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TLR9 Is Localized in the Endoplasmic Reticulum Prior to Stimulation

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In mammals, 10 TLRs recognize conserved pathogen-associated molecular patterns, resulting in the induction of inflammatory innate immune responses. One of these, TLR9, is activated intracellularly by bacterial DNA and synthetic oligodeoxynucleotides (ODN), containing unmethylated CpG dinucleotides. Following treatment with CpG ODN, TLR9 is found in lysosome-associated membrane protein type 1-positive lysosomes, and we asked which intracellular compartment contains TLR9 before CpG exposure. Surprisingly, we found by microscopy and supporting biochemical evidence that both transfected and endogenously expressed human TLR9 is retained in the endoplasmic reticulum. By contrast, human TLR4 trafficked to the cell surface, indicating that endoplasmic reticulum retention is not a property common to all TLRs. Because TLR9 is observed in endocytic vesicles following exposure to CpG ODN, our data indicate that a special mechanism must exist for translocating TLR9 to the signaling compartments that contain the CpG DNA. ***The Journal of Immunology,*** **2004, 173: 1179–1183.**

The TLRs comprise a family of at least 10 proteins that recognize conserved patterns on microorganisms known as pathogen-associated molecular patterns (PAMPs)[2] (1). Exposure of TLRs to PAMPs triggers inflammatory processes that result in enhanced uptake and the direct killing of microorganisms, as well as the generation of primary adaptive immune responses (2, 3). Mice deficient in MyD88 and humans lacking IL-1R-associated kinase 4, two components immediately downstream of TLRs in their signaling pathways, are highly susceptible to infection, demonstrating the critical role that TLRs play in host responses to pathogens (4, 5). Genetic deletion or reconstitution experiments have demonstrated that individual TLRs are both essential and sufficient for specific responsiveness to a wide variety of PAMPs; for example, TLR2 is the receptor for lipopeptides, TLR3 for dsRNA, TLR4 for LPS, and TLR5 for flagellin. TLR9 recognizes bacterial and viral DNA, and studies using synthetic single-stranded oligodeoxynucleotides (ODNs) have defined the immunogenic sequences of bacterial DNA as consisting of unmethylated CpG motifs in the context of species-dependent surrounding sequences (6, 7). TLR9 is expressed primarily on APCs such as B cells and dendritic cells (DC). In human DC, TLR9 is restricted to a subset of DC, plasmacytoid DC, responsible for production of high levels of type I IFN (8). Phagocytes endocytose microorganisms and lyse them in phagolysosomes, where their DNA is released and presumably interacts with TLR9, initiating an inflammatory response. In systemic autoimmune diseases, the stimulation of TLR9 in B cells by endocytosed immune complexes containing hypomethylated CpG DNA has been hypothesized to play a deleterious role in the induction of autoantibodies (9, 10). Thus, the recognition of DNA by TLR9 plays important roles in both host defense and autoimmunity.

Several lines of evidence suggest that the molecular recognition of CpG DNA occurs inside the cell, perhaps in lysosomes. First, in contrast to other TLRs, TLR9 has not been detected on the surface of cells (11, 12). In addition, chloroquine and other inhibitors of endosome maturation inhibit signaling by CpG DNA (11, 13), and MyD88 is recruited to vesicles containing the lysosomal marker, lysosome-associated membrane protein type 1, in cells stimulated with CpG DNA (11). Finally, TLR9 and CpG DNA localize to a common intracellular compartment (12). Studies using fluorescently labeled ODNs indicate that both stimulatory and nonstimulatory DNA are internalized nonspecifically by surface receptors, but only the stimulatory DNA activates TLR9 in the intracellular compartment where both colocalize (11, 12). In this paper, we address the important question of where TLR9 is located within the cell before CpG exposure. We show that transfected and endogenous TLR9 are retained intracellularly in a reticular compartment that we identify as the endoplasmic reticulum (ER). We also propose mechanisms that may explain how TLR9 gets from the ER to the signaling compartment.

