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Superantigen- and TLR-Dependent Activation of Tonsillar B Cells after Receptor-Mediated Endocytosis

Johan Jendholm,* Matthias Mörgelin, † Maria Laura A. Perez Vidakovics,* Michael Carlsson,‡ Hakon Leffler, † Lars-Olaf Cardell,§ and Kristian Riesbeck2*

Classical B lymphocyte activation is dependent on BCR cross-linking in combination with physical interaction with Th cells. Other B cell molecules that contribute to the activation are complement, cytokine, and TLRs recognizing specific pathogen-associated molecular patterns. Moraxella (Branhamella) catarrhalis is a common Gram-negative respiratory pathogen that induces proliferation in human IgD-expressing B cells independently of T cell help. The activation is initiated by the B cell superantigen Moraxella IgD-binding protein (MID) through a nonimmune cross-linking of IgD. However, IgD cross-linking alone is not sufficient to induce proliferation. In this study, we characterized the significance of TLRs in superantigen-dependent B cell activation using whole bacteria or rMID in the presence or absence of TLR ligands. IgD cross-linking by MID sensitized B cells obtained from children with tonsillar hyperplasia for mainly TLR9, whereas TLRs 1, 2, 6, and 7 were less important. The Moraxella-induced activation was inhibited when a dominant-negative TLR9 ligand was added. Interestingly, BCR-mediated endocytosis of whole Moraxella and degradation of live bacteria in naive B cells was observed with fluorescence, confocal, and transmission electron microscopy. This unique observation proved the strong intracellular TLR9 response as well as highlighted the Ag-presenting function of B cells. In conclusion, our findings suggest an important role of TLRs in the adaptive immune response and reveal novel insights into the T cell-independent B cell activation induced by bacteria. The Journal of Immunology, 2009, 182: 4713–4720.
a three-signal model for activation of human naive cells has been suggested involving the following: 1) BCR cross-linking, 2) physical T cell help, and 3) TLR9 stimulation and/or cytokines (22).

*Moraxella (Branhamella) catarrhalis* is an important respiratory pathogen that frequently colonizes the upper respiratory tract (23–25), and accounts for one-third of all pediatric acute otitis media cases. However, this number will most likely rise because an increased nasopharyngeal colonization of *M. catarrhalis* has been suggested to follow in the wake of the use of a conjugated pneumococcal vaccine (26). *M. catarrhalis* is also a common cause of sinusitis as well as an acknowledged pathogen in adults with chronic obstructive pulmonary disease (27). Recent studies have shown that *M. catarrhalis* forms biofilms and hide intracellularly, which may result in an underestimation of the *M. catarrhalis* carriage rate of only 13% (30). Interestingly, it was also shown that *M. catarrhalis* resided in the outer mantel zone of lymphoid follicles, where mainly naive B cells are located.

We have previously isolated and in detail characterized the B cell superantigen *Moraxella* IgD-binding protein (MID) responsible for the IgD binding of *M. catarrhalis* (31–33) (for a review, see Ref. 34). The smallest IgD-binding fragment of MID with comparable affinity as full-length MID 1–2139 has been mapped to residues aa 962-1200 (MID$^{962-1200}$) (35). The MID$^{962-1200}$ binding site on IgD is restricted to the C$_{H}1$ region of the Ig, with aa 198–206 being crucial for the interaction (36). MID has also proven to be responsible for the strong mitogenic effect on B cells by *M. catarrhalis* (37–39). However, IgD binding through MID$^{962-1200}$ alone is not enough to induce proliferation. Apart from cross-linking the BCR, B cells are also dependent on secondary signals such as CD40/CD154 interactions, coreceptor/complement, cytokines, and TLR signaling.

