Current Topics

Metabolism and Functions of Phosphoinositides

Role of Phosphoinositide 3-Kinase in Innate Immunity

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Recent advances in our understanding of the molecular basis of mammalian host immune responses to microbial invasion suggest that the first line of defense against microbes is the recognition of pathogen-associated molecular patterns by Toll-like receptors (TLRs). Phosphoinositide 3-kinase (PI3K) is thought to participate in the TLR signaling pathway. The activation of PI3K is commonly observed after stimulation with various TLR ligands. The resultant activation of a serine-threonine protein kinase Akt leads to the phosphorylation of glycogen synthase kinase (GSK)-3β, which is active in resting cells but is inactivated by phosphorylation. GSK-3β has been linked to the regulation of a multitude of transcription factors, including NF-κB, AP-1, NF-AT, and CREB either negatively or positively. Thus, the altered activity of GSK-3β causes diverse effects on cytokine expression. Generally, activation of PI3K results in the inhibition of proinflammatory events such as expression of IL-12 and TNF-α. Thus, PI3K is a negative regulator of TLR signaling. Among the members of the Class I PI3K family, p85/p110β appears to be the subtype activated on TLR ligation, but the molecular basis for this specificity has yet to be elucidated.

Key words Toll-like receptor; phosphoinositide 3-kinase; Akt; glycogen synthase kinase-3β; NF-κB

1. INTRODUCTION

The innate immune system is the first line of host defense against infectious microbes. Toll-like receptors (TLRs) are a family of receptors that trigger innate immune reactions in response to various microbial products, such as lipopolysaccharide (LPS), flagellin, double-stranded RNA, CpG-DNA, and others. Stimulation of TLRs leads to the activation and maturation of antigen-presenting cells, such as monocytes-macrophages and dendritic cells, which is essential for the subsequent activation of antigen-specific adaptive immune responses.[1,2] Stimulation of TLRs on antigen-presenting cells also induces the production of various cytokines that control the balance between T helper types 1 and 2 (Th1 and Th2) responses.[3] For example, various TLR ligands lead to the production of interleukin (IL)-12, a key inducer of Th1 responses. The amount of IL-12 produced during stimulation is crucial for promoting the inflammatory responses to remove the invading microorganisms.[4]

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that catalyzes the transfer of the γ-phosphate group of ATP to the D-3 position of phosphoinositides. Its product, PtdIns (3,4,5)P_3 (PIP_3), targets Akt/PKB, Bruton's tyrosine kinase (Btk), PDK, atypical PKCs, phospholipase Cγ and others. Numerous studies have implicated PI3K as a regulator of TLR signaling. However, there are conflicting reports concerning the physiological role of PI3K in the signaling pathway; the inconsistent results are partly due to the inadequate use of chemical inhibitors in these investigations. Although there is ample evidence indicating that various TLR ligands activate PI3K, it is yet not clear which subtype of PI3K is activated. It still needs to be determined how TLR stimulation activates the subtype. In this paper, these issues are reviewed so as to consolidate our current understanding of the role that PI3K plays in TLR signaling.

2. PI3K SUBTYPES

Based on their primary sequences, mechanisms of regulation, and substrate specificities, mammalian PI3K can be grouped into three major classes: I, II, and III. Of the class I PI3Ks, class IA subtypes are heterodimers that consist of a catalytic subunit (p110) and a regulatory subunit (p85).[5] These subtypes are thought to be the major *in vivo* source of PIP_3 upon activation of the receptors possessing protein–tyrosine kinase activity or the receptors coupling to Src-type protein–tyrosine kinases. In mammals, there are multiple isoforms of class IA PI3K.[5] Different genes encode class IA catalytic subunits, referred to as p110α, p110β and p1110δ, while two genes encode the associating regulatory subunits, referred to as p85α and p85β. Targeted disruption of either p110α or p110β causes death at the early embryonic stage. Although the regulatory p85α and p85β subunits can compensate for each other during development, disruption of both p85α and p85β genes is lethal.[6]

