The IgD-binding domain of the Moraxella IgD-binding protein MID ($MID^{962-1200}$) activates human B cells in the presence of T cell cytokines

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Abstract: *Moraxella catarrhali*s IgD-binding protein (MID) is an outer membrane protein with specific affinity for both soluble and cell bound human IgD. Here, we demonstrate that mutated *M. catarrhalis* strains devoid of MID show a 75 % decreased activation of human B cells as compared to wild type bacteria. In contrast to MID-expressing Moraxella, the MID deficient Moraxella mutants did not bind to human $CD19^+$ IgD^+ B cells. The smallest MID fragment with preserved IgD-binding capacity comprises 238 amino acids ($MID^{962-1200}$). To prove the specificity of $MID^{962-1200}$ for IgD, a Chinese Hamster Ovary (CHO) cell line expressing membrane anchored human IgD was manufactured. $MID^{962-1200}$ strongly bound to the recombinant IgD on CHO cells. Moreover, $MID^{962-1200}$ stimulated peripheral blood lymphocyte proliferation 5- and 15-fold at 0.1 and 1.0 μg/ml, respectively. This activation could be completely blocked by antibodies directed against the CD40L (CD154). $MID^{962-1200}$ also activated purified B cells in the presence of IL-2 or IL-4. An increased IL-6 production was seen after stimulation with $MID^{962-1200}$ as revealed by a human cytokine protein array. $MID^{962-1200}$ fused to Green Fluorescent Protein (GFP) bound to human B cells and activated peripheral blood lymphocytes to the same degree as $MID^{962-1200}$. Taken together, MID is the only IgD-binding protein in Moraxella. Furthermore, the novel T cell independent antigen $MID^{962-1200}$ may together with $MID^{962-1200}$ – GFP be considered as promising reagents in the study of IgD-dependent B cell activation.

INTRODUCTION

Moraxella catarrhalis is an uncapsulated Gram-negative diplococcus that can be detected in nasopharyngeal cultures in 66 % of children during the first year of life and in approximately 4 % of adults at any given time. Despite *M. catarrhalis* often is considered as a commensal, the bacterium plays an important role in respiratory tract infections in both children and adults [1-4]. More than 80 % of children under the age of three years will be diagnosed with acute otitis media. After *Haemophilus influenzae* and pneumococci, *M. catarrhalis* is the third most common bacterial species causing acute otitis media. In adults and the elderly, *M. catarrhalis* is a common cause of lower respiratory tract infections particularly in those with predisposing conditions, e.g., chronic obstructive pulmonary disease (COPD). Moreover, *M. catarrhalis* is often implicated as a cause of sinusitis in both children and adults.

Despite *M. catarrhalis* is acknowledged as a human pathogen, only a few reports exist on its specific virulence factors. Interestingly, *M. catarrhalis* hampers the host innate immune system by conferring serum resistance [5, 6]. We have recently shown that *M. catarrhalis* interferes with complement activation pathway by binding C4b binding protein (C4BP) and C3 to UspA1 and UspA2 [7, 8].

In addition to the interaction with C4BP, *M. catarrhalis* displays a strong affinity for soluble IgD [9]. IgD-binding at the cellular level explains the strong mitogenic effects on human lymphocytes by *M. catarrhalis* [10-13]. We have previously isolated and characterized the high molecular weight surface protein ”Moraxella IgD-

binding protein" (MID) that displays a high affinity for both soluble and surface-bound IgD [14]. The presence of MID, also designated Hag, has been confirmed by two other laboratories [15, 16]. The apparent molecular mass of monomeric MID is estimated to approximately 200 kDa. The *mid* gene was detected in 98 different strains, and Moraxella isolates from nasopharynx, blood and sputum express MID at approximately the same frequency [17]. Moreover, no variation was observed between strains from different geographical origins.

In addition to the IgD-binding properties of MID, the outer membrane protein is an important adhesin of *M. catarrhalis* [18, 19]. MID-expressing *M. catarrhalis* strains agglutinate human erythrocytes and bind to type II alveolar epithelial cells. In contrast, *M. catarrhalis* isolates with low MID expression levels do not agglutinate erythrocytes and have a 50 % lower adhesive capacity [18]. We have shown that the hemagglutinating and adhesive part of MID is localized within the 150 amino acid residues $MID^{764-913}$. In addition, antibodies against full length MID, $MID^{764-913}$, or a 30 amino acids long consensus sequence ($MID^{775-804}$) inhibited adhesion to alveolar epithelial cells. Furthermore, in a pulmonary clearance model, mice immunized with $MID^{764-913}$ more strongly cleared *M. catarrhalis* wild type bacteria compared with control mice [20].

Downstream of the adhesive $MID^{764-913}$ sequence, we have defined a fragment comprising of 238 amino acid residues ($MID^{962-1200}$) with an essentially preserved IgD-binding when compared to full length MID^{1-2139} [21]. Shorter recombinant proteins gradually loose their IgD-binding capacity, and the shortest IgD-binding fragment comprising 157 amino acids ($MID^{985-1142}$) displays a 1,000-fold reduced IgD-

binding compared to the full length molecule. The truncated MID$^{962\text{-}1200}$ is efficiently attracted to a standard IgD serum and purified myeloma IgD(κ) and IgD(λ) sera, but not to IgG, IgM, or IgA myeloma sera. Results obtained by introducing five amino acids randomly into MID$^{962\text{-}1200}$ using transposons suggested that α-helix structures are important for the IgD-binding. Ultracentrifugation experiments and gel electrophoresis revealed that native MID$^{962\text{-}1200}$ is a tetramer. Interestingly, tetrameric MID$^{962\text{-}1200}$ attracts IgD more than 20-fold efficiently than the monomeric form.