The goal of this study was to characterize TLR signaling in MID-dependent tonsillar B cell activation using bacteria or rMID$^{962-1200}$ in combination with different TLR ligands. Our results indicate that cross-linking of IgD through MID sensitizes human tonsillar B cells for the TLR ligands Pam$_3$CSK$_4$, Pam$_2$CSK$_4$, imiquimod, and in particular CpG. IgD and TLR co-stimulation increased the $[^{3}H]$thymidine incorporation with 6 MID-dependent tonsillar B cell activation using bacteria or complement, cytokines, and TLR signaling.

**FIGURE 1.** *M. catarrhalis*-induced B cell activation is MID dependent.

B cell activation was measured by means of $[^{3}H]$thymidine incorporation after 96 h. B cell cultures were as follows: *M. catarrhalis, M. catarrhalis* Δmid, *M. catarrhalis* plus IgD pAbs, *M. catarrhalis* plus MID$^{962-1200}$ pAbs, MID$^{962-1200}$ *M. catarrhalis* Δmid lysate plus MID$^{962-1200}$, *M. catarrhalis* Δmid lysate, and unstimulated B cells. Data are presented as arbitrary units with B cells activated with *M. catarrhalis* set to 100%. Error bars indicate SEM from three different donors.

InvivoGen. CFSE and ProLong Gold antifade reagent with 4′,6′-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes.

**Bacteria and culture conditions**

*M. catarrhalis* strain BBH18 is a clinical isolate from a sputum sample, and its MID-deficient isogenic mutant *M. catarrhalis* BBH18 Δmid was constructed and killed using formaldehyde, as previously described (38–40). Lysed bacteria were prepared from overnight cultures of *M. catarrhalis* Δmid using ultrasound treatment until no bacterial growth was detected. In some experiments, live bacteria was used. However, no difference in B cell proliferation was found with either alive or formaldehyde-killed *Moraxella*. Genomic *M. catarrhalis* DNA was extracted from BBH18 Δmid using the DNeasy kit (Qiagen), according to the manufacturer’s recommendations. Purified DNA was free of contaminants and enzyme inhibitors. CFSE-stained bacteria was prepared from overnight cultures. Briefly, both *M. catarrhalis* wild type (wt) and *M. catarrhalis* Δmid were precipitated with 10 μM CFSE in 5 ml of PBS and 0.1% BSA for 10 min at 37°C. After repeated washes with ice-cold brain heart infusion, cells were analyzed in flow cytometry and stored in PBS at 4°C.

**Cell preparations**

Tonsils (*n* = 18) were obtained from patients under the age of 12 (age range, 2–12 years old; median 6 years old; 8 boys and 10 girls) undergoing tonsillectomy at the University Hospital (Malmö, Sweden). The Ethics Committee of Lund University approved the study (No. 877/2005), and a signed written informed consent was obtained from the parents of all patients. Surgery was performed due to tonsillous hyperplasia, and apart from the tonsillar symptoms, all patients were healthy and did not receive any medication. Untouched CD19$^+$ B cells were isolated, as previously described (39). In all experiments with lymphocytes, 1 × 10$^6$ cells/ml were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 50 μg/ml gentamicin, and 100 U/ml penicillin in culture plates (Nunc). rMID fragments were coated on flat-bottom plates, as previously described (38). TLRIgD fragments were added in the concentration range 1–10 μg/ml, except for CpG ODN 2006, which was in the range of 0.1–1 μM. Proliferation was measured by [methyl-$^3$H]thymidine incorporation (5 μCi/well; Amersham Biosciences) using an 18-h pulse period.

**Flow cytometry analysis**

Surface expression of IgD and IgM after addition of MID$^{962-1200}$ or MID$^{1000-1200}$ was monitored using flow cytometry (BD Biosciences). Purified B cells were incubated in complete medium with different concentrations of MID$^{962-1200}$ or MID$^{1000-1200}$ ranging from 1 to 10,000 ng/ml and analyzed for IgM and IgD expression at various time points (0–24 h). Harvested cells were washed and incubated in PBS containing 2% BSA.