Materials and Methods

Cell culture

HeLa and HEK293T cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% low endotoxin FBS. B cell lines (gifts from Dr. A. Shaffer, National Cancer Institute (NCI), Bethesda, MD) were cultured in RPMI 1640 containing the same supplements.

Antibodies

Anti-glucose-regulated protein 94 and anti-KDEL Abs were from Stratagene (San Diego, CA), anti-calnexin was from StressGen Biotechnologies (Victoria, British Columbia, Canada), anti-hemagglutinin (HA) epitope (clone 3F10) and anti-HA HRP (clone 12CA5) were from Roche (Indianapolis, IN), and anti-HA-Alexa 488 (clone HA1.1) was from Covance (Berkeley, CA). PE-conjugated anti-TLR4 (HTA125) and unconjugated and PE-anti-TLR9 (eB72-1665) were from eBioscience (San Diego, CA). Alexa 488 and Alexa 546 conjugated secondary Abs (for microscopy) were from Molecular Probes

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[2] Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; CFP, cyan fluorescent protein; DC, dendritic cell; EndoH, endoglycosidase H; ER, endoplasmic reticulum; HA, hemagglutinin; LSC, laser scanning confocal; ODN, oligodeoxynucleotide; PNGaseF, peptide:*N*-glycosidase F; RFP, red fluorescent protein; YFP, yellow fluorescent protein.

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(Eugene, OR), and allophycocyanin secondary Abs (for flow cytometry) were from Caltag Laboratories (Burlingame, CA).

Transfections and constructs

HeLa and HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) and calcium phosphate precipitation, respectively. Human TLR9 and TLR4 tagged at their C termini with enhanced GFP (TLR9-GFP, TLR4-GFP), yellow fluorescent protein (TLR9-YFP, TLR4-YFP), or cyan fluorescent protein (TLR9-CFP) were generated by inserting the TLR in frame into pEGFP-N1, pEYFP-N1, or pECFP-N1, respectively (BD Clontech, Palo Alto, CA). HA-tagged TLR9 was generated by inserting TLR9, lacking its leader sequence but retaining its stop codon, in frame into pDisplay (Invitrogen Life Technologies). All TLR fusion proteins were shown to activate NF-κB in response to CpG (for TLR9) or LPS (for TLR4/MD-2) in HEK293 cells. CFP-tagged constructs used to identify organelles were pECFP-ER and pECFP-Golgi (BD Clontech), pECFP-C1 Rab5, 7, 9, and 11 (gifts from Dr. J. Bonifacino, National Institute of Child Health and Human Development, Bethesda, MD). Monomeric red fluorescent protein (RFP)-tagged CD63 was generated by excising CD63 from CD63-GFP (a gift from Dr. P. Roche, NCI) and ligating it into a monomeric RFP vector (14) modified by Dr. L. Samelson (NCI).

Flow cytometry

HEK293T cells were transfected in 6-well plates and, after 48 h, stained directly for surface expression, or fixed, permeabilized (using Caltag Fix and Perm), and stained for total expression, using a directly conjugated fluorescent Ab. For human B cell lines, cells were fixed, permeabilized, and labeled with anti-TLR9, followed by an Alexa 488 secondary Ab. Data were collected with a FACSCalibur cytometer (BD Immunocytometry Systems, San Jose, CA), and analyzed using FlowJo (Tree Star, Ashland, OR).

Confocal microscopy

HEK293T cells were transfected in 6-well plates and 48 h later were plated in HBSS on Labtek II (Campbell, CA) coverglass chambers precoated with poly-L-lysine. After 1 h, the medium was changed to growth medium containing 10 mM HEPES and 100 μg/ml cycloheximide for 1 h, before visualization. HeLa cells were transfected on coverslips, fixed, permeabilized, and stained. BJAB cells were fixed, permeabilized, and stained for calnexin and TLR9 using anti-calnexin and anti-TLR9 Abs, and counterstained with Alexa 546 anti-rabbit and Alexa 488 anti-rat secondary Abs, respectively. In parallel, cells were stained with isotype control Abs. These control cells were used to set the background levels for image collection. All cells were visualized using a Zeiss LSM510 laser scanning confocal (LSC) microscope (Jena, Germany). Images were processed for presentation in figures using Adobe Photoshop 6.0 (Adobe Systems, San Jose, Ca).

