Receptor Signaling Lymphocyte-activation Molecule Family 1 (Slamf1) Regulates Membrane Fusion and NADPH Oxidase 2 (NOX2) Activity by Recruiting a Beclin-1/Vps34/Ultraviolet Radiation Resistance-associated Gene (UVRAG) Complex*

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Background: The receptor Slamf1 functions as a microbial sensor regulating phagocytosis.

Results: Slamf1 interacts with Vps34/Beclin-1/UVRAG complex in a wide range of cells.

Conclusion: Slamf1 recruits a subset of ubiquitous autophagy-associated proteins, which is involved in membrane fusion and NOX2 regulation.

Significance: These studies provide insight into the regulation of microbialic function allowing for potential therapeutic discoveries.

Phagocytosis is a pivotal process by which macrophages eliminate microorganisms upon recognition by pathogen sensors. Surprisingly, the self-ligand cell surface receptor Slamf1 functions not only as a co-stimulatory molecule but also as a microbial sensor of several Gram-negative bacteria. Upon entering the phagosome of macrophages Slamf1 induces production of phosphatidylinositol 3-phosphate, which positively regulates the activity of the NOX2 enzyme and phagolysosomal maturation.

Here, we report that in *Escherichia coli*-containing phagosomes of mouse macrophages, Slamf1 interacts with the class III PI3K Vps34 in a complex with Beclin-1 and UVRAG. Upon phagocytosis of bacteria the NOX2 activity was reduced in macrophages isolated from Beclin-1−/− mice compared with wild-type mice. This Slamf1/Beclin-1/Vps34/UVRAG protein complex is formed in intracellular membrane compartments as it is found without inducing phagocytosis in macrophages, human chronic lymphocytic leukemia cells, and transfectant HEK293 cells. Elimination of its cytoplasmic tail abolished the interaction of Slamf1 with the complex, but deletion or mutation of the two ITAM motifs did not. Both the BD and CCD domains of Beclin-1 were required for efficient binding to Slamf1. Because Slamf1 did not interact with Atg14L or Rubicon, which can also form a complex with Vps34 and Beclin-1, we conclude that Slamf1 recruits a subset of Vps34-associated proteins, which is involved in membrane fusion and NOX2 regulation.

In recent years, the mammalian class III PI 3-kinase (PI3KC3, also known as Vps34) complex, has emerged as a key regulator of several essential cellular processes through the downstream effects of its catalytic product phosphatidylinositol 3-phosphate (PI3P), including autophagy, phagosome maturation, protein sorting, and cytokinesis. Several of the mammalian Vps34-associated proteins have been identified, and it has been suggested that these proteins assemble during autophagy in a sequential manner. Initially, the regulatory subunit Vps15 associates with specific membranes and recruits the catalytic Vps34 which generates PI3P by phosphorylation of phosphatidylinositol (PI). The function of Vps34 and the downstream signaling of PI3P are regulated by the recruitment of additional Vps34-associated proteins. One such protein, Beclin-1, interacts directly with Vps34, and recent research suggests that it serves as a platform for the recruitment of other proteins. When activated, Beclin-1 is recruited together with other proteins to form macromolecular complexes. One Beclin-1 binding partner is UVRAG (UV radiation resistance-associated gene), which positively regulates Vps34 function. Moreover, UVRAG interacts with Bif-1 (endophilin B1), which has also been shown to enhance Vps34 activity. Ambr1-1 was also found to interact with and to activate Beclin-1. Recently, another Beclin-1-interacting protein, Atg14L/Barkor was identified as the targeting factor that recruits Vps34 to nascent autophagosomes. The mammalian UVRAG and Atg14L bind Beclin-1 in a mutually exclusive manner forming two distinct Vps34 subcomplexes. The other Beclin-1-associated protein Rubicon (Run domain protein as Beclin-1-interacting and cysteine-rich-containing), serves as a negative regulator of autophagosome and endosome maturation through interaction with the UVRAG complex (1, 2).