**Materials and Methods**

**Reagents**

Rabbit anti-human IgD, FITC-conjugated rabbit anti-human IgM or IgD, and R-PE (RPE)-conjugated rabbit anti-human IgD polyclonal Abs (pAbs), CD14, CD16, CD19, CD56, and CD83 mAbs were purchased from DakoCytomation. A FITC-conjugated anti-human TLR6 mAb was from IMGENEX. An anti-human TLRIgD mAb was purchased from InvivoGen. The truncated MID$^{962-1200}$ and MID$^{1000-1200}$ fragments and rabbit anti-MID$^{962-1200}$ antiserum were prepared, as described earlier (35, 38). Chloroquine was obtained from Sigma-Aldrich and dissolved in water, according to the manufacturer’s recommendations. Cell viability was unaffected at the concentrations used. TLRIgD fragments Pam$_3$CSK$_4$, Pam$_2$CSK$_4$, poly(I:C), ultrapure LPS (from *Escherichia coli*), flagellin (from *Bacillus subtilis*), imiquimod, sPolU, CPg oligonucleotide (ODN) 2006, and the suppressive ODN with human-specific CpGs (TATAGGG)$_4$ × 4 from
mAbs against CD3, CD14, CD16, CD56, or CD83 in combination with PBS before analysis. B cells were routinely screened for contaminating Abs directed against either IgD or CD19, followed by repeated washes with 1 h. B cells were washed and incubated for 1 h with RPE-conjugated M. H9262 in the range of 0.1–1 M. Error bars indicate SD from 10 (H), 4 (A), 4 (s) multiple comparison test. [3H]-Thymidine incorporation after 96 h. Error bars indicate SEM from 10 (A), 7 (B), and 4 (A) different donors. Significant values compared with MID962–1200 (A) and M. catarrhalis (B and C) stimulation were calculated using repeated measures ANOVA with Dunnett’s multiple comparison test. FITC-conjugated anti-human CD19 mAbs after isolation and during culture.

**FIGURE 3.** M. catarrhalis DNA is mitogenic for B cells. Purified B cells were cultured with A, four different concentrations (concentration range 10–0.1 µg/ml) of M. catarrhalis DNA with or without the IgD-binding fragment MID962–1200 (1 µg/ml), formalin-killed M. catarrhalis, and B, chloroquine (2.5–10 µg/ml), or C, the dominant-negative TLR9 ligand (TTAGGG)×4 (4 or 8 µM), and analyzed for [3H]thymidine incorporation after 96 h. Error bars indicate SEM from 10 (A), (7 (B), and 4 (C) different donors. Significant values compared with MID962–1200 (A) and M. catarrhalis (B and C) stimulation were calculated using repeated measures ANOVA with Dunnett’s multiple comparison test.

**FIGURE 2.** rMID962–1200-mediated IgD cross-linking sensitizes tonsillar B cells for TLR1 plus 2, TLR2 plus 6, and TLR7 and 9 activation. Purified B cells were cultured with different concentrations of TLR ligands (concentration range 1–10 µg/ml) except for CpG ODN 2006, which was in the range of 0.1–1 M) with or without the IgD-binding fragment MID962–1200 [3H]Thymidine uptake was measured after 96 h. Pam3CSK4 (A), Pam3CSK4 (B), dsRNA (C), LPS (D), flagellin (E), imiquimod (F), ssRNA (G), and Cpg 2066 (H). Error bars indicate SD from 10 (A), 4 (B), 4 (C), 9 (D), 9 (E), 10 (F), 3 (G), and 9 (H) different donors. Significant values compared with MID962–1200 stimulation alone were calculated using repeated measures ANOVA with Dunnett’s multiple comparison test.