The goals of the present paper were to examine the B cell stimulatory capacity of MID$^{1\text{-}2139}$-deficient *M. catarrhalis* and to investigate the interactions between soluble and membrane bound IgD and recombinant truncated MID$^{962\text{-}1200}$. Mutated *M. catarrhalis* strains devoid of MID showed a 75 % decreased activation as compared to wild type counterparts. In parallel, the MID deficient strains did not bind to IgD-expressing B cells. In addition, MID$^{962\text{-}1200}$ specifically bound IgD expressing CHO transfectants. MID$^{962\text{-}1200}$ activated peripheral blood lymphocytes (PBL) up to 15-fold as compared to untreated controls. Interestingly, MID$^{962\text{-}1200}$ fused to GFP was found to bind to human B cells and stimulate PBLs to the same degree as MID$^{962\text{-}1200}$. Finally, the finding that purified B cells were strongly activated by MID$^{962\text{-}1200}$ when co-incubated with T cell cytokines, further strengthen the evidence that the protein functions as a T cell independent antigen.

MATERIALS AND METHODS

Reagents

RPE-conjugated mouse anti-human CD19 mAb, FITC-conjugated rabbit anti-human IgD and swine anti-rabbit pAb were from Dakopatts (Glostrup, Denmark). A human IgD standard serum was from Dade-Behring (Paris, France). For blocking experiments, a mixture of neutralizing mouse anti-human CD154 (CD40L) mAbs (n=8) was obtained from BioLegend (San Diego, CA) and used according to the manufacturer´s instructions. HRP-conjugated goat anti-human IgD pAb were purchased from BioSource International (Camillo, CA). The anti-$MID^{902-1200}$ antiserum was perpared as previously described [21]. Briefly, rabbits were immunized intramuscularly with 200 μg of purified recombinant $MID^{902-1200}$ fragment emulsified in complete Freunds adjuvans (Difco; Becton Dickinson, Heidelberg, Germany) and boosted on days 18 and 36 with the same dose of protein in incomplete Freunds adjuvans. Blood was drawn 3 weeks later. The anti-$MID^{902-1200}$ antiserum reacted with both recombinant $MID^{962-1200}$ and $MID^{1000-1200}$. The Ig-fraction of rabbit anti-human IgD-Fab was prepared as described before [9, 21]. Human recombinant IL-2 was from Roche (Mannheim, Germany), and human recombinant IL-4 and IL-10 were obtained from Peprotech (London, UK). Phytohemagglutinin (PHA) and phorbol myristate actetate (PMA) were purchased from Sigma (St. Louis, MO).

Bacteria and culture conditions

M. catarrhalis strains RH4, BBH18 and Bc5 were clinical isolates as previously described [17]. All bacterial strains were grown on blood agar base solid medium or brain heart infusion (BHI) liquid medium. MID-deficient *M. catarrhalis* were

constructed using a standard protocol. Briefly, a kanamycin resistance gene cassette from pUC4K was amplified by PCR using specific primers introducing the restriction enzyme site for *Eco*RV. The resulting PCR product was digested and ligated into the *Eco*RV site of the *mid* gene [14]. *M. catarrhalis* strains RH4 and BBH18 were transformed by electroporation using a Genepulser apparatus (Bio-Rad, Sundbyberg, Sweden) and the settings 2.5 kV, 25 μF, and 200 Ohm. After transformation, bacteria were first cultured in BHI liquid medium without kanamycin for 6 hrs, and thereafter grown on BHI solid medium supplemented with kanamycin. Resulting mutants were deficient for MID as revealed by Western blot using pAbs directed against full length MID and the truncated MID fragments A to I [18]. Furthermore, the two mutants were devoid of IgD-binding as revealed by Western blots and flow cytometry.

For formaldehyde-killing, bacteria were grown overnight in Nutrient Broth (Oxoid, Basingstoke Hampshire, England), harvested and washed in phosphate-balanced saline (PBS), pH 7.2 by centrifugation. Thereafter, they were immediately resuspended in 0.5 % formaldehyde for 3 h at room temperature followed by heat treatment at 80 °C for 3 min. After being washed in PBS, the bacteria were suspended in RPMI 1640 medium (Gibco, Paisley, UK) and stored in aliquotes at -20 °C.

DNA cloning and protein expression

The truncated MID constructs $MID^{962-1200}$ and $MID^{1000-1200}$ were amplified by PCR using specific primers introducing the restriction enzyme sites *Bam*HI and *Hin*dIII [21]. The primers were: 5'-GGATCCTGACCAAACCAAAGGCTTAAC-3' (forward primer $MID^{962-1200}$), 5'-GCCAAGCTTGGTTTGGGCTTGGGCGACC-3' (forward primer $MID^{1000-1200}$), and 5'-CAAGGATCCAACCGATGCCACCAAC-3' (reverse

primer for both constructs). The open reading frame of the *mid* gene from *M. catarrhalis* (pET26-MID) was used as template [14]. The resulting PCR products were cloned into pET26b(+) (Novagen, Darmstadt, Germany). To avoid presumptive toxicity, the resulting plasmids were first transformed into the host *E. coli* DH5α. Thereafter, the plasmids encoding for the MID fragments were transformed into the expressing host BL21(DE3) (Novagen). To produce recombinant proteins, bacteria were grown to mid-log phase (OD_{600} 0.5-1.0) followed by 3.5 hrs of induction with 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) resulting in overexpression of the proteins.

For construction of the fusion protein $MID^{962-1200}$ - GFP, the $MID^{962-1200}$ cassette was amplified using the specific primers 5'-AAGGCATGCTGACCAAACCAAAGGCTTAACCACGCC-3' and 5'-GGTCGCCCAAGCCCAAACCCCGTCGACCAC-3' introducing the *Sph*I and *Sal*I restriction enzyme sites. The resulting product was thereafter cloned into pGFP (Clontech, Palo Alto, CA). To be able to purify the recombinant fusion protein, a histidine tag was introduced in the C-terminal of GFP using the oligonucleotide 5'-CGGCATGGACGAGCTGTACAAGCTTCACCACCACCACCACCACTG ATCAACGAATTCCC-3'. The histidine tag was also introduced in pGFP. The resulting plasmids were transformed into DH5α. To produce recombinant proteins, bacteria were grown overnight.

To purify the recombinant proteins, bacteria were sonicated and the proteins were applied to columns containing a nickel resin (Novagen) according to the manufacturer's instructions for native conditions. The concentrations of eluted

proteins were determined using the BCA Protein Assay kit (Pierce, Rockford, IL). The resulting proteins were examined by SDS-PAGE and further confirmed by Western and dot blots [21].