A substantial body of work has implicated all of the aforementioned Vps34-interacting proteins in the regulation of autophagy. In contrast, although there is evidence that Vps34 is

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1 The abbreviations used are: PI3P, phosphatidylinositol 3-phosphate; CLL, chronic lymphocytic leukemia; EAT, EWS/FLI1-activated transcript; ITAM, intracellular tyrosine-based activating motif; NOX2, NADPH oxidase; Rubicon, Run domain protein as Beclin-1-interacting and cysteine-rich-containing; SLAM, signaling lymphocyte-activation molecule; Slamf, SLAM family; UVRAG, UV radiation resistance-associated gene; BD, Bcl-2 homology 3 (BH3)-only domain; CCD, coiled coil domain; ECD, evolutionarily conserved domain.
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required for phagocytosis, no attempts have been made to assess the roles of the different Vps34-interacting proteins in this process. It is interesting from cellular and evolutionary perspectives that autophagy and phagocytosis are ancient, highly conserved processes controlled by a similar mechanism. Not only do both processes function in the degradation of targets within a series of sequestered membranes, they also share a common mechanism, the lysosomal degradation pathway. Recent studies have demonstrated the interactions of autophagy proteins and immune signaling molecules (3, 4). There is also overlap between the components involved in phagosome maturation and autophagy as Toll-like receptor-dependent triggering of phagocytosis recruits the autophagy proteins Beclin-1 and LC3 to the phagosome (5).

Receptors of the signaling lymphocyte-activation molecule family (Slamf), encoded by Slamf1—Slamf9 in the mouse, are adhesion molecules on the surface of hematopoietic cells that serve as co-stimulatory molecules which initiate distinct signal transduction networks in T cells, natural killer cells, and anti-gen-presenting cells (6). Additionally, our recent findings show that Slamf1 is also a microsensor that regulates bacterial phagosome functions in macrophages by recruiting Vps34 and Beclin-1 to the phagosome (7). We therefore sought to elucidate the interaction of Vps34-associated proteins with Slamf1, an important molecule in phagocytosis. The results indicate that a complex containing Vps34, Beclin-1, and UVRAG is important in Slamf1-modulated immune responses. These results allow a better understanding of the functional specificity of the Vps34-associated proteins and suggest alternative innate immune regulation mechanisms (other than autophagy) for these proteins.

EXPERIMENTAL PROCEDURES

Mice—Slamf1<sup>−/−</sup>, EWS/FL11-activated transcript 2a and 2b (EAT2a/b)<sup>−/−</sup> mice were generated as described previously (8, 9). Beclin-1<sup>−/−</sup>;Beclin-1-GFP mice were kindly provided by Dr. Zhenyu Yue (Mount Sinai School of Medicine, New York). Beclin-1<sup>+/−</sup> mice and Beclin-1<sup>+/+</sup>;Beclin-1-GFP mice were generated by breeding Beclin-1<sup>−/−</sup>;Beclin-1-GFP mice with wild-type B6 mice (The Jackson Laboratory). All mice were males between 8 and 12 weeks of age. The Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center approved all animal care and experimental procedures.

Cell Culture—HEK293 and RAW264.7 cells were cultured in DMEM. Human chronic lymphocytic leukemia (CLL) MEC-1 cells (a gift from Dr. Silvia Deaglio, University of Turin, Turin, Italy) were cultured in RPMI 1640 medium. Peritoneal macrophages were obtained from mice 72 h after intraperitoneal injection of sterile Brewer’s thioglycollate medium and cultured in DMEM. For nutrient deprived starvation, cells were washed three times with PBS and cultured in Earle’s Balanced Salt Solution for 2 h (HEK293) or 3 h (macrophages).