with FITC-conjugated rabbit anti-human IgM and RPE-conjugated rabbit anti-human IgD pAb at 4°C for 1 h. After two washes in PBS, B cells were screened for IgM and IgD using flow cytometry. Binding of CFSE-stained bacteria to IgD-expressing B cells was also tested in flow cytometry before confocal microscopy. Briefly, 2 × 10⁷ purified B cells were incubated with 1 × 10⁷ CFSE-stained M. catarrhalis wt or M. catarrhalis Δmid on ice for 1 h. B cells were washed and incubated for 1 h on ice with RPE-conjugated Abs directed against either IgD or CD19, followed by repeated washes with PBS before analysis. B cells were routinely screened for contaminating monocytes, T cells, NK cells, or dendritic cells using RPE-conjugated mAbs against CD3, CD14, CD16, CD56, or CD83 in combination with

For immunohistochemistry and TEM, ultrathin sections were mounted on gold grids and subjected to Ag retrieval with sodium metaperiodate (41, 42). For immunostaining, the grids were floated on top of drops of immune reagents displayed on a sheet of paraffin. Free aldehyde groups were blocked with 50 mM glycine, and the grids were then incubated with 5% (v/v) goat serum in incubation buffer (0.2% AURION BSA-c (Electron Microscopy Sciences) in PBS (pH 7.6)) for 15 min. This blocking procedure was followed by overnight incubation with primary Abs (dilution 1/50–1/100) at 4°C. After washing the grids in 200 ml of incubation buffer, floating on drops containing the gold conjugate reagents (diluted 1/10–1/20
in incubation buffer) was performed for 60 min at room temperature. After further washes in incubation buffer, the sections were postfixed in 2% glutaraldehyde. Finally, sections were washed in distilled water, poststained with uranyl acetate and lead citrate, and examined under the electron microscope. Specimens were observed in a JEOL JEM 1230 electron microscope (JEOL) operated at 60 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera (Gatan).

**Statistical analysis**

Statistical analysis was performed using GraphPad PRISM 5, and significance was calculated with one-way repeated measures ANOVA with Dunnett’s multiple comparison test (for comparisons of more than two data sets). Significant values were defined as \( p \leq 0.05 \) (*), \( p \leq 0.01 \) (**), and \( p \leq 0.001 \) (***).

**Results**

IgD cross-linking sensitizes tonsillar B cells for TLR activation

We have demonstrated previously that MID is a powerful B cell superantigen by its unique ability to target human IgD (37, 38, 43). The nonimmune IgD cross-linking by MID-expressing *Moraxella catarrhalis* leads to B cell proliferation and IgM production (39). In Fig. 1, the importance of MID for *Moraxella*-induced T cell-independent tonsillar B cell activation is demonstrated using the formalddehyde-killed *M. catarrhalis* strain BBH18 and its MID-deficient isogenic mutant (*M. catarrhalis Δmid*). The proliferation was dramatically reduced with *M. catarrhalis Δmid* as compared with the *M. catarrhalis* wt. Preincubation with Abs blocking either the IgD BCR or MID completely inhibited the *M. catarrhalis*-induced activation. However, IgD cross-linking with only rMID962–1200 did not induce cell proliferation in purified B cells as compared with the whole bacteria. Furthermore, when B cells were incubated with the MID-deficient mutant in addition to the IgD-binding fragment MID962–1200, only a minor increase in [3H]thymidine uptake was detected. The low level of activation suggested either additional B cell targets of the full-length MID molecule or differences in Ag recognition between rMID962–1200 and MID expressed on the bacterial surface. The later hypothesis was strengthened when the MID962–1200 segment was combined with lysed *M. catarrhalis Δmid* (Fig. 1; B). This combination resulted in vigorous proliferation, indicating that no other part of MID than the IgD-binding segment (i.e., MID962–1200) was needed for proliferation, but rather that intracellular bacterial factors were required for optimal stimulation.