IgD-expressing cell-line

To manufacture the membrane bound human IgD expressing CHO cell line (CHO IgD), the IgD coding sequences were amplified by PCR from the vector pAZ6812 (kindly provided by Dr Morrison, Dept. of Microbiology and Molecular Genetics, University of California, LA, CA) using the specific primers 5'-GAGCGCTAGCCAGTGTGATGGATATCCACCATGTACTTGGGAC-3' and 5'-GAGGGATCAGGGGGGTCATGGCCAGATAGCTGACTTCTAGGCTCCG GC-3'. The membrane anchor was amplified from B cell cDNA using the primers 5'-GCCGGAGCCTAGAAGTCAGCTATCTGGCCATGACCCCCCTGATCCC TC-3' and 5'-CGCGGATCCCTACTTCACCTTGATGAAAGTGACAATGCCGCTGTAG-3'. These two products were spliced together using overlap extension PCR. After digestion with the restriction enzymes *Nhe*I and *Bam*HI, the spliced product was cloned into the pcDNA5/FRT vector (Invitrogen, Carlsbad, CA). The CHO Flp-In cell line (Invitrogen) was transfected with cDNA encoding the human κ-light-chain, resulting in a cell line designated CHO-KLC. The CHO-KLC line was supertransfected with the pcDNA5/FRT vector harbouring cDNA encoding for IgD heavy chain including the transmembrane part. All transfections were done using Lipofectin Reagent (Invitrogen).

Gel-electrophoresis

SDS-PAGE (12 %) was run as described [14]. Briefly, samples of purified $MID^{962-1200}$, $MID^{1000-1200}$ and $MID^{962-1200}$ – GFP were mixed with SDS-sample buffer at 100 °C and applied on a SDS-PAGE. Gels were stained with Coomassie Brillant Blue R-250 (Bio-Rad, Hercules, CA).

Enzyme linked immunosorbent assay (ELISA)

The interactions between IgD and the MID derived proteins were analysed in ELISA. Microtiter plates (F96 Maxisorp, Nunc-Immuno Module, Roskilde, Denmark) were coated with 50 µl of 100 nM recombinant $MID^{962-1200}$, $MID^{1000-1200}$, or $MID^{962-1200}$ – GFP in 0.1 M Tris-HCl (pH 9.0) at 4 °C overnight. The plates were washed four times with PBS containing 0.05 % Tween 20 (PBS-Tween) and blocked for 2 hrs at room temperature with PBS-Tween supplied with 1.5 % ovalbumin (blocking buffer). After four washings, the plates were incubated for 1 hr at room temperature with recombinant human IgD standard serum diluted in three-fold steps in blocking buffer. Thereafter, HRP-conjugated goat anti-human IgD pAb was added and incubated at room temperature for 45 min. After four additional washings, the plates were developed and measured at 450 nm.

Conjugation of $MID^{962-1200}$, $MID^{1000-1200}$ and $MID^{962-1200}$ – GFP to CNBr Sepharose

The $MID^{962-1200}$, $MID^{962-1200}$ and $MID^{962-1200}$ – GFP was conjugated to CNBr-Sepharose according to the manufacturer´s instructions. Briefly, 1.0 mg each of the recombinant proteins was diluted in 1 ml coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl,

pH 8.3). CNBr-activated Sepharose 4B (0.5 g; Amersham Pharmacia Biotech, Uppsala, Sweden) was pre-swelled and washed in 1 mM HCl. The recombinant proteins and CNBr-Sepharose were mixed and rotated overnight at 4 °C. Excess ligand was quantitated by the BCA Protein Assay Kit and thereafter discarded. The remaining active groups were blocked with 0.1 M Tris-HCl, pH 8.0 for 2 hrs. Finally, the conjugated proteins were washed with three cycles of 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0 and 0.1 M Tris-HCl containing 0.5 M NaCl, pH 8.0. The final product was diluted in 4 ml of 0.1 M NH_4CO_3 (pH 8.0). The estimated concentration of the proteins bound to CNBr-Sepharose was 180 μg/ml for $MID^{962-1200}$, 250 μg/ml for $MID^{1000-1200}$, and 200 μg/ml for $MID^{962-1200}$ – GFP.

Cell preparations

Human peripheral blood mononuclear cells (PBLs) were isolated from healthy donors by Lymphoprep (Nycomed, Oslo, Norway) density-gradient centrifugation as described [22]. $CD19^+$ B lymphocytes were isolated using anti-CD19 conjugated magnetic beads and a VarioMACS magnetic cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer´s instructions. $CD19^+$ B cells isolated by positive selection were routinely > 97 % $HLA\text{-}DR^+$ as assessed by flow cytometry. A series of experiments was also performed by using negatively selected B cells (B cell isolation kit; Miltenyi Biotec) with an additional step using anti-CD3 conjugated magnetic beads (Miltenyi Biotec). Cells were cultured in RPMI 1640 medium (Life Technologies, Paisley, Scotland) supplemented with 10 % FCS, 2 mM glutamine and 10 μg/ml gentamicin (complete medium). A total of $2.5x10^5$ cells were cultured in 96-well round-bottomed plates (Nunc, Roskilde, Denmark) in triplicates in

a final volume of 200 μl of complete medium supplemented with various reagents. In CD40L blocking experiments, PBLs were preincubated with 50 μg/ml anti-CD40L mAb for 1 hr at 37 °C. In some experiments, $MID^{962-1200}$ was immobilized on flat-bottomed plates, which gave the same results as $MID^{962-1200}$ conjugated to CNBr-Sepharose. To analyze whether the folding of $MID^{962-1200}$ affected the proliferation, $MID^{962-1200}$ was incubated at various temperatures (20, 37, 60 and 100 ºC). After 15 min, $MID^{962-1200}$ was immediately coated on flat-bottomed plates in 0.1 M Tris-HCl (pH 9.0) at 4 °C over night. The plates were thoroughly washed before addition of PBLs. Proliferation was measured by [methyl-^{3}H]-thymidine incorporation (5 μCi/well; Amersham Pharmacia Biotech) using an 18 hrs pulse period.