Class I includes another member, PI3Kγ, which is mainly expressed in hematopoietic cells.[7—11] This subtype consists of a catalytic subunit (p110γ) and a regulatory subunit (p101), and is classified as belonging to Class IB. PI3Kγ can be activated by the $\beta\gamma$ subunits of G proteins, and thus mediates the signal from G protein-coupled receptors.[12] This property was originally thought to be the specific function of PI3Kγ. However, this opinion has been challenged by the finding that p110β is also activated by $\beta\gamma$ subunits.[9,13,14] Mice lacking PI3Kγ have been successfully generated.[10,11,15] Phagocytes from these mice have defects in their response to chemoattractants, such as formyl-Met-Leu-Phe or complement component C5a. Class II PI3Ks are 170—210 kDa pro-

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Table 1. Pathogen Recognition of TLRs

TLRs	Ligands	Adaptor
TLR1	Triacyl lipopeptides (bacteria)	MyD88
TLR2	Peptidoglycan, lipoprotein, lipopeptides, atypical LPS (bacteria). Zymosan, phospholipomannan (fungi). GPI anchor (protozoa). Envelope protein (virus)	MyD88, TIRAP/Mal
TLR3	Poly(I:C), dsRNA (virus)	TICAM1/TRIF
TLR4	LPS (bacteria). Mannan, glucuronoxylomannan (fungi). Glycoinositolphospholipids (protozoa). RSV fusion protein (virus)	MyD88, TIRAP/Mal, TICAM1/TRIF, TICAM2/TRAM
TLR5	Flagellin (bacteria)	MyD88
TLR6	Diacyl lipopeptides (bacteria)	MyD88
TLR7/8	Synthetic imidazoquinoline-like molecules, ssRNA (virus)	MyD88
TLR9	CpG DNA (bacteria, protozoa, virus). Hemozoin (protozoa)	MyD88
TLR11	Component of uropathogenic bacteria (bacteria). Profilin like molecule (protozoa)	MyD88

teins with an *in vitro* substrate specificity restricted to PtdIns and PtdIns 4-phosphate.[16] Class III PI3Ks are homologues of *S. cervisiae* Vps34p and exclusively phosphorylate PtdIns.[17]

3. TLR FAMILY AND THEIR ADAPTORS

The mammalian family of TLR includes 13 members. The ligand specificity of each member is shown in Table 1.[18] The cytoplasmic portion of TLR has a homology to that of the IL-1 receptor family. It is therefore called the Toll/IL-1 receptor (TIR) domain. When stimulated, the TIR domain of TLR binds to adaptor molecules that also contain the TIR domain as a result of the TIR/TIR interaction.[19,20] The TLR adaptor family has four known members, MyD88, TIRAP/Mal, TICAM-1/TRIF, and TICAM-2/TRAM. Studies with mice lacking individual adaptors have defined specific roles for each adaptor. MyD88 is shared by all TLRs except TLR3, while TIRAP/Mal only binds to TLR2 and TLR4,[21] TICAM-1/TRIF binds exclusively to TLR3 and TLR4,[22] and TICAM-2/TRAM binds only to TLR4.[23,24] The diversity of the adaptor molecules may partially account for the variety of inflammatory responses that occur to different ligands.

4. TLR SIGNALING

Two major pathways are activated by TLRs. The first pathway culminates in the activation of transcription factor NF-κB, which plays an important role in promoting inflammation. The second pathway results in the activation of IRFs, which regulate the expression of type I interferons (IFNs) (Fig. 2). In the first pathway, ligand binding to TLRs induces the association of a common adaptor, MyD88, with the cytoplasmic TIR domain of the receptor. MyD88 recruits IRAKs (IRAK1 and 4) to TLRs[25,26] and allows IRAK1 to bind TRAF6, a key event leading to the activation of TAK1.[27] TAK1 activates the IKK complex consisting of IKKα, IKKβ, and NEMO/IKKγ.[28] The activated IKK complex phosphorylates IκB leading to its ubiquitination. The resultant degradation of IκB enables the bound NF-κB to translocate into the nucleus and to induce the expression of inflammatory cytokines. This signaling pathway bifurcates at TAK1. TAK1 has the ability to act as a MAPK kinase kinase and is known to activate MAPK kinases, including MKK3/6, which in turn activate p38MAPK and SAPK/JNK.[28] This branch is important in the expression of some inflammatory cytokines and in the induction of apoptosis. Another MAPK kinase kinase, Cot/Tpl2, which phosphorylates MEK1/2, is also activated