Glycosidase treatment

HEK293 cells stably expressing HA-TLR9 or BJAB cells were lysed as described (11), and immunoprecipitated with anti-HA or anti-TLR9 mAbs, respectively. The immunoprecipitates were treated with endoglycosidase H (EndoH) or peptide:*N*-glycosidase F (PNGaseF) according to manufacturer instructions (New England Biolabs, Beverly, MA). Treated samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-HA or anti-TLR9.

Pulse chase

After transfection with untagged TLR9, 5×10^5 HeLa cells were pulsed with 0.2 mCi [^{35}S]methionine for 30 min in methionine-free medium. The cells were washed twice with culture medium supplemented with 300 mg/L methionine, and chased for various times in culture medium with 75 mg/L methionine. Cells were harvested in PBS with 20 mM EDTA, lysed, immunoprecipitated with anti-TLR9 mAb, and treated with glycosidase before separation by SDS-PAGE. Gels were fixed, treated with Enlightening (NEN, Boston, MA), and exposed to film for 48 h.

Results

TLR9 is retained intracellularly

Although most TLRs are expressed at the cell surface, recent studies have shown that TLR9 is located in intracellular compartments. To determine the extent of intracellular localization of endogenously expressed TLR9, permeabilized or nonpermeabilized Ramos B cells were stained with an anti-TLR9 mAb and examined by flow cytometry. As seen in Fig. 1*A*, the permeabilized cells ("Total") stained positive for TLR9, but no TLR9 was detected on