In some experiments, a T cell cytokine rich supernatant was used. To produce T cell cytokines, T lymphocytes that were obtained from the ”flow-through” during positive B cell selection (as described above) were incubated with 1 μg/ml PHA and 50 ng/ml PMA for 24 hrs. After centrifugation, the supernatant was thoroughly dialysed (dialysis filter: molecular weight cut off 6-8000 kDa); (Spectrum Laboratories, Rancho Dominguez, CA) for 24 hrs at 4 °C in order to remove the mitogens. For the cytokine protein arrays, 1 x 10^{6} negatively selected B cells were cultured with or without $MID^{962-1200}$ in 12-well flat-bottomed plates (Nunc) in a final volume of 1 ml of complete medium. The cell free supernatant was harvested after 96 hrs and the cytokine protein arrays were performed according to the manufacturer’s instructions (RayBio Human antibody array VI, RayBiotech, Inc. Norcross, GA).

Fluorescence labeling of *M. catarrhalis*

Bacteria (OD_{600} 0.4) were washed in phosphate buffered saline (PBS), pH 7.4, and then resuspended in 1 ml PBS containing 1 % bovine serum albumin (BSA). FITC (Sigma) was added at a final concentration of 0.2 mg/ml and the suspension was incubated at room temperature. After 20 min under rotation, Moraxella was washed three times in PBS-BSA and resuspended in PBS before addition to the cells. The bacterial viability was not affected by the labeling as judged by counting colony forming units on agar plates.

Flow cytometry analyses

For flow cytometry analyses, bacteria (10^8) were washed twice with PBS containing 1 % BSA and incubated with a human IgD standard serum or rabbit anti-$MID^{902-1200}$ polyclonal antibodies in a final volume of 100 µl PBS, 1 % BSA on ice for 30 min. After washing, the bacteria were incubated with FITC-conjugated anti-IgD pAb or FITC-conjugated swine anti-rabbit polyclonal antibodies for 30 min on ice. After two additional washes, the bacteria were analysed by flow cytometry (EPICS, XL-MCL flow cytometer, Coulter, Hialeah, FL). In another set of experiments, the FITC-labeled *M. catarrhalis* strain BBH18 or its derived MID mutant was incubated with 5 x 10^5 PBLs in 100 µl of PBS containing 1 % BSA for 30 min on ice. After washings, a RPE-conjugated anti-CD19 mAb was added for 30 min on ice followed by additional washings and analysis by flow cytometry.

To determine the ability of $MID^{962-1200}$ – GFP fusion protein to bind to human B cells, 10 µg of $MID^{962-1200}$ – GFP was incubated with 5 x 10^5 PBLs in 100 µl of PBS-BSA.

After 30 min on ice and washings, anti-CD19-RPE mAb was added for another 30 min on ice followed by additional washings and flow cytometry analysis. In blocking experiments, PBLs were pre-incubated with 30 μg anti-human IgD-Fab pAb for 1 hr on ice before incubation with the $MID^{962-1200}$ – GFP fusion protein. After two additional washes, the cells were analysed by FACS Calibur Flow Cytometer (Becton Dickinson, NJ). FITC-conjugated rabbit anti-human IgD and GFP was included as controls.

To investigate the presence of membrane anchored IgD on the surface of CHO-IgD, 3 x 10^5 cells were incubated with an anti-human IgD FITC mAb for 1 hr on ice. Thereafter, the cells were washed in PBS-BSA and analyzed in the FACS Calibur Flow Cytometer (Becton Dickinson, NJ). The binding of $MID^{962-1200}$ to CHO-IgD was investigated by incubating 3 x 10^5 cells with 100 μg $MID^{962-1200}$ for 1 hr on ice. After washing in PBS-BSA, anti-$MID^{962-1200}$ pAb were added. Cells were washed and incubated with FITC-conjugated goat anti-rabbit pAB. Cells were analyzed by flow cytometry. CHO-KLC was used as a negative control.

Immunohistochemistry

For fluorescence microscopy, 2 x 10^6 PBLs were incubated with 0.5 μg $MID^{962-1200}$ – GFP or GFP together with RPE-conjugated anti-CD19 mAb in PBS containing 1 % BSA for 30 min on ice. Thereafter, PBLs were then washed twice in PBS. Samples were examined using a Nikon Eclipse E800 fluorescence microscope (Nikon, Osaka, Japan), and images analyzed using Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

RESULTS

M. catarrhalis-dependent activation of human peripheral blood lymphocytes is caused by the outer membrane protein MID

M. catarrhalis stimulates B cells to proliferate and MID purified from Moraxella bacteria has been demonstrated to induce B cell proliferation [11, 13]. To further examine the importance of MID in *M. catarrhalis*-dependent activation, two Moraxella clinical isolates (BBH18 and RH4) were chosen for inactivation of MID. A kanamycin resistance gene cassette was introduced into the chromosome and the resulting mutants were screened for the absence of MID expression and further confirmed by PCR. Flow cytometry analysis of the two MID deficient mutant *M. catarrhalis* strains using an anti-$MID^{902\text{-}1200}$ polyclonal antibody proved the absence of MID at the cell surface. In **Figure 1A**, the flow cytometry analysis of *M. catarrhalis* BBH18 strain is exemplified**.** The wild type BBH18 strongly bound soluble IgD, whereas the corresponding MID deficient mutant was negative for IgD-binding **(Fig. 1B).**

To demonstrate the specificity of the MID-dependent binding to human B cells, the *M. catarrhalis* wild type and the MID-deficient *M. catarrhalis* mutant were FITC-conjugated followed by incubation with PBLs. B lymphocytes were visualized by RPE-conjugated anti-CD19 mAbs. The high MID-expressing wild type *M. catarrhalis* BBH18 bound to $CD19^+$ B cells (**Fig. 1C**), whereas the MID-deficient mutant did not (**Fig. 1D**). Furthermore, neither the MID expressing Moraxella strain nor the mutant bound to the T cells (the $CD19^-$ cell population). A FITC-conjugated anti-human IgD

pAb was used as positive control (**Fig. 1E**). Similar results were obtained with the *M. catarrhalis* RH4 strain and the corresponding RH4Δ*mid* mutant (data not shown).