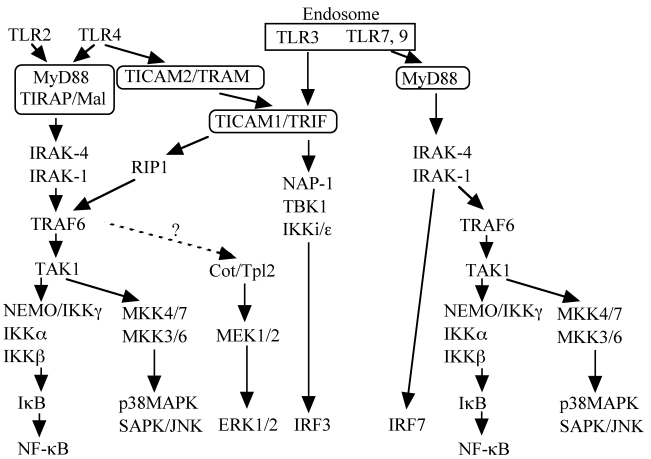

Fig. 1. TLR Signaling Pathways

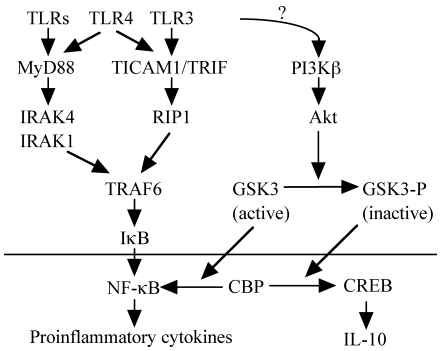

Fig. 2. Mechanism of PI3K-Dependent Regulation of TLR-Mediated Cytokine Production

downstream of TLR, although the mechanism is yet unknown.[29] This branch leads to the activation of Erk1/2 and is indispensable for the expression of some cytokines and cyclooxygenase-2.

The stimulation of TLR3 on endosomal membranes or the stimulation of TLR4 on cell surfaces activates an additional pathway that induces type I IFNs.[30] Treatment of macrophages and dendritic cells (DCs) with dsRNA or LPS results in IRF3 activation, which leads to the expression of IFN-β and the IFN-inducible gene products. This pathway requires a TIR domain-containing adaptor TICAM1/TRIF that interacts with TBK1, IKK*i*, RIP1, and TRAF6. The nuclear translocation of IRF3 is triggered by phosphorylation with TBK1 and IKK*i*. In the case of TLR4, an additional

adaptor, TICAM2/TRAM, is also required for IRF3 activation. Activation of endosomal receptors, such as TLR7 and TLR9, induces the secretion of large amounts of IFN-α and IFN-β. The signal derived from these receptors depends entirely on MyD88. Formation of a signaling complex consisting of MyD88, IRAKs, and TRAF6 induces the phosphorylation of IRF7 by IRAK-1 and allows the nuclear translocation of this transcription factor.[31)]

5. GLYCOGEN SYNTHASE KINASE (GSK)-3 AS A TARGET OF PI3K

Before discussing the role of PI3K as a regulator of TLR signaling, we will briefly describe some properties of GSK-3β, which is one of the downstream targets of PI3K. GSK-3 is a multifunctional serine/threonine kinase found in all eukaryotes. The two highly homologous GSK-3 proteins, GSK-3α and GSK-3β are critical factors in the proper regulation of a wide variety of signaling proteins and transcription factors, including cyclin D1, c-Jun, NF-AT, and β-catenin.[32)] Due to the TNF-α-dependent apoptosis of hepatocytes, genetic targeting of GSK-3β[33)] is lethal to embryos. This phenotype is similar to that of mice lacking IKKβ or the p65 subunit of NF-κB.[34,35)] In fibroblasts lacking GSK-3β, no defects in degradation of IκB or nuclear translocation of p65 were detected. However, in these cells, DNA binding of NF-κB in gel shift assay, as well as the NF-κB-dependent response in luciferase reporter assays, was diminished.[33)] It is proposed that GSK-3β is required for the efficient localization of p65 to the promoter regions of a specific subset of the NF-κB-regulated genes.[36)]

