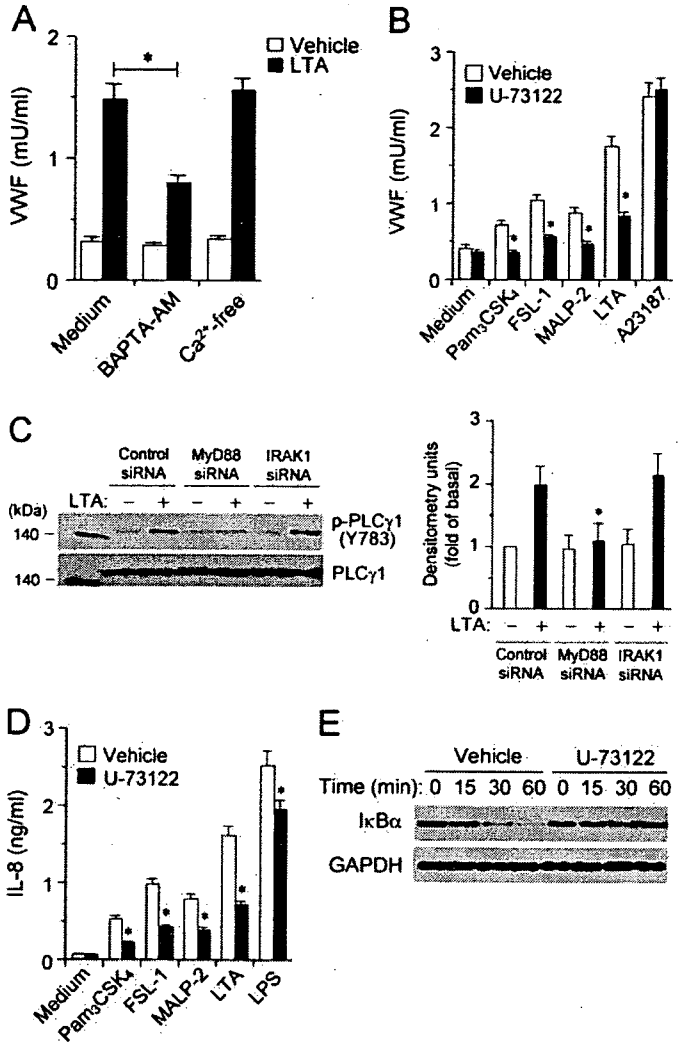

FIGURE 5. **PLCγ-mediation of TLR2-activated Weibel-Palade body exocytosis and NF-κB activation.** *A*, confluent HAECs were pretreated with 20 μM BAPTA-AM for 30 min or incubated in Ca^{2+}-free media. Then the cells were washed and stimulated with 10 μg/ml LTA for 60 min in Ca^{2+}-free media. The amounts of VWF released into the media were measured by ELISA. Each value is the mean ± S.D. ($n = 3$). *, $p < 0.01$. *B*, HAECs were pretreated with 10 μM U-73122 for 30 min and then washed and stimulated with Pam_3CSK_4 (10 μg/ml), FSL-1 (1 μg/ml), MALP-2 (1 μg/ml), LTA (10 μg/ml), and A23187 (1 μM) for 60 min. The amounts of VWF released into the media were measured by ELISA. Each value is the mean ± S.D. ($n = 3$). *, *versus* vehicle group, $p < 0.01$. *C*, HAECs transfected with MyD88- or IRAK1-specific or control siRNA were stimulated with 10 μg/ml LTA for 90 min. Immunoblot analysis was then performed to examine the expression of phosphorylated PLCγ1 (Y783) and total PLCγ1 (*left*). Immunoreactive bands were quantified by a densitometer (*right*). Results are expressed as means ± S.D. of three independent experiments. *, *versus* control group, $p < 0.01$. *D*, HAECs were pretreated with 10 μM U73122 for 30 min and then washed and stimulated with Pam_3CSK_4 (10 μg/ml), FSL-1 (1 μg/ml), MALP-2 (1 μg/ml), LTA (10 μg/ml), and A23187 (1 μM) for 4 h. The amounts of IL-8 released into the media were measured by ELISA. Each value is the mean ± S.D. ($n = 3$). *, *versus* vehicle group, $p < 0.01$. *E*, HAECs were pretreated with 10 μM U73122 for 30 min and then washed and stimulated with 10 μg/ml LTA for the indicated period. Immunoblot analysis was then performed to examine the expression of IκBα and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