Transient Transfections—Transfection of HEK293 cells was performed using a standard calcium phosphate precipitation procedure. RAW264.7 cells were transfected with Xfect transfection reagent according to the manufacturer’s protocol (Clontech).
RESULTS

**Slamf1 Interacts with Vps34, Beclin-1, and UVRAG in Primary Murine Macrophages and E. coli-containing Phagosomes**—As Slamf1 is involved in the regulation of phagocytosis by murine macrophages, to investigate this process in more detail, we assessed the interactions of Slamf1 with Vps34-associated proteins in these cells. Crude LPS was given to primary macrophages to drive Slamf1 expression as a result of the low detection level of endogenous Slamf1 (supplemental Fig. 1). Slamf1 co-precipitated with Vps34, Beclin-1, and UVRAG in primary macrophages from wild-type, but not Slamf1−/− mice (Fig. 1A and supplemental Fig. 2A). To exclude the artifact of IgG heavy chain which has a molecular mass similar to that of Beclin-1, we performed the immunoprecipitation analysis in mice that express the slightly heavier Beclin-1-GFP protein (12). Slamf1 also co-precipitated with Beclin-1-GFP, confirming that the interaction was specific between Slamf1 and Beclin-1 (Fig. 1B and supplemental Fig. 2B). Currently, EAT2a/b are the only known Slamf1 adapters expressed in macrophages. To our surprise, we found that EAT2a/b are not required for the interaction of Slamf1 with Vps34, Beclin-1, or UVRAG (Fig. 1B and supplemental Fig. 2B), suggesting that Slamf1 interacts with these proteins in an EAT2-independent manner.

Our recent studies have demonstrated that Slamf1 acts as a vital regulator in the innate immune defense against Gram-negative bacteria in macrophages by independently regulating two main bactericidal processes: phagosome maturation and the production of free radical species by the NOX2 complex (7). These Slamf1-dependent mechanisms require the entry of Slamf1 into phagosomes. To test whether the interaction between Slamf1 and Vps34-interacting proteins could occur in the phagosomes, we initiated phagocytosis by adding 1.5-μm E. coli-containing magnetic particles to RAW264.7 macrophages transfected with Slamf1-GFP, GFP, or Slamf1 tailless. Slamf1 interacted with Vps34, Beclin-1, and UVRAG in phagosomes isolated from Slamf1-GFP-transfected macrophages but not from GFP or Slamf1 tailless transfected macrophages (Fig. 1C and supplemental Fig. 2C). Slamf1 did not interact with Atg14L or Ambra-1 (data not shown). In early endosomes and phagosomes, Vps34 binds to and is seemingly activated by GTP-bound Rab5. Accordingly, Rab5 is known to promote formation of PI3P on endosomes and phagosomes (13, 14). Our previous cell biology experiments showed that Vps34 co-localizes in the early phagosomes with Rab5 (7). In this study, Rab5 was also found in the Slamf1-precipitated phagosome components (Fig. 1C and supplemental Fig. 2C). These data suggest that the cytoplasmic tail of Slamf1 recruits the intracellular Vps34 complex to the phagosomes/endosomes, which is responsible for inducing the accumulation of PI3P, a regulator of both NOX2 function and phagosomal/endosomal membrane fusion.

**NOX2 Activity Is Reduced in Beclin-1 Heterozygous Macrophages**—Vps34 produces PI3P, which resides transiently in the outer layer of the phagosomal lipid bilayer, where it binds to the tethering molecule EEA1, which is involved in phagosomal/endosomal maturation, and to p40phox, which is part of the NOX2 enzyme. Our studies showed that primary Slamf1−/− macrophages produced less reactive oxygen in response to E. coli compared with wild-type macrophages (7). Next, we tested the hypothesis that, by recruiting Vps34, Beclin-1 affects phagosomal PI3P production in primary macrophages, which in turn regulates NOX2 activity. Beclin-1 heterozygous mice were used for this study because of the embryonic lethality of homozygous deficient mice (15, 16). We found that Beclin-1 heterozygous disruption resulted in decreased NOX2 activity in primary macrophages in response to E. coli. In contrast, NOX2 activity induced by S. aureus was not affected significantly in the Beclin-1 heterozygous macrophages (Fig. 2). These results are in parallel with the findings in Slamf1−/− macrophages (7), indicating that Slamf1 recruits an enzymatically active Vps34/Beclin-1/UVRAG complex, which governs NOX2 function.