GSK-3 is constitutively active in resting cells but can be inactivated through phosphorylation with other protein kinases. The consensus sequence for GSK-3 substrates is (S/T)-X-X-X-(S/T), where the first S/T is the target residue and X is any amino acid. The efficacy of catalysis is greatly increased when the last S/T of the substrate has been phosphorylated by another priming kinase.[32)] The mechanism of this acceleration has been found to be related to phosphorylation of the last S/T, which allows this residue to bind a pocket of positive charge that is located near the catalytic site of GSK-3. When a Ser residue in the N-terminal region of GSK-3 (Ser-21 in GSK-3α or Ser-9 in GSK-3β) is phosphorylated, the phosphate-binding pocket is occupied by an intramolecular interaction with this phosphorylated Ser residue. This interaction effectively inhibits the activity of GSK-3 by changing the conformation of the catalytic site and also by preventing the binding of the primed substrate. The N-terminal Ser residue of GSK-3 is within the consensus sequence of the substrates for protein kinase Akt. Thus, GSK-3 can act as a downstream player of PI3K-Akt signaling.[37)] In addition to Akt, several other protein kinases, including cyclic AMP-dependent kinase and certain PKCs, are known to phosphorylate the inhibitory N-terminal site of GSK-3.[38,39)]

6. DIFFICULTIES IN STUDYING THE ROLE OF PI3K IN TLR SIGNALING

The role of PI3K in a certain signaling pathway is often examined by the use of pharmacological tools. Two inhibitors, wortmannin and LY294002, have been successfully used to clarify the physiological roles of PI3K. However, pharmacological studies always face the problem of specificity. Wortmannin has been shown to inhibit myosin light chain kinase and PI3K-related protein kinase, while LY294002 is an effective inhibitor of casein kinase 2. It is difficult to use wortmannin when high concentrations of cysteine and dithiothreitol are included in the assay mixture, due to the formation of adducts with nucleophils that inactivate wortmannin. This issue is particularly prominent when the effects of the inhibitors are determined after prolonged incubation. It often takes several hours to see their effects on the production of cytokines and the activation of transcription factors. Their effects on autocrine/paracrine factors also have to be considered. Due to these limitations, pharmacological studies that use PI3K inhibitors sometimes report conflicting results. Typically, we have reported that wortmannin enhances TLR-mediated cytokine production, while LY294002 inhibits these responses.[40)] The inhibition caused by LY294002 can be observed even in the presence of wortmannin. On the other hand, transfection of shRNA targeting p110β of PI3K or a dominant negative mutant of p85 shows similar effects to wortmannin exposure.[40)] These results indicate that the inhibitory effect of LY294002 on cytokine production is not the result of PI3K inhibition. This is confirmed by the fact that the effect of LY294002 on the TLR response is mimicked by its inactive analogue, LY303511, which does not inhibit PI3K.[41)]

The use of the mutants of PI3K and Akt is considered to be a more specific way to analyze the PI3K-dependent pathways. However, it is not easy to examine the direct effects of these mutants on cytokine production in antigen-presenting cells, since plasmids harboring these mutants are difficult to transfect into hematopoietic cells. Thus, many studies use HEK293 cells and examine the mutants' effects on transcription factors with a luciferase reporter assay. However, the results of this powerful approach should be carefully verified, because the intracellular environment in these artificial cells may be different from those of antigen-presenting cells. A recent intriguing approach is the use of mice that lack the α-subtype of the regulatory subunits of class IA PI3K (p85α).[42)] The alternative regulatory subunit p85β can rescue p85α functions in most tissues. However, the hematopoietic cells from these mice are thought to have impaired class IA PI3K function, because p85α is dominantly expressed in these cells.

Another problem that obscures the function of PI3K in TLR signaling is derived from the fact that some TLR ligands are first incorporated into the cells by endocytosis. Irrespective of the target particle, PI3K is indispensable for this process.[43—45)] Therefore, the role that PI3K plays in TLR signaling is further obscured in these cases; the function of PI3K in engulfment must be distinguished from its role in TLR signaling. Finally, the action of PI3K/Akt might be varied and complex even after artificial results are carefully excluded. In fact, it has recently been shown that production of most cytokines is down-regulated, while the production of other cytokines is up-regulated by the PI3K/Akt pathway.