To characterize *Moraxella*-dependent B cell activation signals in addition to IgD cross-linking, we focused on TLR signaling. Purified human tonsillar B cells were incubated with different TLR ligands in the presence or absence of MID962–1200, followed by

![FIGURE 4. IgD binding by MID causes a down-regulation of surface-expressed IgD in a dose-dependent manner. Change in surface expression of IgD and IgM after MID binding in purified human B lymphocytes was analyzed by flow cytometry. B cells were cultured in plates coated with different concentrations of MID962–1200 ranging from 1 ng to 10 μg/ml and monitored for IgD and IgM expression after 24 h (A). B. The kinetics of changes in IgD expression was surveyed using 1 μg/ml MID962–1200 or MID1000–1200 at 37°C. The surface expression data are shown as arbitrary units comparing expression at different time points with expression at time 0 h set to 100%. Error bars indicate SEM from 4 and 10 different donors in A and B, respectively.](image)

![FIGURE 5. MID binding results in clustering of IgD receptors. B cells were incubated with overnight cultures of *M. catarrhalis* wt for 1 h on ice and analyzed by TEM without (A–C) or with (D–F) gold-labeled Abs directed against IgD (large granules, white arrows) or the IgD-binding part of MID (small granules, black arrows). The scale in A and D represents 1 μm, and in the magnified pictures B and C and E and F, 100 nm.](image)
The strong mitogenic effect of M. catarrhalis on tonsillar B cells is dependent on IgD BCR cross-linking and TLR9 signaling

To further investigate the role of TLR9 in M. catarrhalis-induced B cell activation, we isolated genomic DNA from M. catarrhalis ∆mid and incubated human tonsillar B cells with different concentrations of DNA (0.1–10 μg/ml) in the presence or absence of the IgD-binding MID[962–1200] fragment. A clear synergistic effect was seen with combined IgD and TLR9 signaling (Fig. 3A). To further test the involvement of TLR9 in M. catarrhalis-induced B cell activation, we added various concentrations of chloroquine (ranging from 2.5 to 10 μg/ml) to our B cell cultures. Chloroquine prevents acidification of the endosomes and lysosomes needed for TLR9 activation, and was found to be a powerful inhibitor of M. catarrhalis-induced B cell activation (Fig. 3B). To specifically target the TLR9 signaling pathway, the dominant-negative TLR9 inhibitory oligonucleotide (TTAGGG)×4 was added to purified B cells that were stimulated with M. catarrhalis. Interestingly, B cell activation was significantly reduced by 50% in the presence of the dominant-negative TLR9 inhibitor, indicating the importance of TLR9 for the M. catarrhalis-dependent mitogenic effect on B cells (Fig. 3C).

M. catarrhalis is internalized by tonsillar B cells via IgD-mediated endocytosis

B cell stimulation by recombinant superantigens normally leads to internalization of the targeted receptor (44). To analyze the expression of IgD and IgM after MID binding, B cells were incubated in microtiter plates coated with different concentrations of the IgD-binding fragment MID[962–1200]. Cells were harvested after 24 h and screened for changes in IgD and IgM expression using flow cytometry analysis. Cell viability after MID[962–1200] stimulation was routinely tested using propidium iodine in combination with annexin V (45) and was found to be unaffected. The surface-expressed IgD was strongly down-regulated in the presence of increasing concentrations of MID[962–1200] (Fig. 4A). In contrast, the IgM expression was not affected by MID[962–1200]. The kinetics of the MID-induced IgD down-regulation was further analyzed comparing IgD expression on B cells incubated with either the IgD-binding fragment MID[962–1200] or the nonbinding fragment MID[1200–1200] (Fig. 4B). The number of surface-expressed IgD was significantly reduced as early as after 1 h of incubation with MID[962–1200], and the lowest level of expression was detected after 5 h.

The MID/IgD interaction was further studied by TEM using B cells incubated with live bacteria. The TEM analysis clearly demonstrated the interplay between M. catarrhalis and B cells. In Fig. 5A, two Moraxella diplococci were bound to a B cell, and Fig. 5B, C, and D, shows that this particular B cell simultaneously had it.