We also incubated human PBLs with the *M. catarrhalis* BBH18 and RH4 wild type bacteria and their MID deficient counterparts. Cells were pulsed with [^{3}H]-thymidine and the uptake was analysed by scintillation counting. Interestingly, PBLs incubated with BBH18Δ*mid* and RH4Δ*mid* showed a 75 % reduced proliferation as compared to cells stimulated with the wild type MID-expressing bacteria (**Fig. 2**). Thus, the IgD-binding protein MID was the major Moraxella protein responsible for binding to and consequently activating human B cells.

MID$^{962\text{-}1200}$ fused to GFP retains its IgD- and B cell binding capacity

The smallest fragment with essentially preserved soluble IgD-binding, as compared to the full-length molecule MID, comprises 238 amino acid residues (MID$^{962\text{-}1200}$) (**Fig. 3A**). MID$^{962\text{-}1200}$ was recombinantly produced in *E. coli* and purified by affinity chromatography. In addition, a non-IgD-binding fragment (MID$^{1000\text{-}1200}$) was manufactured to be included as a negative control (**Fig. 3A and B**). To further characterize the interaction between MID$^{962\text{-}1200}$ and B-cells, a fusion protein of MID$^{962\text{-}1200}$ and GFP was constructed. MID$^{962\text{-}1200}$ as well as the MID$^{962\text{-}1200}$ – GFP fusion protein bound soluble recombinant IgD in a dose-dependent manner as revealed by an IgD ELISA, whereas MID$^{1000\text{-}1200}$ did not bind IgD (**Fig. 3C**). Thus, the ability of MID$^{962\text{-}1200}$ - GFP to bind soluble IgD was not affected by the GFP fusion partner.

To analyse whether the $MID^{962-1200}$ – GFP fusion protein bound to the B cell receptor (BCR) IgD, human PBLs were isolated. PBLs were incubated with $MID^{962-1200}$ – GFP and anti-human CD19 RPE followed by flow cytometry analyses and fluorescence microscopy. As can be seen in **Figure 4B**, $MID^{962-1200}$ – GFP efficiently bound to the $CD19^+$ B cells. The interaction was completely blocked when the PBLs were preincubated with anti-human IgD-Fab (**Fig. 4C**). Furthermore, the specificity for the IgD receptor was verified by staining B cells with anti-CD19 mAb and FITC-conjugated anti-IgD pAb in the presence of $MID^{962-1200}$ – GFP **(Fig. 4E). This combination of antibodies in addition to $MID^{962-1200}$ – GFP did not further increase the fluorescence (mfi) excluding other surface molecules involved in $MID^{962-1200}$ – GFP binding.** The binding of $MID^{962-1200}$ – GFP to B cells was also confirmed by fluorescence microscopy. $MID^{962-1200}$ – GFP (green fluorescence) bound to $CD19^+$ cells (red fluorescence), whereas $CD19^-$ cells did not bind $MID^{962-1200}$ – GFP (**Fig. 4H and I**). In contrast, any green fluorescence could not be detected when PBLs were incubated with GFP alone (**Fig. 4L**). Taken together, $MID^{962-1200}$ – GFP specifically bound to IgD-expressing B cells as revealed by flow cytometry and immunohistochemistry.

$MID^{962-1200}$ binds recombinantly expressed membrane anchored IgD

To further prove that $MID^{962-1200}$ binds to the IgD BCR, IgD-expressing CHO cells were manufactured. A cell line was established by transfecting cDNA encoding κ-light-chains (KLC). Thereafter, the CHO-KLC cell line was supertransfected with cDNA coding for the IgD heavy chain containing the cell membrane bound anchor. The resulting CHO-IgD transfectants were confirmed by flow cytometry using a mAb

directed against human IgD (**Fig. 5A**). The negative control, i.e., the CHO-KLC cell line did not express IgD. To analyse binding of $MID^{962-1200}$ to the IgD BCR, IgD expressing CHO cells were incubated with $MID^{962-1200}$ followed by rabbit anti-$MID^{902-1200}$ pAb and FITC-conjugated goat anti-rabbit pAb. Flow cytometry profiles revealed that $MID^{962-1200}$ bound to the CHO-IgD transfectants (**Fig. 5B**). In contrast, $MID^{962-1200}$ did not bind to CHO-KLC indicating that the binding was specific for IgD. The apparent binding of $MID^{962-1200}$ to CHO-KLC in **Figure 5B** was background binding of the anti-$MID^{902-1200}$ polyclonal antibody to the CHO-KLC cells. Thus, our results further strengthen the specificity of $MID^{962-1200}$ for membrane anchored IgD.

$MID^{962-1200}$ and $MID^{962-1200}$ – GFP activates human PBLs, whereas $MID^{1000-1200}$ is inert

To analyse the capacity of $MID^{962-1200}$ and the GFP-fusion protein to stimulate human lymphocytes, PBLs were isolated and incubated for various times with $MID^{962-1200}$ or $MID^{962-1200}$ – GFP at increasing concentrations. In these experiments, $MID^{962-1200}$ or $MID^{962-1200}$ – GFP was conjugated to Sepharose beads in order to achieve an efficient crosslinking of the IgD BCRs. During the last 18 hrs of incubation, PBLs were pulsed with [^{3}H]-thymidine. As can be seen in **Figure 6A**, $MID^{962-1200}$ at 0.5 μg/ml was optimal to induce a vigorous proliferation. Interestingly, similar results were obtained with the $MID^{962-1200}$ – GFP fusion protein (**Fig. 6B**). However, since the GFP protein is almost twice as large as $MID^{962-1200}$, more molecules were required to achieve the same level of proliferative response. Analysis of the kinetics revealed that the strongest proliferation was observed at 96 hrs (**Fig. 6C**). A similar kinetics was seen with $MID^{962-1200}$ – GFP (not shown). In contrast, the recombinant non-IgD-binding

$MID^{1000-1200}$ fragment, which was included as a negative control, did not induce any proliferation. Thus, $MID^{962-1200}$ and the GFP – fusion protein both induced lymphocyte proliferation.