**Slamf1 Interacts with Endogenous Vps34, Beclin-1, and UVRAG in Human CLL Cells and Transfectant HEK293 Cells**—Whereas Slamf1 is only expressed on the surface of a range of hematopoietic cells, Vps34, Beclin-1, and UVRAG are univer-
Slamf1 recruits a Beclin-1/Vps34/UVRAG complex

**Slamf1**

- **Figure 2.** Defective NOX2 activity in primary macrophages derived from *Beclin*−/− mice. NOX2 activity in wild-type (wt) and *Beclin*−/− peritoneal macrophages were stimulated for 0–90 min with *E. coli* or *S. aureus* and assessed with lucigenin. Pooled data from three independent experiments are shown (mean and S.D. (error bars)).

- **Figure 3.** Slamf1 associates with endogenous Vps34, Beclin-1, and UVRAG in human CLL cells or Slamf1-transfected HEK293 cells; starvation has no effects on the interaction. A, human CLL cells were stimulated with or without 100 ng/ml crude LPS overnight. Cell lysates were immunoprecipitated (IP) with anti-hSLAMF1. B, Slamf1-transfected HEK293 cells were cultured under normal or starvation conditions. Cell lysates were immunoprecipitated with anti-Slamf1 or anti-UVRAG. WCL, whole cell lysate. Data are representative of three independent experiments.

- **Figure 4.** Distinct Vps34 subcomplexes can potentially confer different functional specificity. It was reported that the UVRAG complex is important in mediating autophagy, receptor degradation, and cytokinesis, whereas Atg14L is not required for receptor degradation or cytokinesis (17). Therefore, we speculated that Slamf1 could interact with UVRAG, but we were not certain whether Atg14L would be involved in Slamf1 signal events. Given the negative role of Rubicon in endosome and autophagosome maturation, while Slamf1 is a positive regulator of phagosome maturation, we anticipated that Slamf1 would not interact with Rubicon.

- Consistent with our findings in primary cells, Slamf1 formed a complex with Vps34/15, Beclin-1, and UVRAG (Fig. 4A). In contrast, anti-Slamf1 did not pull down Atg14L or Rubicon and vice versa (Fig. 4, B and C). However, to our surprise, Slamf1 did not co-prefect with Beclin-1 in the presence of Atg14L. The interaction occurred only when co-expressing Vps34/15. Similarly, Atg14L also significantly inhibited the interaction between Slamf1 and Vps34/15. Co-expression with Beclin-1 improved this interaction. On the contrary, the association of Atg14L with Beclin-1 or Vps34/15 was not influenced by Slamf1. We reason that excess Atg14L could compete with Slamf1 for Beclin-1 binding, and Atg14L has a stronger affinity to Beclin-1 than Slamf1. The co-expression of Vps34/15 together with Beclin-1 may have enhanced the interaction of Slamf1 with Beclin-1/Vps34/15 complex. As predicted, Rubicon did not interact with Slamf1. Therefore, Rubicon may not be involved in the regulation of Slamf1-mediated signal events. Taken together, these results suggest that Slamf1 regulates NOX activity and phagosome and possibly endosome functions using a subset of the components of the ubiquitous autophagy machinery.

- Slamf1 complexes with Vps34-associated proteins is not regulated by activation of ITAM motifs—isoforms of both human and mouse Slamf1 have been described (6), including two membrane-bound forms that differ in the length of their cytoplasmic tails, a secreted form that lacks the transmembrane region, and a cytoplasmic form lacking the leader peptide. CD46 is another receptor for measles virus in addition to Slamf1. Due to alternative splicing, two groups of CD46 isoforms differing by their C-terminal intracytoplasmic domain, Cyt-1 and Cyt-2 were reported. Cyt-1 was found to interact...
with Vps34/Beclin-1 via its scaffold protein GOPC but not Cyt-2 (18). Therefore, we assessed whether the full-length and short forms of Slamf1 function differently. Slamf1 short (Tyr-288) was constructed by ablating of the downstream sequence of the first tyrosine motif mimicking the human SLAMF1 short sequence. We found that the removal of the last two tyrosine motifs had no effect on the interaction of Slamf1 with Beclin-1, whereas Slamf1 tailless completely abolished this interaction (Fig. 5A).