FIGURE 6. MID-expressing M. catarrhalis is bound and internalized in IgD-expressing B cells. M. catarrhalis wt and M. catarrhalis ∆mid were analyzed in flow cytometry for IgD binding (A) and UspA1/2 expression (B) before CFSE staining (C). D–I, Purified B cells were stained with RPE-conjugated anti-human CD19 or IgD, followed by incubation with CFSE-labeled M. catarrhalis and flow cytometry analysis. Internalization of CFSE-labeled M. catarrhalis in purified B cells was monitored with fluorescence (J, K, and L) and confocal microscopy (L and M). F–N, Show merged pictures from light microscopy, DAPI, and FITC filters after addition of M. catarrhalis. The scale in J–L is 15 μm, whereas the scale in M and N is 20 μm. Arrows indicate internalized bacteria.
The scale in confocal microscopy (Fig. 6). Internalization of whole bacteria was thereby monitored for 12 h using fluorescence and confocal microscopy. Characteristic *M. catarrhalis* diplococci could be detected in B cells incubated with the MID-expressing strain using fluorescence microscopy already after 10 min (Fig. 6K). As expected, uptake of the *M. catarrhalis* Δmid mutant was not detected (data not shown). In addition, the confocal microscopy confirmed internalization of *M. catarrhalis* after 10 min (Fig. 6M). A notable increase of large lymphocytes was also observed over time, proving that the MID-activated B cells developed from naive cells into lymphoblasts (Fig. 6N). Finally, the bound and internalized CFSE-stained bacteria were gradually degraded, and green fluorescence in the cytoplasm was only detected as a tinge of green in late lymphoblast.

The uptake of live bacteria was further proven using TEM, and the entire course of events is visualized in Fig. 7. After 2-h incubation of B cells with live *M. catarrhalis*, several different stages could be seen and suggested that the IgD BCR-mediated recognition and endocytosis was an ongoing and active process within the same B cell. The various stages, including Ag binding (Fig. 7B), uptake/endocytosis (Fig. 7C), and finally engulfed bacteria (Fig. 7D), are highlighted. The clustering of IgD and TLR9 on the vacuolar membrane-containing bacteria was also observed (Fig. 7E).

Thus, by using different methods (Figs. 6 and 7), we have shown that whole bacteria were taken up by B cells and degraded, supporting the activation through the intracellularly located TLR9.

### Discussion

*M. catarrhalis* is an important human respiratory pathogen with a high affinity for human IgD (31). Binding of surface-expressed IgD by MID explains the strong mitogenic effect of *M. catarrhalis* on human lymphocytes (37–39, 43). In this study, we demonstrate the importance of IgD cross-linking in *M. catarrhalis*-induced T-independent B cell activation by blocking the IgD and MID interactions with Abs against IgD or the IgD-binding part of MID. These manipulations lowered the [³H]thymidine incorporation to levels comparable to the MID-deficient counterpart. However, IgD BCR cross-linking alone or in combination with formaldehydde-killed *M. catarrhalis* Δmid was not enough to drive B cells into cell cycling. Moreover, IgD cross-linking combined with a sonicated *M. catarrhalis* Δmid preparation resulted in vigorous proliferation, suggesting involvement of immune-stimulatory PAMPs released after bacterial lysis. This triggered us to investigate the role of TLRs in *M. catarrhalis*-induced B cell activation.