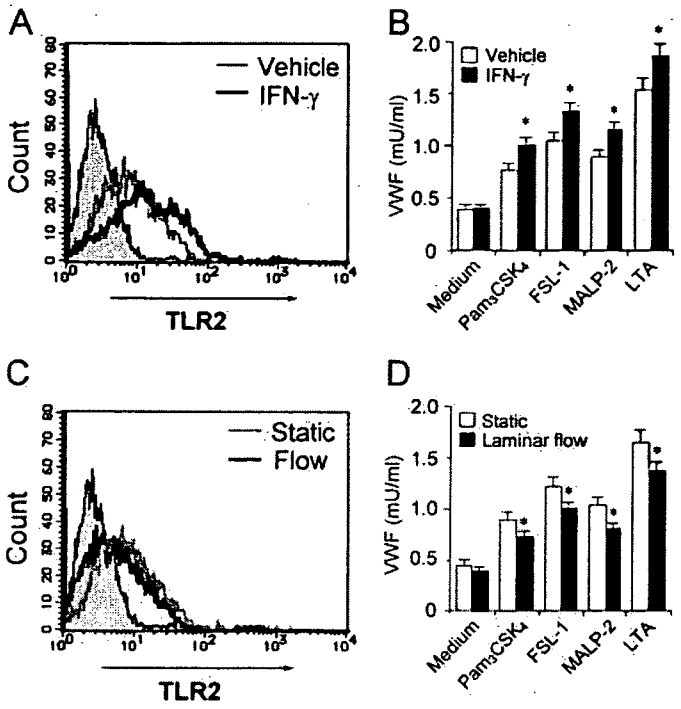

FIGURE 6. **Regulation of TLR2-mediated Weibel-Palade body exocytosis.** *A* and *B*, HAECs were treated with IFN-γ for 12 h. Surface TLR2 expression was detected by flow cytometry (*A*). The *shaded histogram* indicates cells stained with control antibody. The cells were washed and stimulated with Pam_3CSK_4 (10 μg/ml), FSL-1 (1 μg/ml), MALP-2 (1 μg/ml), and LTA (10 μg/ml) for 60 min. The amounts of VWF released into the media were measured by ELISA (*B*). Each value is the mean ± S.D. ($n = 3$). *, *versus* vehicle group, $p < 0.05$. *C* and *D*, HAECs were incubated under laminar flow for 12 h. Surface TLR2 expression was detected by flow cytometry (*C*). The *shaded histogram* indicates cells stained with control antibody. The cells were then stimulated with Pam_3CSK_4 (10 μg/ml), FSL-1 (1 μg/ml), MALP-2 (1 μg/ml), and LTA (10 μg/ml) for 60 min. The amounts of VWF released into the media were measured by ELISA (*D*). Each value is the mean ± S.D. ($n = 3$). *, *versus* static group, $p < 0.05$.

lipopeptide FSL-1.[3] Sequentially or simultaneously, both PLCγ- and IRAK1-mediated signaling pathways activate NF-κB, by which production of various proinflammatory cytokines, and expression of adhesion molecules such as ICAM-1 are induced to promote adherence and activation of platelets and leukocytes (37). The delayed Weibel-Palade body exocytosis with *de novo* protein synthesis is further activated in the cells. Therefore, endothelial TLR2 may be able to function as a primary initiator and a modulator of artery inflammation through these early-phase endothelial responses after recognition of cognate agonists.

We investigated the responsiveness of HAECs toward common bacterial constituents. For the TLR2 agonists, we prepared several compounds that have already been proposed to function as TLR2 agonists, because TLR2 forms a complicated recognition system and because human endothelial cells from different vascular beds show different degrees of responsiveness to TLR2 agonists (32, 38, 39). Unexpectedly, PGN, unlike other TLR2 agonists, could not activate either Weibel-Palade body exocytosis or IL-8 production (Figs. 1*D* and 3*A*). The issue of recognition of PGN by TLR2 is still controversial. The existence of an intracellular receptor for PGN (NOD2) further complicates this matter. However, Gupta's group recently concluded that PGN is in fact recognized by TLR2 by showing that muramidase treatment of PGN abolished the TLR2-stimulating activity (8). We showed that recognition of our PGN was at least dependent on TLR2 (Fig. 3*A*). It has been shown that PGN directly binds TLR2 *per se* (40), whereas bacterial lipopeptides

[3] T. Into, Y. Kanno, J.-i. Dohkan, M. Nakashima, M. Inomata, K.-i. Shibata, C. J. Lowenstein, and K. Katsushita, unpublished data.

are thought to directly interact with TLR2-associated molecules such as CD14 and LBP but not with TLR2 *per se* (7, 41, 42), suggesting the existence of different ligand-recognition mechanisms by TLR2. Furthermore, a novel family of PGN-binding proteins such as peptidoglycan recognition proteins has been found (43) and might enable discrimination of PGN from other TLR2 agonists. Thus, PGN may be recognized by a TLR2 recognition system different from that for LTA and lipoproteins/lipopeptides. Collectively, HAECs express functional TLR2 to respond to several TLR2 agonists, including lipopeptides and LTA, but may lack a PGN-recognition system resulting in an inability to respond to PGN. Moreover, aortic endothelial cells may particularly recognize diacylglyceride-containing bacterial lipid derivatives (LTA and bacterial lipopeptides), recognition of which has recently been reported to depend on TLR6 and CD36 (11).

