition, IRAK-mediated phosphorylation of Pellino-1 was noted to be accompanied by subsequent reductions in Pellino-1 protein levels, suggesting that certain phosphorylation events can target Pellino proteins for K48-linked polyubiquitination via unknown E3 Ub ligases and degradation via the proteasomal pathway.

Given known associations of Pellinos with disease, e.g. association of Pellino-1 and Pellino-2 with multiple sclerosis, coronary vascular lesions in children with Kawasaki disease and asthma, respectively, and linkage of Pellino-3 with Crohn's disease and lymphomagenesis, it will be of importance to continue genome-wide association studies in efforts to link Pellino SNPs with inflammatory, allergic, infectious diseases and cancer. It is also plausible to suggest that development of new peptide-based or small molecule chemical inhibitors of individual members of the Pellino family could represent a perspective new therapeutic intervention. Thus, comprehensive studies into the biology of the Pellino family proteins is warranted, as Pellino proteins could represent valuable targets in disease and improved understanding of the molecular mechanisms governing their expression and activation will provide important insights to use to develop new therapeutic strategies.

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Figure 1. TLR Signaling Pathways

TLRs signal activation of the MyD88-dependent pathway from the plasma membrane (TLR2, TLR4, TLR5) or from endosomes (TLR7, TLR9) to recruit adapter/kinase complexes involving MyD88-IRAKs-TRAF6-TAK1 to mediate activation of NF-κB and expression of pro-inflammatory cytokines and chemokines. TLR7 and TLR9 also utilize the MyD88-IRAK4/1 module to phosphorylate and activate IRF7, leading to induction of type I IFNs. TLR3 and TLR4 employ TRIF to signal from endosomes and to trigger induction of type I IFNs via engagement of the TRAF3/TBK1/IKK-ε that phosphorylate and activate IRF3, and signal induction of pro-inflammatory cytokines by RIP-1-mediated activation of TRAF6-TAK1 module to activate MAPKs and NF-κB.

Figure 2. The Ubiquitination Pathway

Ubiquitylation is initiated by a ubiquitin-activating enzyme (E1) that forms a labile thioester bond with Ub in an ATP-dependent manner, and presents UB to a ubiquitin-conjugating enzyme (E2). An E3 Ub ligase binds to substrates and to the E2-Ub complex and catalyzes the ligation of the C-terminal residue of Ub to a lysine residue on the target protein, initiating monoubiquitination that promotes endocytic trafficking and DNA repair. In many signaling systems, this process follows by subsequent attachment of multiple Ub residues, initiating the formation of Ub polymers whereby an internal lysine residue of one Ub is attached to the C-terminal residue of another. Seven lysine residues of Ub (K6, K11, K27, K29, K33, K48 and K63) could provide different Ub–Ub linkages in branched Ub polymers; and Ub molecules can be linked linearly. K48-linked Ub chains mark proteins for proteasomal degradation, while K63-linked and linearly-linked Ub moieties facilitate assembly of protein complexes and promote downstream signaling.

Figure 3. Structural Features and Post-translational Modifications of Pellinos

Pellino proteins share a common structural organization, expressing a cryptic N-terminal forkhead-associated (FHA) domain with two inserts that form a ‘wing’ or appendage in a close proximity, and a RING-like domain with the characteristic pattern of cysteine and histidine residues. Ubiquitination (Ub) and sumoylation (Su) sites are indicated, the numbered lysines correspond to the sites identified in Pellino 1, and Ser and Thr phosphorylation acceptor sites for Pellino 1 phosphorylated by IRAK1/4 and TBK1 are depicted. The stars mark phosphorylation sites that are required for the full E3 Ub ligase activity of Pellino-1.

Figure 4. Pellino-1 and TLR Signaling

A: In macrophages, TRIF signaling activates TBK1 and IKKε that activate expression of Pellino-1 via the IRF3-dependent mechanism and also phosphorylate Pellino-1. Pellino-1 promotes K63-linked polyubiquitylation of RIP1 (dashed arrow), leading to recruitment of TAK1 and TAK1-mediated phosphorylation (p) and activation of IKK kinases that mediate activation of NF-κB. B: In microglia cells, TLR2 or TLR4 signal activation of TRAF6 via the MyD88-dependent pathway to activate MAPK and the transcription factor AP-1 that co-operate with NF-κB to initiate transcription of pro-inflammatory cytokine genes. TRAF3 negatively regulates MAPK activation, which is blocked via Pellino-1-mediated K63-linked polyubiquitination of IAP2, leading to IAP2-mediated K48-linked polyubiquitination and degradation of TRAF3.

Figure 5. Pellino-1 Acts as a Negative Regulator of T Cell Activation

Recognition of cognate antigen presented by MHC proteins through TCR along with costimulatory signals via CD28 and cytokine receptors in T cells induces downstream signaling involving adapter-kinase modules ZAP70/linker for activation of T cells/ lymphocyte cell-specific protein-tyrosine kinase, culminating in activation of the NF-κB subunit c-Rel. c-REL translocates to the nucleus and binds to the CD28-responsive element of the IL-2 promoter, driving expression of IL-2. CD28 signals downstream to up-regulate Pellino-1, which promotes K48-linked polyubiquitination of c-Rel, targeting it for proteasome-mediated degradation. This negative feedback mechanism promotes the induction of T cell anergy and self-tolerance.

Figure 6. Pellino-3 Regulates TLR3 and NOD2 Signaling

A: TLR3 exclusively employs TRIF to recruit RIP1 in order to activate TAK1-driven activation of NF- κ B and to trigger activation of TBK1/IKK ϵ kinases to phosphorylate and activate IRF3 and IRF7, leading to the induction of type I IFNs. TRAF6 promotes K63-linked polyubiquitination of IRF7, facilitating its activation and nuclear translocation. TBK1/IKK- ϵ pathway up-regulates expression of Pellino-3, leading to Pellino-3-mediated polyubiquitination of TRAF6, which blocks TRAF6-mediated ubiquitination and activation of IRF7 and impaired induction of type I IFNs. B: Upon microbial infection of the gut, bacterial-derived MDP, a component of peptidoglycan, is recognized by NOD2, resulting in receptor oligomerization and signal transduction. The signaling cascade involves recruitment of RIP-2 to NOD2, RIP2-Pellino-3 association and Pellino-3-mediated K63-linked polyubiquitination of RIP2, promoting recognition of polyUb moieties via Ub recognition domains of TAB1/TAB2 associated with TAK1 and by NEMO (IKK γ) associated with IKK α /IKK β , facilitating their recruitment and activation. TAK1 subsequently activates MAPKs, AP1 and NF- κ B to drive expression of cytokines, chemokines and antimicrobial peptides.