The cytoplasmic tail of Slamf1 contains three tyrosine sites, two of which are embedded in the intracellular tyrosine-based activating motif (ITAM), which has a high affinity for the single SH2-domain signaling molecules SLAM-associated protein (SAP) and EAT2a/b. After receptor ligation, the tyrosines present in the ITAM motifs are phosphorylated to trigger biochemical signals that are crucial for SLAM-dependent functions. To evaluate the role of the ITAM motifs in Slamf1/Beclin-1 interaction, we constructed a series of Slamf1 mutants by deleting one or two tyrosine motifs (Fig. 5B and supplemental Fig. 5A) or by a point mutation from tyrosine to phenylalanine at one or two tyrosine sites (Fig. 5C and supplemental Fig. 5B). The interaction was not disrupted by any of these mutations. These results indicate that the interactions between Slamf1 and Beclin-1 are not mediated by the activation of the ITAM motifs, which is consistent with the finding in EAT2a/b (18).

Slamf1 Binds Beclin-1 at BD and CCD Domains—Next, we mapped the binding regions of Beclin-1. We used a series of deletion mutants of Beclin-1 to analyze the domains required for Slamf1/Beclin-1 association, including mutants containing BD, CCD, ECD, BD and CCD, or CCD and ECD domains from full-length Beclin-1. The interaction of these mutants with Slamf1 was evaluated by Slamf1 immunoprecipitation assay. We found that both the BD and CCD domains were required for efficient binding of Beclin-1 to Slamf1. The surrounding region could also assist with the interaction because the binding was weaker compared with Slamf1 binding with full-length Beclin-1 (Fig. 6A).

Here, we propose a model of Slamf1 interacting with Vps34, Beclin-1, and UVRAG on the phagosome/endosome (Fig. 6B). Slamf1 interacts with the Vps34/Beclin-1/UVRAG complex by binding to the BD and CCD domains of Beclin-1, which in turn activates Vps34 to catalyze PI to PI3P, thus regulating NOX2 activity and phagosome/endosome fusion events.

DISCUSSION

Although phosphoinositides play a key role in many fundamental cell functions, specific phosphatidylinositol lipids are required to initiate microbicidal events in the phagosome. Vps34 is thought to be essential for phagolysosome formation. Its product, PI3P, which transiently accumulates in the phagosome membrane, recruits EEA1, which partakes in phagosome maturation and fusion events. Thus, Vps34 participates in the complex mechanisms that also initiate recruitment of Rab5, and possibly Rab7, to the phagosome (19). Vps34 also partakes in autophagy, an essential, homeostatic process by which cells break down their own components, which orchestrates diverse aspects of cellular and organismal responses to stimuli such as starvation or infection (1).

Whereas several Beclin-1/Vps34 protein networks actively participate in many aspects of autophagy, the interaction with Slamf1 focuses on the function of one of the Beclin-1 protein complexes in phagosome/endosome fusion and NOX2 activity. We find that Slamf1 binds to a protein complex comprised of autophagy-associated proteins Beclin-1/Vps34/UVRAG, but not to Beclin-1 associated with Atg14L or Rubicon. In the phagosomes of macrophages Slamf1 controls the killing of Gram-negative bacteria by a signaling mechanism that appears to be distinct from its signaling as an adhesion molecule. Specifically, in the E. coli phagosomes Slamf1 interacts with a Vps34 complex, which only resides in intracellular membrane compartments, but not in the plasma membrane. This interaction was also found without induction of phagocytosis in macrophages, transfectant HEK293 cells, and human CLL cells. These studies suggest that Slamf1 uses ubiquitous autophagy proteins to control phagosome and possibly endosome functions.