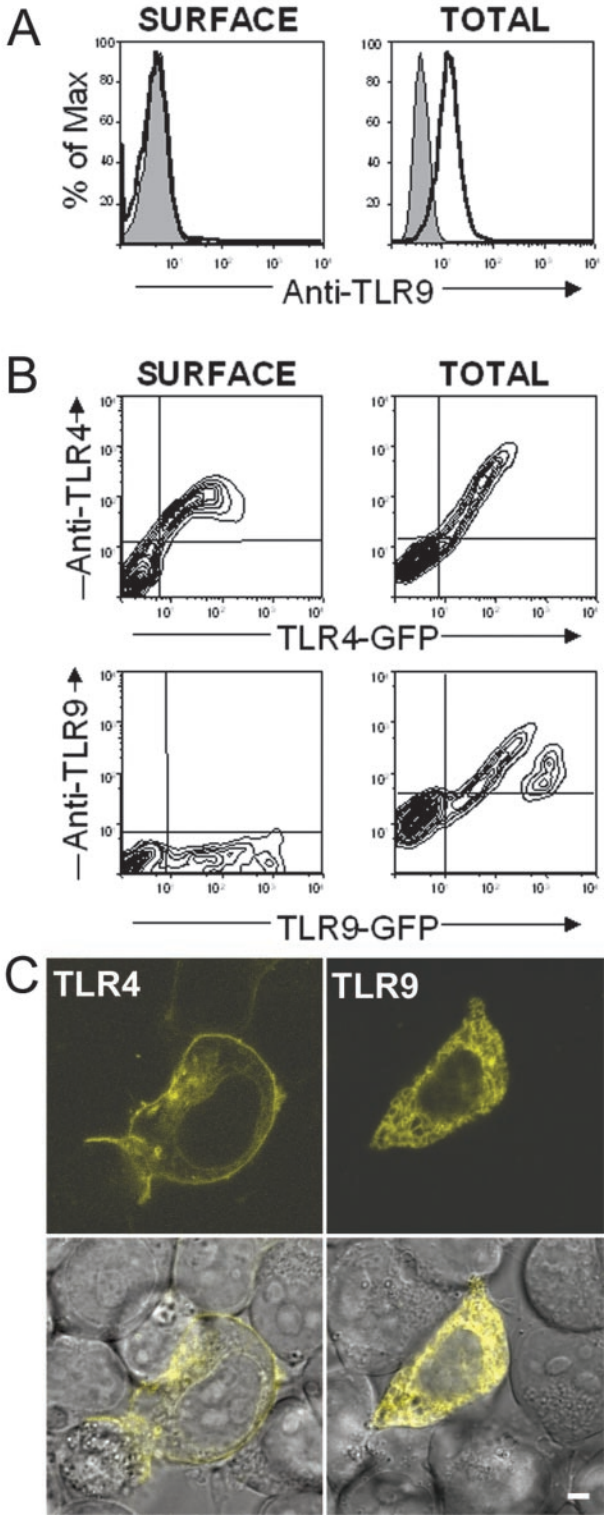

FIGURE 1. TLR9 is retained intracellularly, TLR4 traffics to the cell surface. *A* and *B*, Flow cytometric analyses. *A*, Ramos B cells were stained directly with an anti-TLR9 mAb (Surface) or were fixed and permeabilized before staining (Total). Shaded histograms represent cells stained with an isotype-matched control mAb. *B*, HEK293T cells transfected with TLR4-GFP (*upper panels*) or TLR9-GFP (*lower panels*) were stained for surface or total expression, as in *A*, using PE-conjugated anti-TLR4 or anti-TLR9 mAbs. *C*, LSC microscopy analysis. Live HEK293T cells transfected with TLR4-YFP (*left panels*) or TLR9-YFP (*right panels*) were treated with 100 μg/ml cycloheximide for 1 h before visualization to block protein synthesis. A single section (0.8 μm) is shown for YFP (*upper panels*) and as an overlay of YFP with the differential interference contrast image (*lower panels*). Scalebar, 4.5 μm.