7. POSITIVE REGULATION OF TLR SIGNALING BY PI3K

Although in human fibroblasts, TLR3 is located both on the cell surface and the endosome, it is preferentially located on the endosome in plasmacytoid DC (pDC) and macrophages. Similarly, TLR7 and TLR9 exist in cytosolic compartments in the vicinity of the plasma membrane in pDC.[30] Therefore, to stimulate their receptors, ligands of these TLRs, such as nonmethylated CpG DNA, should be incorporated into phagocytes by endocytosis. The engulfment of diverse targets, such as IgG-coated particles,[45] both opsonized and unopsonized zymosans (unpublished data), apoptotic cells,[46] and fluid phase reagents,[47] is susceptible to PI3K inhibitors, though the mechanism is not fully understood. Since class III PI3Ks have been implicated in phagosome maturation,[46] it is expected that TLR3-, TLR7-, and TLR9-mediated events are impaired by PI3K inhibitors at an initial step before receptor ligation. Although this mechanism is not related to "the inhibition of TLR-derived signaling", it provides one possible explanation for reports that CpG-induced events are ultimately dependent on PI3K.[48,49] On the other hand, in several studies, inhibition of PI3K was reported to enhance TLR3- and TLR9-mediated cytokine production (Table 2). As described in the following sections, class IA PI3K often acts as a powerful negative regulator of TLR signaling (negative regulatory role of PI3K). Thus, it is possible in these cases that the residual incorporation of ligands, even in the presence of PI3K inhibitors, triggers downstream signals, which is markedly augmented due to the inhibition of class IA PI3K activity.

In several species, the inhibition of PI3K is reported to impair TLR signals other than TLR3, TLR7, and TLR9 (Table 2). In human clonic epithelial cells, the dominant negative forms of Akt and PI3K (Δp85) inhibit TLR5-mediated cytokine production.[50] In lung neutrophils, LPS-induced activation of NF-κB and the production of IL-1β and TNF-α are inhibited by PI3K inhibition.[51] The inhibition of NF-κB by wortmannin has been reported in U937 (granulocytes), Jurkat (T cells), HeLa cells, and H4 glioma cells.[52] In contrast, most studies, especially those dealing with macrophages and DCs, indicate that PI3K inhibition augments the TLR-in-

Table 2. Regulation of TLR-Mediated Events by PI3K

TLR	Cell	Action of PI3K	Refs.
TLR4	Mouse macrophages	Augmentation of NO production by wort.	57
TLR4	Raw264.7	Augmentation by wort and suppression by LY of NO production.	79
TLR4	Mouse macrophages	Inhibition of iNOS expression by co-activation of receptor tyrosine kinase in a manner dependent on PI3K.	69
TLR4	Raw264.7	Augmentation by wort, LY or p110(DN) and suppression by p110 (CA) of iNOS expression.	58
TLR4	C6 glial cells	Augmentation by wort or p85 (DN) of iNOS production.	68
TLR2	THP1, 293-TLR2	Inhibition of NF-κB by wort, LY, p85 (DN), or Akt (DN).	74
TLR4	U937, Jurkat, Hela, H4 glioma	Inhibition of NF-κB and AP-1 by wort.	52
TLR4	β-pancreatic cells	Inhibition of iNOS expression by IGF-1 in a manner susceptible to wort, LY or p85 (DN). Inhibition of iNOS promoter by p110 (CA).	67
TLR4	Mouse peritoneal neutrophils	Inhibition of IL-1β and TNF-α production through suppression of NF-κB in PI3Kγ−/− cell. Inhibition by wort or LY.	51
TLR9	BMDC, 293-TLR9	Inhibition of CpG uptake and IL-12 production by wort.	49
TLR4	Human alveolar macrophages	Stabilization of cyclooxygenase 2 mRNA by LY or p38MAPK inhibitor.	62
TLR4	THP1, human PBMC	Augmentation by wort, LY or Akt (DN) and suppression by Li (GSK3 inhibitor) of TNF-α expression and NF-κB activation.	59
TLR2, 4, 9	p85α−/− DCs	Augmentation of IL-12 production and suppression of IL-4 production in p85−/− mouse DC and also in wort- or LY-treated cell.	61
TLR4	Raw264.7	Inhibition of IL-1β production and NF-κB translocation by wort.	80
TLR2	Human PBMC	Augmentation of IL-12 production and suppression of IL-10 production through inhibition of NF-κB p65 by wort or LY.	81
TLR3	293-TLR3	Inhibition of IRF3 by wort, LY, p110 (KD) or siRNA targeting p110.	75
TLR4	THP-1	Inhibition of LPS tolerance by wort through inhibition of IRAK-1 degradation.	64
TLR4	Raw264.7	Augmentation of NF-κB activation by overexpression of SHIP. Lower IL-6 and TNF-α production in SHIP−/− cells.	65
TLR4	Raw264.7	PI3K-independent inhibition of NF-κB by LY.	41
TLR3, 4	Human DC, 293-TLR3, 4	Augmentation by wort, LY or p85 (DN) and suppression by p110 (CA) of NF-κB activation and IFN-β synthesis.	60
TLR4	Human monocytes	C5a-mediated inhibition of IL-12 production in a manner susceptible to wort or Akt inhibitor.	66
TLR2, 4, 5, 9	Human PMN, monocytes	Augmentation of IL-10 production and inhibition of IL-12 production by LY, wort or siRNA targeting GSK3.	71
TLR9	Raw264.7, 293-TLR9	Inhibition of CpG uptake and IL-12 production by wort or Vps34p (KD).	48
TLR9	CD4+ T cells	Inhibition of IL-2 production in defect of activation of PI3K, Akt and GSK3.	54
TLR5	Human epitherial cells	Inhibition of IL-8 production by LY, Akt (KD) or p85 (DN). Association of p85 and MyD88.	50
TLR5	Epitherial cells	Augmentation by wort, LY or p85−/− and suppression by p85 (CA) of proinflammatory gene expression (NO, IL-6, IL-8).	70
TLR2	Human monocytes	Augmentation of IL-23 production and suppression of IL-10 production by LY, wort or rapamycin.	82
TLR2, 3, 4, 9	Macrophages (mouse, human)	PI3K-dependent augmentation by wort and -independent suppression by LY of IL-12 and NO production (using siRNA targeting PI3Kβ).	40