Our previous studies showed that Slamf1 regulated phagocytosis upon recognizing the bacterial outer membrane proteins Omp C/F (7). In this study, Omp C/F was also the main stimulator to drive Slamf1 expression in primary mouse macrophages because pure LPS had no effect. Slamf1 interacted with the Vps34/Beclin-1/UVRAG complex even without induction.
of phagocytosis in the presence of LPS. Thus, it is likely that LPS triggered Slamf1-mediated signaling events as a microbial component in addition to driving Slamf1 expression on murine macrophages. It has been reported that LPS stimulation increases the number of autophagosomes in primary human monocytes, but it fails to induce autophagy in primary mouse macrophages (20, 21). Our study showed that induction of autophagy did not enhance the interaction between Slamf1 and the Vps34/Beclin-1/UVRAG complex, and Slamf1 deficiency did not have any effect on autophagy. Therefore, Slamf1 recruits the Vps34 complex to regulate membrane fusions other than autophagy.

Slamf1 is a positive regulator of NOX2 activity in macrophages. NOX2 has been identified as one of the key sources of reactive oxygen species in immune cells. It plays multiple roles in host immunity, functioning as a source of antimicrobial reactive oxygen species, an activator of many signaling pathways, a participant in chemotaxis, an immune modulator, and a critical player in the initiation of antigen cross-presentation (22). Beclin-1 regulates NOX2 activity in parallel with Slamf1; that is, Beclin-1 deficiency disrupted the NOX2 response by macrophages to *E. coli* but not *S. aureus*. Although there is no direct coupling of Slamf1 with Beclin-1 in the NOX2 regulation, we hypothesize that Slamf1 positively regulates NOX2 function by recruiting an active Vps34/Beclin-1/UVRAG enzyme complex.

The finding that Slamf1 signals through Vps34-interacting proteins in B cells suggests that Slamf1 probably plays an important role in B cell activation through reactive oxygen species production. CLL is the most common human leukemia, accounting for ~10,000 new cases diagnosed each year in the United States. For a number of years it was generally accepted that CLL is caused by an inherent defect in apoptosis. However, studies in the last 10 years have shown a high lymphocyte count in CLL patients and B cell immune incompetence (23). Our study provides a new insight that the Slamf1-activated Vps34 signaling pathway could function as a critical event in CLL pathogenesis.

However, the mechanism and sequestration order of Slamf1 recruiting the Vps34 complex to the membrane vesicles are not clear and need to be further explored. We propose two hypotheses. First, once Slamf1 engages the pathogen, it is actively dragged into the developing phagosome, where it is responsible for recruiting a Vps34 complex to the early phagosomes/endosomes. The recruitment of active Vps34 catalyzes the conversion of PI to PI3P, a key regulator of phagosomal/endosomal maturation and NOX2 function. Second, Slamf1 has a more
general role where it sequesters Vps34-related proteins intra-cellularly, and then the Slamf1 complex is recruited to the phagosomes/endosomes.

Together, our studies indicate for the first time a connection between a cell surface receptor and the class III PI3K Vps34, which provides convincing evidence that Slamf1 positively regulates NOX2 function, phagosome/endosome maturation, and major cellular processes in a wide range of cells. All of these processes are dependent upon the transient recruitment of the Vps34 complex to the outer leaflet of the lipid bilayer of Slamf1-containing phagosomes/endosomes because the cytoplasmic tail of Slamf1 binds first to Beclin1, which, together with UVRAG, becomes the scaffold upon which the Vps34 enzyme is activated. Thus, the results support our overall hypothesis that signaling networks induced by the positive regulator Slamf1 control innate immune responses to several pathogenic bacteria and parasites. Information about the positive and negative control elements in microbial functions will allow us to explore ways to manipulate these functions for potentially therapeutic purposes in appropriate infectious bacterial diseases. Therefore, our studies can serve as a basis for human studies that are directed at intervening with Slamf1 receptor signaling upon infections with pathogens.

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