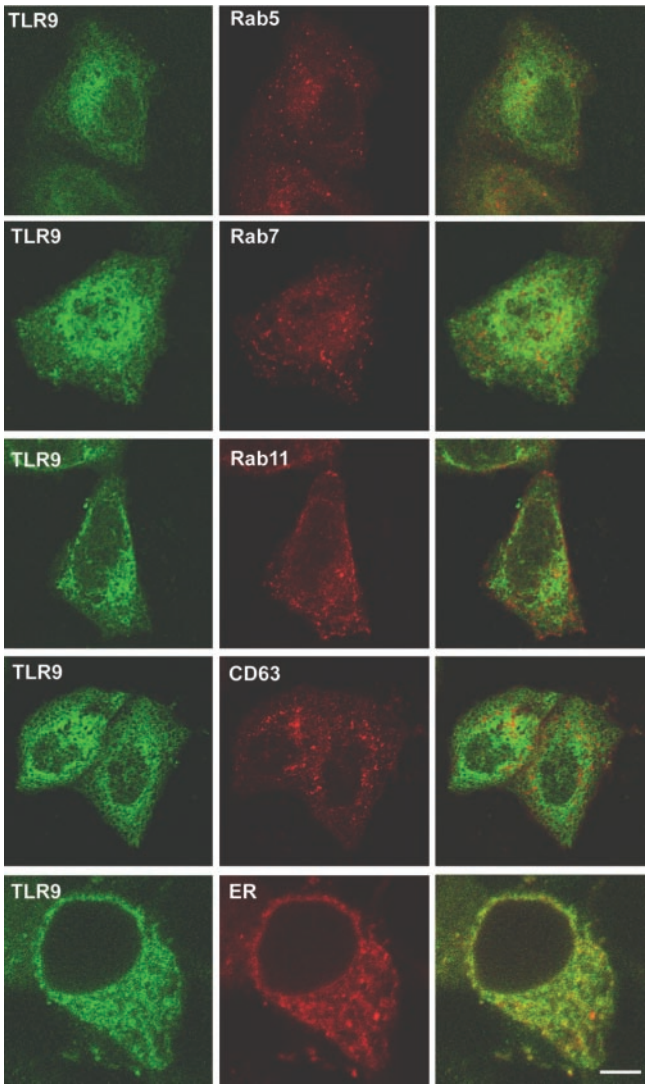

FIGURE 2. TLR9 colocalizes with ER markers. HeLa cells were cotransfected with TLR9-YFP and either Rab5-CFP (early endosome), Rab7-CFP (late endosome), or Rab11-CFP (recycling endosome); with TLR9-GFP and CD63-mRFP (lysosome); and with TLR9-CFP and pEYFP-ER (a commercial marker for ER). Cells were fixed, mounted, and examined by LSC microscopy. Single 0.8-μm sections pseudocolored green (TLR9, *left panels*) and red (organelle marker, *middle panels*) are shown. An overlay of the two channels is shown in the *right panels*. Scalebar, 9 μm.

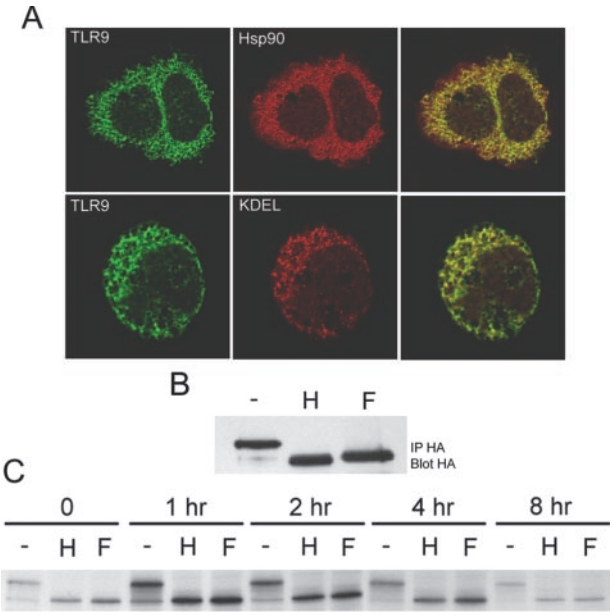

FIGURE 3. Stably expressed TLR9 resides in the ER. *A*, HEK293 cells stably expressing HA-TLR9 were stained for TLR9 (with anti-HA-Alexa 488) and the ER markers, heat shock protein 90 (*upper panels*, two connected cells) and KDEL (*lower panels*), using Alexa 546 secondary Abs. Single 0.8-μm sections from LSC microscopy are shown for TLR9 (*left panels*) and ER markers (*middle panels*). Overlays are shown in the *right panels*. *B* and *C*, TLR9 does not enter the Golgi. *B*, HA immunoprecipitates (from the same cells as in *A*) were incubated with medium alone (−), EndoH (H), or PNGaseF (F). The proteins were analyzed by SDS-PAGE and immunoblotting using an anti-HA-HRP mAb. *C*, Untagged TLR9, labeled with [^{35}S]methionine and chased for the indicated times, was immunoprecipiated and treated with glycosidase as in *B*. Shown is the autoradiograph of the SDS-PAGE gel.

the cell surface (nonpermeabilized cells). Then, we analyzed the expression of TLR4-GFP and TLR9-GFP in transiently transfected HEK293T cells. Fig. 1*B* shows that only TLR4 was surface labeled with a PE-conjugated mAb, whereas both TLR4 and TLR9 were labeled with mAbs in permeabilized cells. Finally, HEK293T cells were transfected with TLR4-YFP or TLR9-YFP and examined live by LSC microscopy. After treatment with cycloheximide to block protein synthesis and allow newly synthesized protein to reach its final destination, TLR4-YFP was observed on the cell surface and in a perinuclear area, most likely the Golgi. In contrast, TLR9-YFP remained in an intracellular compartment (Fig. 1*C*) even after a 24-h incubation with cycloheximide (data not shown). Thus, TLR9 is retained intracellularly and is not detected on the cell surface in either TLR9 transfectants or in cells endogenously expressing TLR9.