To determine the importance of the CD40/ CD40L interaction between T cells and $MID^{962-1200}$-induced B cells, PBLs were preincubated with a mixture of neutralizing anti-CD40L mAb. As can be seen in **Figure 6D**, blocking of the CD40L completely inhibited the proliferation. This result proved that the physical B and T cell interaction was crucial for $MID^{962-1200}$-induced PBL activation.

We recently demonstrated that $MID^{962-1200}$ is a tetramer under native conditions and that the tetramer bound IgD 23-fold more efficiently as compared to the monomeric form [21]. To analyse whether the folding of $MID^{962-1200}$ affected its ability to stimulate human lymphocytes, $MID^{962-1200}$ was incubated at increasing temperatures in order to create monomers. Thereafter, cell culture plates were coated with $MID^{962-1200}$ followed by incubation with PBLs. Interestingly, the proliferation of the PBLs dramatically decreased when $MID^{962-1200}$ had been incubated at 60 and 100°C (**Fig. 7**)**, i. e., when $MID^{962-1200}$ were denaturated and existed as a monomer [21].** This implies that a stable tetramer formation of $MID^{962-1200}$ is required for efficient activation.

$MID^{962-1200}$ stimulates B cells in a T cell independent manner requiring T cell cytokines

To investigate the importance of T cell cytokines for the $MID^{962-1200}$-dependent activation of human B lymphocytes, purified B cells were incubated with $MID^{962-1200}$ in the absence or presence of a cell-free supernatant obtained from T lymphocytes, which had been stimulated with phorbol myristate acetate (PMA) and phytohemagglutinin (PHA) for 24 hrs. Only a minor stimulation of purified B cells was seen in the presence of 0.5 or 1.0 μg/ml $MID^{962-1200}$ without cytokines (**Fig. 8A**). In contrast, when the T cell cytokine-rich supernatant was supplemented together with $MID^{962-1200}$, a strong proliferation was observed, demonstrating the need for a co-stimulatory signal. When B lymphocytes were incubated with T cell cytokines only (in the absence of $MID^{962-1200}$), no proliferation was found. This control ensured that the T cell supernatant did not contain trace amounts of the mitogens PHA and PMA. Furthermore, the non-IgD-binding recombinant fragment $MID^{1000-1200}$, which in addition to $MID^{962-1200}$ also was produced in *E. coli,* did not activate B cells in the presence of the T cell cytokine-rich supernatant, excluding that trace amounts of *E. coli* lipopolysaccharide (LPS) induced the B lymphocytes (**Fig. 8A**).

Experiments with the T cell supernatant revealed that cytokines were crucial for optimal B cell activation in the presence of $MID^{962-1200}$. To further examine the specific need for T cell cytokine co-stimulatory activation, the cytokines IL-2, IL-4, and IL-10 were separately incubated with $MID^{962-1200}$. Interestingly, when IL-4 or IL-2 was supplemented together with $MID^{962-1200}$, an increased proliferation was observed as compared to cells incubated with the truncated $MID^{962-1200}$ fragment only (**Fig. 8B**). $MID^{962-1200}$ at 0.5 μg/ml together with IL-4 increased the proliferation up to 9-fold, whereas $MID^{962-1200}$ together with IL-2 resulted in a 4.5-fold increase. Addition of IL-10 slightly inhibited the proliferative response, and when IL-10 was added separately

together with MID$^{962-1200}$ no significant proliferation could be detected (**Fig. 8B**). Furthermore, B cells did not proliferate when incubated with cytokines in the absence of MID$^{962-1200}$. The data shown represent experiment with positively selected B cells, but similar results were obtained with untouched (negatively selected) B cells. Taken together, MID$^{962-1200}$-dependent B cell activation required the T cell cytokines IL-2 or IL-4, but no direct T cell contact.

MID$^{962-1200}$ induces IL-6 production

We recently showed that full-length MID stimulates B cell to produce IL-6 [13]. In order to determine the cytokine production of B cells after stimulation with MID$^{962-1200}$, B cells were isolated using negative selection with an additional anti-CD3 mAb purification step reducing the T cell population to < 0.1 %. The final preparation contained > 96 % pure B cells. A human cytokine array containing antibodies directed against 60 different growth factors, chemokines, and interleukins including IL-10, IL-6, TNF-α, TGF-β, and IL-1 was used. A strongly increased IL-6 production was detected when B cells were activated with MID$^{962-1200}$ as compared to unstimulated cells (**Fig. 9**). A slightly upregulated IL-1β synthesis was also observed with stimulated B cells. Finally, a low level of macrophage chemoattractant protein-2 (MCP-2) was found irrespectively of activation. Taken together, amongst all cytokines and chemokines tested, IL-6 was the main interleukin produced by purified B cells incubated with MID$^{962-1200}$.

DISCUSSION

The respiratory pathogen *M. catarrhalis* is a strong IgD-binding bacterium [14, 17]. In the present paper we show, by using MID-deficient Moraxella mutants, that MID was the only *M. catarrhalis* outer membrane protein interacting with IgD (**Fig. 1**). Furthermore, the truncated protein $MID^{962-1200}$ activated human B cells in the range 0.1 to 5 μg/ml when either T cells (**Fig. 6**) or T cell cytokines (**Fig. 8**) were supplied. Importantly, the non-IgD binding fragment $MID^{1000-1200}$, which in addition to $MID^{962-1200}$ was produced in *E. coli*, did not induce any B cell proliferation. $MID^{1000-1200}$ also failed to attract soluble IgD when analysed in ELISA **(Fig. 3)**. $MID^{962-1200}$ was shown to recognize membrane anchored IgD on the surface of transfected CHO cells **(Fig. 5)**. Finally, $MID^{962-1200}$ fused to GFP was found to bind and stimulate B cells in the same order as $MID^{962-1200}$.

In addition to the IgD-binding *M. catarrhalis*, only a few examples of nonimmune Ig-binding to Gram-negative bacteria are known. Binding of bovine IgM to *Brucella abortus,* equine IgG to *Taylorella equigenitalis*, and bovine IgG and IgM to *Haemophilus somnus* have been reported [23-25]. An Ig-Fc-receptor has also been purified from *H. somnus* [26]. Furthermore, the respiratory pathogen *H. influenzae* strongly attracts human IgD, albeit the particular Ig-binding protein has not yet been found [27].