LY; LY294002, wort; wortmannin, DA; dominant negative mutant, CA; constitutive active mutant, KD; kinase dead, DC; dendritic cell.

duced activation of NF-κB. These conflicting results suggest that the effect of PI3K on NF-κB activation may depend on the cell species. As described above, the PI3K-induced activation of Akt can lead to the inhibition of GSK-3, which is required for the efficient expression of several NF-κB-regulated genes.[36] Thus, in these cells, the inhibition of PI3K may augment the activation of NF-κB through GSK-3 activation. On the other hand, a considerable number of studies have reported that PI3K inhibition leads to the down-regulation of NF-κB in T cells,[53,54] B cells,[55,56] and many other cell types. One possible explanation for this discrepancy is the fact that PI3K has multi-functional roles that may regulate various signaling pathways leading to NF-κB activation. Alternatively, GSK-3 is regulated by a variety of protein kinases, which may control each other to keep an appropriate balance.

8. NEGATIVE REGULATION OF TLR SIGNALING BY PI3K

Prior to the discovery of TLR, wortmannin was reported to enhance LPS-induced NO production in macrophages.[57] The role of PI3K in this regulation was confirmed by the finding that a dominant negative mutant of PI3K (p110DN) increased, while a constitutively active form of PI3K (p110CAAT) suppressed the expression of inducible NO synthase (iNOS).[58] In human monocytic THP1 cells, as well as in human peripheral blood monocytes, inhibitors of PI3K and a dominant negative Akt enhance the LPS-induced activation of AP-1, Egr-1, and NF-κB, which results in increased production of TNF-α.[59] The effects of PI3K inhibition are antagonized by lithium-induced GSK-3 inhibition. The negative regulation of TLR signaling by PI3K is not restricted to TLR4. In human DCs, the inhibitors of PI3K enhance TRIF-dependent NF-κB activation and IFN-β synthesis downstream of TLR3 and TLR4.[60] The role of PI3K was confirmed in HEK293 cells expressing TLR4 or TLR3 using mutant forms of PI3Ks. In addition, TLR2-, TLR4-, TLR5-, and TLR9-mediated production of IL-12, IFN-γ, and TNF-α was found to be increased in DCs obtained from p85α−/− mice.[61] The effects of PI3K depletion are reproduced by wortmannin and by LY294002 administration. TLR-mediated synthesis of proteins other than iNOS and cytokines is also increased by PI3K inhibition. The induction of cyclooxygenase by LPS is enhanced in the presence of wortmannin.[62] IRAK-1, which plays a central role in TLR signaling by connecting MyD88 and TRAF6 or by phosphorylating IRF7, is ubiquitinated and degraded in proteosomes upon TLR ligation.[63] The degradation of IRAK-1 is relevant to LPS tolerance, which is demonstrated by unresponsiveness to second or prolonged LPS stimulation. Interestingly, PI3K inhibitors abrogate LPS-induced degradation of IRAK-1 and result in the inhibition of the tolerance.[64] It is likely that re-synthesis of IRAK-1 may be regulated through the Akt/GSK-3 pathway.