TLR9 is located in the ER

To identify the compartment(s) that TLR9 localizes to, HeLa cells, cotransfected with fluorescently tagged TLR9 and markers for selected vesicles and organelles, were examined by LSC microscopy. TLR9 localized to a reticular compartment that did not contain Rab5, an early endosome marker; Rab7 or Rab9, late endosome markers; Rab11, a recycling endosome marker; CD63, a lysosome marker; or a commercial Golgi marker (Fig. 2, and data not shown). In stark contrast, TLR9 colocalized with a commercial ER marker (Fig. 2). In additional experiments, HEK293 cells stably expressing HA-TLR9 also showed reticular staining for TLR9. In these cells, the TLR9 localized to a compartment that was stained with Abs to the ER-resident KDEL motif, the ER-retained heat shock protein 90 family member, glucose-regulated protein 94, and MHC class II invariant chain, which is retained in the ER in the absence of the α- and β-chains (Fig. 3*A*, and data not shown).

After leaving the ER, most newly synthesized proteins enter the Golgi stack where their *N*-linked carbohydrates are processed and become resistant to digestion with EndoH (15). We have previously shown that TLR4 contains an EndoH-resistant form that is located on the cell surface (16). In contrast, HA-TLR9 is sensitive to both EndoH and PNGaseF (an enzyme that digests both immature and mature *N*-linked carbohydrates) at steady-state (Fig. 3*B*), and for at least 8 h following synthesis (Fig. 3*C*). Although previous studies have indicated that TLR9 localizes in vesicles after treatment with CpG ODN, our current data show that TLR9 resides in the ER before treatment.

ER localization of endogenously expressed TLR9

Previous studies have shown that B cells express TLR9 and respond to TLR9-dependent ligands (6, 17). To determine whether

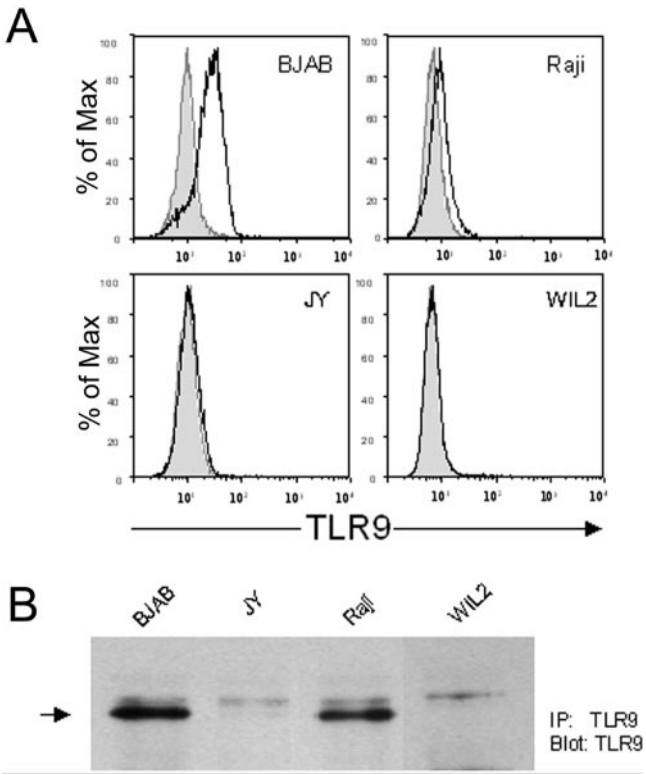

FIGURE 4. Endogenous TLR9 is retained intracellularly in B cell lines. *A*, Four B cell lines (BJAB, Raji, WIL2, and JY) were permeabilized, labeled with anti-TLR9 Ab, and stained with an Alexa 488 secondary Ab. Shaded profiles represent isotype controls. *B*, Endogenous TLR9 from the four B cell lines was immunoprecipated and analyzed by SDS-PAGE. After transferring to nitrocellulose, TLR9 was detected with the same Ab. In addition to the TLR9 band (→), a faint nonspecific band migrating immediately above TLR9 is evident in all samples.