The expressed TLR repertoire of human B cells is to date not fully defined. mRNA corresponding to all 10 TLRs have been detected in peripheral B cells, with a dominant expression of TLR1, 2, 6, 7, 9, and 10 (47). We have previously examined the TLR expression in both human peripheral and tonsillar B cell subsets (naive, germinal center, and memory B cells), combining mRNA analysis with protein expression in flow cytometry and immunohistochemistry (48). Our results showed the presence of TLR1, 2, 7, 9, and 10 in B cells, with only minor differences between the various B cell subsets. Furthermore, our study also showed that it is possible to activate B cells to secrete IL-6 and up-regulate MHC class II when incubated with ligands for TLR1 plus 2, 7, and 9. A higher degree of variation in TLR expression between naive, IgM-memory, and switch-memory peripheral B cells has been reported by Bernasconi et al. (49). These authors also showed an up-regulation of TLRs 9 and 10 that were induced by IgM cross-linking, indicating an infection-induced variation (49).
In this study, we show a prominent synergetic effect when combining IgD binding through MID962–1200 and a series of different TLR ligands. Interestingly, we also monitored activation via TLR2 plus 6 using diacylated lipoproteins (Pam3CSK4). The activation via TLR2 plus 6 was later explained when examining changes in TLR6 expression during cell culture (data not shown). TLR6 was not detectable at the initiation of cultures, but was up-regulated during culture in complete medium. This increased TLR6 density was particularly prominent in B cells incubated with rMID962–1200. However, this does not only explain the detected activation via TLR2 plus 6, but also differences shown in previous publications on TLR expression in human B cells, suggesting a serum-dependent alteration of the TLR expression in B cells. Naïve B cells are relatively insensitive to TLR-ligand binding, except for TLR9, which showed a comparable activation to IgD cross-linking alone. This indicates that B cells need BCR signaling to be able to fully respond to TLR ligands, a strategy of directing innate signals to Ag-stimulated B cells to prevent unspecific B cell activation.

The most potent combination of IgD BCR and TLR signaling was seen in B cell cultures with CpG-ODN 2006, in which the activation increased over 10-fold as compared with IgD binding or TLR9 signaling alone. Interestingly, a similar result was obtained when combining MID962–1200 and control CpG-ODN 2006. This can be explained by a previous study put forward by Bartz et al. (50), who showed that the differences in B cell activation between CpG-ODN 2006 and control CpG-ODN 2006 are based upon uptake. In our experimental setup, the MID fragment induced endocytosis of both oligonucleotides, thus explaining the similar results between the oligonucleotides. Furthermore, experiments using genomic M. catarrhalis DNA with or without the IgD-binding fragment proved that M. catarrhalis DNA has the potential to activate B cells via TLR9. However, the activation with M. catarrhalis DNA was lower compared with the activation induced with commercial CpG motifs, reflecting either a lower frequency of CpG motifs in the genomic DNA or differences in uptake of the larger genomic fragments. The B cell-activating potency of TLR9 could also explain the differences in B cell proliferation when cultured with either whole or lysed M. catarrhalis Δmid in microtiter plates coated with rMID962–1200. This experimental setup separated the IgD binding from the bacterium, hence avoiding receptor-mediated uptake of MID-containing bacterial fragments, and therefore excluding activation via intracellular B cell PRRs. Whole M. catarrhalis Δmid resulted in only minor synergistic effects between IgD binding (MID962–1200) and surface B cell PRR signaling. However, when repeating the experiment with a sonicated MID mutant preparation and thereby releasing intracellular PAMPs into the culture, the proliferative response was even higher than with whole M. catarrhalis wt. The strong activation could be explained by the exposure of bacterial DNA after lysis. This indicated both the importance of intracellular PAMPs in B cell superantigen coactivation and that the modest response was not caused by a lower IgD cross-linking efficiency with rMID962–1200 compared with full-length bacterial expressed MID.