SHIP is a lipid phosphatase that metabolizes PtdIns-3,4,5-P_3 (PIP_3) to produce PtdIns-3,4-P_2. Transfection of SHIP into the macrophage cell line Raw264.7 up-regulates LPS-induced activation of NF-κB.[65] This phenomenon is explained by the inhibition of Akt due to a decrease in PIP_3. This notion is supported by the finding that macrophages from SHIP−/− mice produce less TNF-α and IL-6.[65] The negative regulation of PI3K in TLR signaling is also suggested by the co-stimulation of TLR and other receptors. In mouse macrophages, stimulation of Gi-coupled receptors (C5a receptors) results in the inhibition of LPS-induced IL-12 production.[66] This effect of C5a is susceptible to wortmannin. The activation of a receptor tyrosine kinase, RON, inhibits the LPS-induced expression of iNOS in murine peritoneal macrophages; this inhibition is recovered by wortmannin administration.[67]

In C6 glial cells, LPS or wortmannin alone has no effect, but their combination induces iNOS production.[68] The effect of wortmannin can be reproduced by transfection of the dominant negative PI3K (Δp85). In β-pancreatic cells, LPS induces the expression of iNOS, which is abrogated by insulin-like growth factor-1 (IGF-1).[69] The effect of IGF-1 is inhibited by wortmannin, LY294002, and the overexpression of Δp85. In epithelial cells, flagellin-induced (TLR5-mediated) production of NO, IL-6, and IL-8 was increased in the presence of wortmannin or LY294002[70]; a similar enhancement was observed in the p85α−/− cells. Thus, the negative regulation of TLR signaling by the PI3K/Akt pathway is considered to be operative in diverse cell types.

9. THE NEGATIVE AND POSITIVE ROLE OF PI3K IN TLR-MEDIATED CYTOKINE PRODUCTION THROUGH GSK-3 REGULATION

Fukao was the first to report the specific regulatory role of PI3K with respect to cytokine species in TLR signaling. The production of IL-12 induced by TLR2-, TLR3-, or TLR9-stimulation is increased, while the production of IL-4 and IL-5 is decreased in DCs obtained from p85−/− mice[61]; the researchers concluded that Th-1 responses are increased, while Th-2 responses are decreased in the p85−/− cells.[42] Recent work by Martin *et al.* indicated that the inhibitors of PI3K and Akt suppress IL-10 production but augment IL-12 production in monocytes or peripheral blood mononuclear cells stimulated with agonists of TLR2, TLR4, TLR5, or TLR9.[71,72] They also show that GSK-3 inhibition by siRNA and certain pharmacological reagents increases IL-10 production but suppresses the release of pro-inflammatory cytokines, such as IL-12, TNF-α, IL-6, IFN-γ and IL-1β. These results suggest that the PI3K-Akt pathway's ability to inactivate GSK-3 is responsible for the differential regulation of cytokine production. GSK-3 can differentially regulate the production of pro-inflammatory and anti-inflammatory cytokines by affecting the relative amounts of active transcription factors, CREB and the p65 subunit of NF-κB, that interact with a common coactivator CBP (CREB-binding protein). Since GSK-3 inactivates CREB, the inhibition of GSK-3 augments the binding of CREB to CBP and competitively suppresses the binding of NF-κB p65 to the coactivator.[71] This mechanism explains how the TLR-mediated inhibition of GSK-3 increases the CREB-dependent production of IL-10 while it decreases NF-κB-dependent production of pro-inflammatory cytokines.

10. THE PI3K SUBTYPE INVOLVED IN TLR SIGNALING

Previous reports indicate that the class IA subtype of PI3K is responsible for the TLR-mediated activation of Akt. Most of these studies used a dominant negative mutant of the regulatory subunit p85 (Δp85) to demonstrate the involvement of PI3K. It was also shown that TLR-mediated cytokine production is enhanced in macrophages obtained from p85−/− mice.[61] Recently, using an shRNA technique, we established Raw 264.7 macrophages that lack either p110α or p110β.[73] The results obtained using these cells indicate that p110β is involved in TLR signaling, while p110α is not. Interestingly, p110β-deficient cells show enhanced expression of iNOS in response to stimulation with TLR2, TLR3, TLR4, and TLR9, and perhaps other TLRs (in preparation).