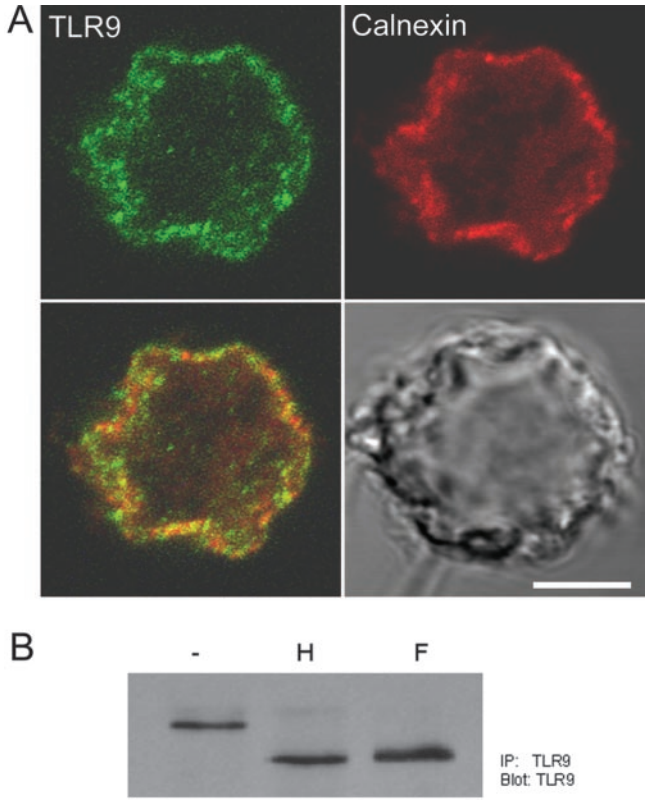

FIGURE 5. Endogenous TLR9 is in the ER. *A*, BJAB cells were stained for calnexin, an ER marker (red), and TLR9 (green). Shown is a 0.7-μm section of TLR9 (*upper left*), calnexin (*upper right*), an overlay of the two (*lower left*), and the differential contrast image (*lower right*). Scalebar, 4.5 μm. *B*, TLR9 was immunoprecipitated from BJAB cells and digested with EndoH (H) or PNGaseF (F) as in Fig. 3*B*. The trace *upper band* in the EndoH digest corresponds to the nonspecific band seen in Fig. 4*B*.

endogenously expressed TLR9 also localizes to the ER, we first analyzed a panel of B cell lines for TLR9 expression. By flow cytometry, BJAB expressed the highest level of TLR9, followed by Ramos and Raji. JY expressed barely detectable levels of TLR9, and no TLR9 was detected in H929 or WIL2 (Figs. 1*A* and 4*A*). Consistent with our earlier results, permeabilization was required for staining, indicating that TLR9 was located intracellularly. Immunoprecipitation and blotting of TLR9 from the B cell lines showed a 160-kDa protein whose expression level correlated with flow cytometry results and was the correct molecular mass of the glycosylated form (Fig. 4*B*). The anti-TLR9 Ab also detected a faint, slower migrating band that was also seen in the isotype control (data not shown) and, therefore, was not specific. Because BJAB cells expressed the highest amount of TLR9, we used these cells to examine the intracellular location of TLR9. B cells contain a large nucleus and very little cytoplasm, therefore, the different organelles are not resolved with high precision in these cells. However, by LSC microscopy, TLR9 gave a clearly discernable staining that colocalized with the ER marker calnexin (Fig. 5*A*). In addition, TLR9 was sensitive to both EndoH and PNGaseF digestion (Fig. 5*B*), resulting in the expected 113-kDa unglycosylated form, indicating that it did not reach the Golgi. Taken together, these data provide strong evidence that endogenously expressed TLR9, like transfected TLR9, resides solely in the ER in unstimulated cells.