We have previously shown that B cells cultured with formaldehyde-killed M. catarrhalis result in non-M. catarrhalis-specific IgM production, suggesting an important role for MID in M. catarrhalis pathogenesis redirecting the adaptive humoral response (39). In this study, we investigated the response of human tonsillar B cells from children (ages 2–12 years), and as a consequence of the high rate carriage of M. catarrhalis in this age group (51), it is therefore most likely that all of these children have encountered M. catarrhalis. Moreover, our experiments showed that the B cell activation induced by M. catarrhalis seemed to be independent of the heterogeneity in maturation stages of the tonsillar B cell population because MID-deficient M. catarrhalis would have activated the same memory B cell population as the Moraxella wt. However, MID stimulation alone is not enough to drive B cells into IgM secretion (37, 52). Data from Bekerjian-Ding et al. (53) have suggested a synergy in B cell activation between the Staphylococcus aureus B cell superantigen protein A (SpA) and TLR2 signaling. However, SpA and TLR2 signaling is not sufficient to induce IgM production, as seen with SpA-expressing bacteria (45) or rSpA with either ligands directed against TLR7 or TLR9. The present study also highlights the contribution of TLR signaling, especially TLR9 in M. catarrhalis-induced B cell activation. TLR1, 2, and 6 most likely participate in B cell activation because experiments with the dominant-negative TLR9 ligand showed a reduced activation to approximately half of what was seen in the control cultures. TLR9 signaling requires uptake of M. catarrhalis or at least DNA-containing M. catarrhalis fragments. We demonstrate a down-regulation of surface-expressed IgD without affecting IgM after IgD cross-linking by rMID962–1200 in a dose-dependent manner. The kinetics of the IgD down-regulation using MID962–1200 is displayed in Fig. 4B and shows a rapid IgD internalization. A similar reduction of IgD density was earlier observed in naive tonsillar B cells when cultured with M. catarrhalis (39). The MID-IgD interaction was highlighted in TEM with gold-labeled Abs against IgD, and the IgD-binding part of MID. The TEM image showed clustering of IgD receptors upon MID binding and formation of an immunological synapse between the B cell and bacteria. These membrane rafts (also referred to as lipid rafts) are formed within seconds after BCR cross-linking and enhance the signaling (for a review, see Ref. 54). The clustering between IgD and TLR9 on the vacuolar membrane containing M. catarrhalis was also shown by TEM. Our findings are in agreement with a recently published report by Chaturvedi et al. (55), in which TLR9 recruitment to autophagosome-like compartments after BCR cross-linking by DNA-containing Ags was proven.

Furthermore, CFSE-stained MID expressing live M. catarrhalis bound to IgD+ B cells, which resulted in internalization after 10 min of incubation. The number of large lymphocytes increased during culture, suggesting that the naive B cells developed into lymphoblasts after MID activation. In the early lymphoblasts, internalized bacteria were visualized by a faint green fluorescence, suggesting degradation of the bacteria. The entire process of binding, endocytosis, and complete internalization of live bacteria is shown in TEM after 2 h. Interestingly, Fig. 7A shows one B cell in which all these different stages are present, indicating that Ag binding and internalization is an ongoing process and that a B cell is capable of internalizing multiple Ags at the same time. Detection of bacteria in the cytoplasm and clustering of IgD and TLR9 on the vacuolar membrane clearly supported that intracellular PRRs like TLR9 play an important costimulatory role in superantigen-dependent B cell activation. Uptake of live bacteria also excludes that TLR9 signaling is an in vitro artifact restricted to formaldehyde-killed bacteria. Moreover, flow cytometry and fluorescence microscopy analyses showed that the MID-deficient isogenic mutant was neither recognized by B cells nor detected intracellularly. This suggested that MID-expressing M. catarrhalis was internalized in a BCR-dependent manner.

To summarize, in this study, we have examined B cells from children with tonsillar hyperplasia and interactions with the IgD-binding species Moraxella. Our findings highlight the importance of signaling via PRR in superantigen-mediated B cell activation, as well as for the first time demonstrate the existence of IgD-mediated endocytosis of whole bacteria in primary tonsillar B cells, supporting the strong TLR9 signals observed in the presence of Moraxella.