We also showed that the TLR4 agonist LPS did not activate Weibel-Palade body exocytosis (Fig. 1*D*). Although the reason for this is not clear, several lines of evidence obtained in previous studies may provide an explanation. For example, TLR4 expression has been reported to localize intracellularly in artery endothelial cells (44). This observation suggests that TLR4 in artery endothelial cells may be lacking in induction of phospholipid-dependent signaling events, including PLCγ activation, which are commonly intrinsic to the signaling receptors spanning the cell membrane. Further investigation is needed to determine the reason.

Several properties of endothelial TLR2 have been proposed to be involved in the development of atherosclerosis. First, endothelial TLR2 expression is enhanced by proinflammatory stimuli, such as TNF-α, IFN-γ, and LPS (32), and by SP-1-dependent machinery in areas of disturbed blood flow such as lesion predilection within the aortic tree and heart (33). The expression level of TLR2 is indeed increased in an atherosclerotic lesion in humans (45). Furthermore, a recent study has revealed that complete deficiency of TLR2 in atherosclerosis-prone LDLR-null mice leads to an apparent reduction in the formation of lesions (46). Proinflammatory signaling pathways downstream of TLR2 have been thought to be activated through TIRAP/Mal, MyD88, IRAK-1, and TRAF6 in endothelial cells. Other pathways involving PI3K and the downstream protein kinase Akt/PKB (47), the Rho family GTPase Rac1 (48), and the redox-activated mitogen-activated protein kinase kinase kinase ASK1 (49) also link TLR2 signaling to the NF-κB pathway. In this study, we showed that PLCγ also mediated the NF-κB pathway downstream of TLR2 in HAECs, although involvement of PLCγ in the TLR2 proinflammatory signaling has been described in several reports (27, 50). Because PLCγ isoforms are thought to be activated by both generation of phosphatidylinositol 3,4,5-triphosphate by PI3K and tyrosine phosphorylation, we found the latter process downstream of TLR2 was dependent on MyD88 but not IRAK-1 (Fig. 5*C*). Recent studies have suggested a linkage of TLRs and tyrosine kinases, including Syk via MyD88-STAP-2 interaction (51) and Btk via direct interaction with TIR domain (52), both of which have been shown to activate PLCγ isoforms. Moreover, Btk-induced phosphorylation of TIRAP/Mal has recently been reported to play an important role in TLR signal transduction

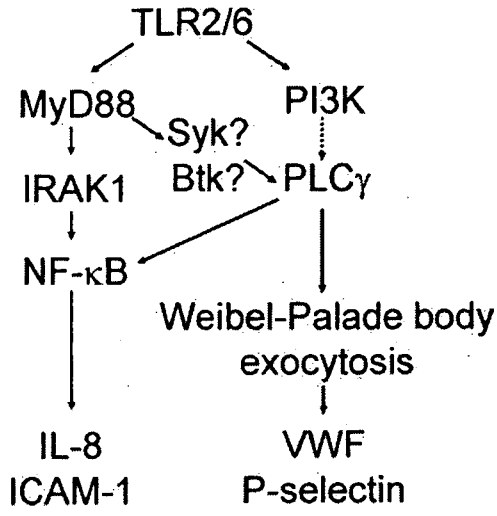

FIGURE 7. **The proposed schematic for TLR2 regulation of early-phase inflammatory signaling in human aortic endothelial cells.**

(53), which may occur at a phosphatidylinositol diphosphate-rich membrane compartment after recruitment of MyD88 to membrane-localized TIRAP/Mal (54). A schematic of signaling pathways proposed here is shown in Fig. 7.

Endothelial activation by several proinflammatory agents has been shown to increase endothelial responsiveness toward TLR2 agonists via up-regulation of TLR2 expression (32). Increased endothelial TLR2 expression increased the magnitude of TLR2-mediated exocytosis of Weibel-Palade bodies (Fig. 6*B*) and endothelial responses (38), suggesting enhanced responsiveness of endothelial cells to pathogens in inflamed lesions. In contrast, fluid shear decreased the magnitude of TLR2 ligand-stimulated Weibel-Palade body exocytosis (Fig. 6*D*). Physiological fluid shear stress has been suggested to have atheroprotective effects *in vivo*, because atherosclerosis preferentially occurs in an area of disturbed flow or a low level of shear stress, whereas regions with steady laminar flow and physiological shear stress are protected. Disturbed flow or a low level of shear stress has been reported to regulate expression of various regulatory molecules of endothelial activation, by which atherosclerotic processes may be accelerated in the sites. These observations are consistent with the previous finding that physiological fluid shear stress decreases endothelial TLR2 expression via impaired activity of the transcriptional factor SP1 (33). Thus, our results raise the possibility that bacterial constituent-induced Weibel-Palade body exocytosis can be physiologically or pathologically regulated in particular circumstances of the vessel wall.

In conclusion, our study focused on endothelial exocytosis induced by bacterial pathogens and showed a linkage between endothelial innate recognition of pathogens and early-phase endothelial inflammatory responses. Our results may provide a new insight into the role of endothelial TLR2 in the initiation and modulation of vascular inflammation or atherogenic responses.

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