Discussion

Recognition of bacterial DNA induces a potent innate immune response and affects the development of adaptive responses. It has been demonstrated that bacterial DNA or synthetic ODNs containing unmethylated CpG motifs are recognized only after endocytosis and not at the cell surface (12, 13). Recently, several studies have shown that TLR9 colocalizes with CpG ODN in a vesicular compartment, identified as a lysosome-associated membrane protein type 1-positive lysosome, where signaling is initiated (11, 12). What remained unclear was where TLR9 resides before stimulation. In this study, we show that both endogenously expressed and transfected TLR9 are retained in the ER.

In agreement with previous results, we were unable to detect TLR9 on the surface of transfected cells or B cell lines, although these same cells clearly expressed TLR9 intracellularly. When TLR9 was either labeled with a fluorescent mAb or fused to a fluorescent protein, it was located in a perinuclear reticular compartment that colocalized with ER markers, but not with markers specific for endocytic vesicles or, more importantly, lysosomes where it has been detected after stimulation with CpG ODN. Endogenously expressed TLR9 in B cells or ectopically expressed TLR9 in transfectants was sensitive to digestion with EndoH, an enzyme that digests immature *N*-linked sugars that are added in the ER but are modified to an EndoH-resistant form in the Golgi. In fact, newly synthesized TLR9 remained sensitive to EndoH digestion even after 8 h. Because most proteins traffic directly to the Golgi from the ER, the EndoH sensitivity of TLR9 in unstimulated cells suggests that the TLR9 has never left the ER. Although we have observed that TLR9 is located in the ER in several cell types, we cannot rule out that in other cells, TLR9 may reside in a different cellular compartment. The precedence for this is TLR4,

which has been shown to reside in a distinct compartment depending upon the cell type examined (18–20).

It is unclear how TLR9 reaches the lysosome where stimulation by CpG DNA occurs. One possibility is suggested by recent studies of Desjardins and colleagues (21, 22) who demonstrated that the ER supplies membrane and ER proteins to phagosomes during particle engulfment. Because bacterial DNA is released in phagosomes following lysis, TLR9 might enter the phagosome directly from the ER. According to this hypothesis, the ER is the predicted location of TLR9 in resting cells. In contrast to phagocytosis, soluble CpG DNA is taken up in nonphagocytic cells such as B cells, by receptor-mediated and fluid phase endocytosis. This process also has important biological consequences; it stimulates responses that protect against pathogens (23) but may also contribute to systemic autoimmune diseases (9, 10). Endocytosis of soluble molecules such as CpG DNA leads to their entry into early and late endosomes and lysosomes. Unlike phagosomes, these vesicles are formed from membranes that bud off from the plasma membrane or Golgi, and not by direct fusion with the ER. Nevertheless, the direct fusion between endosomes and the ER may occur by a previously unknown pathway. In favor of the direct pathway of ER to endosomes, we have not detected an EndoH-resistant form of TLR9 following stimulation of cells with CpG ODN (C.A.L., unpublished observations). Indeed, Latz et al. (24) suggest that an EndoH-sensitive form of TLR9 reaches early endosomes, where CpG DNA is localized soon after endocytosis. Biotinylation studies demonstrated that upon CpG DNA stimulation, a small amount of TLR9 becomes accessible to the extracellular environment, possibly by ER to endosome fusion. However, if a small fraction of TLR9 molecules trafficked to vesicles by the conventional pathway (via the Golgi), then our current studies, and those of Latz (24), may not be sensitive enough to detect the presence of mature sugars on TLR9. Thus, we cannot rule out the possibility that TLR9 may pass through the Golgi en route to the CpG-containing vesicles by a conventional mechanism. Studies are currently underway to identify which mechanism(s) are involved.

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