Class III PI3K is known to regulate CpG-induced cytokine production and NF-κB activation,[48] during which the process of endocytosis may also be regulated. Class III PI3K acts upstream of MyD88 and regulates CpG uptake; this is in sharp contrast to class I PI3K, which acts downstream of MyD88. Although we have observed that PI3K activity within macrophages obtained from PI3Kγ−/− mice responds normally to various TLR ligands (unpublished), one report indicates that the LPS-induced activation of Akt is abrogated in lung edema neutrophils obtained from PI3Kγ−/− mice.[51] This may be due to the loss of chemotactic activity of these neutrophils.

11. MECHANISM OF PI3K ACTIVATION IN TLR SIGNALING

PI3Ks of the Class IA subtype are activated when the SH2 domains of p85 bind the tyrosine-phosphorylated proteins that possess a pYXXM motif.[5] The signaling molecules that are recruited to p85 upon stimulation of TLRs are not completely understood. Several reports indicate that p85 binds the receptor itself to activate PI3K. For example, TLR2 is tyrosine-phosphorylated within the YXXM motif of its TIR domain and directly binds p85.[74] Likewise, the TIR domain of TLR3 is phosphorylated on the tyrosine residue. Although the tyrosine residue is not within the optimal consensus sequence for p85 binding (YXXM), phosphorylation is reported to recruit p85.[75] It was shown that most TLRs, including at least TLR2, TLR3, TLR4, TLR5, and TLR9, activate PI3K. This fact suggests that adaptor proteins shared by TLRs may also play a role in activating PI3K. One candidate protein is MyD88, an adaptor shared by all TLRs except TLR3. In fact, MyD88 associates with p85 in response to flagellin and LPS.[50] MyD88 with a point mutation in the YXXM motif (Y257F) fails to interact with p85.[54] It is reported that IL-1β-, IL-18-, and LPS-induced activation of PI3K is diminished in IRAK-1 deficient cells.[76] Since IRAK-1 is known to directly bind MyD88, the protein kinase may play a role in MyD88-dependent PI3K recruitment. Upon TLR3 stimulation, another adaptor, TICAM1/TRIF, may act in the recruitment of p85. The association of the catalytically active form of p110α (p110αCAAT) with TRIF has been reported when both are transfected into 293T cells.[60] RIP1 is another candidate for the adaptor of p85 in TLR signaling, since neither LPS nor CpG activates Akt in cells lacking RIP1.[77] However, the mechanism by which RIP1 links TLR and PI3K is completely obscure.

Stimulation of TNF receptors induces the binding of TRAF6 to Src-family tyrosine kinases to activate PI3K.[78] Since TLRs share several properties with TNF receptors, it is intriguing to speculate that the TRAF6/Src-dependent mechanism of PI3K activation functions in TLR signaling pathways as well. This hypothesis may explain the loss of TLR-mediated activation of Akt in macrophages that lack MyD88, RIP1, or IRAK-1. It is interesting to note that TRAF6 is shared by all TLRs without exception. Further studies are needed to elucidate the mechanism by which PI3K is activated in TLR signaling.

12. CONCLUDING REMARKS

Although stimulation of TLRs results in the activation of PI3K, this activation is rather moderate. As determined by Western blotting, TLR-mediated Akt activation is less than 10% of that which occurs with other well-known stimuli, such as insulin and IGF-I in various cell types or C5a in macrophages (unpublished data). Furthermore, activation of PI3K is not an indispensable event in most of the TLR-mediated responses. PI3K is a negative feedback regulator that suppresses excessive immune responses. The most unique characteristic of the regulation is the selective suppression of pro-inflammatory responses and the augmentation of anti-inflammatory responses. In fact, an inhibitor of GSK-3 can protect mice against septic shock.[71] However, given the diverse roles of PI3K, Akt, and GSK-3, drugs that disrupt these signaling molecules may be difficult to use clinically. The putative molecule connecting TLR and PI3K may be a possible target for drug therapy. Further studies are required to fully understand how PI3K is integrated in TLR signaling pathways.

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