Introduction
Vascular endothelial cells are critical targets for microbial products and are directly exposed to them. A series of toll-like receptors (TLRs), which are innate immune pattern recognition receptors, recognize a variety of microbial products and activate vascular endothelial cells in response to microbial products. The TLR signaling is reported to cooperate the interferon (IFN)-γ signaling pathway by influencing the activity of transcription factors, such as signal transducer and activator of transcription (STAT) 1. However, the precise action of other TLR ligands on the IFN-γ signaling in vascular endothelial cells is unknown. In this study, we examined the effect of a series of TLR ligands on IFN-γ-induced nitric oxide (NO) production by using murine vascular endothelial END-D cells and found their enhancing effect on the NO production. Among TLR ligands tested, we focused on the enhancing mechanism of Pam3CSK4, a TLR2 ligand.

Materials and Methods
The murine aortic endothelial END-D cells and the murine macrophage RAW 264.7 cells were pretreated with or without Pam3CSK4 and then stimulated with IFN-γ. Nitrite, the end product of NO metabolism, was measured using the Griess reagent. The expression of protein and mRNA was determined by immunoblotting and reverse transcription-polymerase chain reaction, respectively. The phosphorylation and expression of various signaling molecules were analyzed with immunoblotting. MyD88-specific or non-targeting small interfering RNA was transfected into the cells. The physical association was analyzed with immunoprecipitation and immunoblotting.

Results
Pretreatment or post-treatment with Pam3CSK4 augmented IFN-γ-induced NO production via enhanced expression of an inducible NO synthase (iNOS) protein and mRNA. Pam3CSK4 augmented phosphorylation of Janus kinase (JAK) 1 and 2, followed by enhanced phosphorylation of STAT1 at tyrosine 701. Subsequently, the enhanced STAT1 activation augmented IFN-γ-induced interferon-regulatory factor (IRF) 1 expression leading to the iNOS expression. There was no augmented IFN-γ receptor (IFN-γR) expression on Pam3CSK4-treated cells. Pam3CSK4 also induced the activation of p38 and subsequent phosphorylation of STAT1 at serine 727. A pharmacological p38 inhibitor abolished the augmentation of IFN-γ-induced NO production by Pam3CSK4. Pam3CSK4 enhanced a physical association of MyD88 and IFN-γR.

Conclusion
We have demonstrated that Pam3CSK4, a TLR2 ligand, upregulates IFN-γ-induced NO production via enhanced IFN-γ signaling in vascular endothelial cells. It is supported by the augmentation of IFN-γ-induced JAK1/2 and STAT1 phosphorylation by Pam3CSK4. Interestingly, Pam3CSK4 pretreatment accelerates IFN-γ-induced STAT1 phosphorylation, suggesting that IFN-γ more rapidly activates the JAK/STAT signaling in Pam3CSK4-pretreated cells. Therefore, Pam3CSK4 may upregulate the IFN-γ signaling more rapidly and strongly, followed by higher expression of IFN-γ-induced genes. The augmentation of IFN-γ signaling by Pam3CSK4 is mediated by two different mechanisms; one is a physical association between IFN-γRα and MyD88; the other is p38-dependent phosphorylation of STAT1 at S727. TLR signaling is suggested to regulate IFN-γ signaling in the expression of IFN-γ-induced genes in vascular endothelial cells.