In contrast to Gram-negative species, several examples exist of Gram-positive bacteria that attract immunoglobulins in a nonimmune manner. *Staphylococcus aureus* protein A (SpA) is the most characterized protein and binds to the Fc part of IgG [28, 29]. SpA also binds a fraction of Ig-molecules of all classes due to the so-

called ‘alternative’ binding to VH3 [30]. In addition to SpA, an *S. aureus* gene encoding another Ig-binding protein has been found [31]. Protein G isolated from group C and G streptococci of human origin has a distinct affinity for the same site on the human Fc-fragment of IgG as SpA, but also interacts with IgG Fab fragments [32, 33]. Protein H, isolated from *Streptococcus pyogenes*, is able to compete for the same region of IgG-Fc with SpA and protein G [34]. In addition, *S. pyogenes* produces an antigenically and functionally heterogenous group of M-like proteins with different Ig-binding specificities [35]. Protein Bac or the B-antigen is an IgA-binding protein expressed by certain strains of group B streptococci [36]. Finally, protein L, a surface component of *Peptostreptococcus magnus*, has affinity for all classes of Ig through an interaction with determinants present in the variable region of κ-light-chains [37].

The outer-membrane glycolipids of *M. catarrhalis* lack the repeating O-antigen polysaccharides of lipopolysaccharides (LPS) and are hence designated lipo-oligosaccharides (LOS) [38]. These occur commonly in non-enteric Gram-negative bacteria, such as those that colonise the mucosal surfaces of the upper respiratory tract [39]. The LOS of *M. catarrhalis* consists of lipid A plus a core polysaccharide and one oligosaccharide [40]. The lipid A component is similar to that of other Gram-negative bacteria [41] and cross-reacts with lipid A of the *Enterobacteriaceae*, but it lacks the 3-hydroxytetradecanoic acid that normally is present in enteric bacteria [40]. We show that lymphocyte activation in the presence of MID-deficient *M. catarrhalis* mutants was significantly decreased compared to when cells were incubated with wild type bacteria expressing high levels of MID (**Fig. 2**). These experiments did not only demonstrate the importance of MID as the major B cell stimulus of moraxella´s but also that *M. catarrhalis* LOS plays a minor role in human B lymphocyte activation.

When B cells are activated through IgD an intracellular signal is evoked resulting in cell division and an improved humoral response. $MID^{962-1200}$ strongly activates human B cells both in the presence and absence of T cells. This is in parallel to full length MID^{1-2139} or MID^{1-2139} conjugated to Sepharose that stimulates human peripheral B cells, but not T cells [13]. IgM-secretion was detected in B cell cultures stimulated with MID and IL-2, whereas secretion of IgG and IgA was induced in cultures with the combination of MID and IL-4, IL-10 and soluble CD40L. These findings suggest that Th2-derived cytokines are required for MID-dependent class-switch [13].

MID and other antigens that can induce antibody responses without obvious ("physical") T cell help are classified as T cell independent (TI) antigens [13, 42]. This phenomenon was initially evaluated by the ability of antigens to elicit antibody responses in T cell-deficient nude mice. In general, TI antigens have repeating determinants that can be recognized by antibody receptors on B lymphocytes. TI antigens have been subdivided into two types based upon their differential ability to induce Ab responses in neonates and in CBA/N mice, the later having an X-linked immune deficiency [43]. Dextran-conjugated anti-IgD antibodies can at very low concentrations induce a B cell signal and have as a representative TI-2 antigen been used in several experimental models [42, 44]. In contrast to other TI-2 antigens, the dextran-conjugated anti-IgD antibodies do not result in antigen specific antibodies but induce a polyclonal activation [45]. Activation by MID most likely also results in a polyclonal antibody production and by analogy with dextran-conjugated anti-IgD antibodies MID, as an activator through the IgD BCR, may also be regarded as a TI-2 antigen.

In spite of the fact that $MID^{962-1200}$ activated the B cells without the presence of physical T cell help, addition of cytokines was required to achieve similar B cell proliferation as compared to cell cultures with PBLs. The increased proliferation in cultures with PBLs can be explained by co-stimulatory activation via the CD40-CD40L and cytokines. Indeed, blocking of the CD40L prior to stimulation with $MID^{962-1200}$ resulted in a decreased proliferation (**Fig. 6D**). It has also been shown that recombinant CD40L strongly enhances MID-dependent B cell proliferation [13]. Since $MID^{962-1200}$ was covalently attached to Sepharose beads or in some experiments bound to the microtiter plate plastic surface, $MID^{962-1200}$ was most likely not internalized, processed, or presented to T cells on MHC class II. The T cells might, however, be activated through the release of B cell cytokines, e.g., IL-6 (**Fig. 9**). Finally, it cannot be excluded that monocytes in the PBL preparations also were activated by lymphocyte cytokines.

An interesting fact is that a high number of IgD-producing plasma cells have been observed in the lymphoid tissue from nasopharyngeal tonsils and lacrimal, parotid, and lactating mammary glands as compared with spleen lymph nodes and glandular tissue of the gastrointestinal tract [46, 47]. A substantial local IgD synthesis is found both in nasopharynx and in the middle ear cavity [48, 49]. In approximately 20 % of middle ear effusions examined, a content of more than 600 mg/l of IgD can be calculated. This finding indicates that IgD may have a crucial role in the humoral immunity of secretions from the upper respiratory tract. *M. catarrhalis* frequently colonizes the respiratory tract and also is an important pathogen in, for example, acute otitis media. It is an enigma why *M. catarrhalis* binds IgD, especially in the light of a

much higher bacterial turnover rate compared to the human host, and thus would mutate or turn off a non-beneficial IgD-binding protein within a short period of time. Is it of value for the bacterium to bind IgD and/ or does the nonimmune IgD binding play a role in the innate (humoral) immune response? A possibility would be that *M. catarrhalis* by inducing the BCR signalling pathway entices the B cells to polyclonal Ig synthesis, which perhaps would be potentially harmless for the bacterium itself, and thus excluding production of specific monoclonal anti-moraxella antibodies. It is tempting to speculate that a bacteria-IgD interaction on lymphocytes and in secretions of the mucous membranes may play an important role in both the pathogenesis and host defence in upper respiratory tract infections. Thus, further studies on bacterial IgD-binding proteins are required to fully explain the *M. catarrhalis*/ host interactions and $MID^{962-1200}$ will most likely be a basis of further studies.

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LEGENDS TO FIGURES

Fig. 1. MID-deficient *M. catarrhalis* does not bind soluble or membrane bound IgD. Flow cytometry profiles of *M. catarrhalis* BBH18 and the MID-deficient mutant BBH18Δ*mid* showing MID expression (A), or IgD-binding (B). PBLs were incubated with the FITC-conjugated BBH18 wild type (C), the MID-deficient mutant BBH18Δ*mid* (D), or anti-human IgD pAb (E). In (A and B), the bacteria were grown on solid medium overnight. After incubation with anti-$MID^{902-1200}$ pAb or human IgD standard serum followed by a FITC-conjugated anti-rabbit pAb or anti-human IgD pAb and several washings, bacteria were analysed by flow cytometry. In (C to E), PBLs were isolated from heparinized human blood using Lymphoprep one-step gradients, incubated with an RPE-conjugated anti-CD19 mAb, washed and further incubated with FITC-conjugated *M. catarrhalis* or FITC-conjugated anti-human IgD pAb. After final washings, PBLs were analysed by flow cytometry.

Fig. 2. MID-expressing *M. catarrhalis* strongly activates peripheral blood lymphocytes as compared to MID-deficient *M. catarrhalis*. PBLs were incubated with formaldehyde-killed *M. catarrhalis* BBH18 or RH4 wild type strains or their MID mutant counterparts. After 3 days, lymphocyte proliferation was analysed by determination of [^{3}H]-thymidine uptake. Control indicates the background proliferation without any added bacteria. Three separate experiments with different donors were done. Error bars indicate SD.

Fig. 3. Schematic map of the recombinant proteins used in this study. $MID^{962-1200}$ and $MID^{962-1200}$ – GFP binds soluble human IgD in a dose-dependent manner, whereas

$MID^{1000-1200}$ does not. Localization of the two truncated protein fragments $MID^{962-1200}$ and $MID^{1000-1200}$ within full-length MID^{1-2139} (A) (16). Coomassie stained gel demonstrating the purity of the recombinant proteins (B). A molecular weight standard is indicated to the left. Soluble IgD bound to $MID^{962-1200}$ and $MID^{962-1200}$ – GFP, but not to $MID^{1000-1200}$ as analysed by ELISA (C). Recombinant proteins were expressed in *E. coli* and purified using His-tags followed by analysis in SDS-PAGE. In (C), an immunoplate was coated with $MID^{962-1200}$, $MID^{962-1200}$ – GFP or $MID^{1000-1200}$ followed by incubation with human IgD diluted in three-fold steps and added in duplicates. After incubation and washings, bound IgD was analysed by HRP-conjugated goat anti-human IgD pAb. The experiment was repeated twice and error bars show SD.

Fig. 4. The fusion protein $MID^{962-1200}$ – GFP specifically bound to $CD19^+$ IgD^+ B cells. Flow cytometry profiles demonstrate PBLs incubated with RPE-conjugated anti-CD19 mAbs (A-F) together with $MID^{962-1200}$ – GFP (B, C and E) or FITC-conjugated anti-human IgD (D and E). In (C), PBLs were preincubated with anti-IgD-Fab pAb before addition of $MID^{962-1200}$ – GFP. GFP was used as a negative control (F). In (E), anti-IgD-FITC mAb and $MID^{962-1200}$ – GFP were added simultaneously. Panels with immunohistochemistry show bright fields (G and J) and filters for the emission wavelengths of RPE (575 nm) (H and K) or GFP (508 nm) (I and L). PBLs without added mAbs or recombinant proteins were used to exclude autofluorescence (not shown). In (G-I), PBLs were incubated with RPE-conjugated anti-CD19 mAbs and $MID^{962-1200}$ – GFP. Moreover, cells were also incubated with RPE-conjugated anti-CD19 mAbs and GFP without the IgD-binding protein $MID^{962-1200}$ (J-L). PBLs were purified from heparinized human blood using Ficoll gradients, incubated with with

RPE-conjugated anti-CD19 mAbs, washed and further incubated with $MID^{962-1200}$ – GFP or GFP as indicated. After final washings, PBLs were analyzed by flow cytometry or fluorescence microscopy. Representative results from two separate experiments are shown.

Fig. 5. $MID^{962-1200}$ specifically binds to membrane anchored recombinant IgD. (A) CHO cells expressing KLC (CHO-KLC) or KLC in addition to the IgD heavy chain (CHO-IgD) were analysed by flow cytometry using FITC-conjugated anti-human IgD. (B) Flow cytometry profiles of the two CHO cell lines pre-incubated with $MID^{962-1200}$ followed by detection with rabbit anti- $MID^{902-1200}$ and a FITC-conjugated goat anti-rabbit pAb.

Fig. 6. $MID^{962-1200}$ and $MID^{962-1200}$ – GFP stimulates untouched PBLs to vigorous proliferation and blocking with anti-CD40L mAbs inhibits the activation. $MID^{962-1200}$ (A) or $MID^{962-1200}$ – GFP (B) activates human PBLs in a dose-dependent manner (range 0.1 to 5 μg/ml). (C) Kinetics of PBL proliferation. (D) PBLs preincubated with anti-CD40L mAbs (50 μg/ml). PBLs were isolated and incubated with formaldehyde-killed *M. catarrhalis* or the indicated recombinant proteins conjugated to CNBr-Sepharose for 96 hrs (A, B and D) or 48 to 120 hrs (C). The last 18 hrs, the PBLs were pulsed with [^{3}H]-thymidine. Results are presented as means of three separate experiments with different donors. In (D), the control with *M. catarrhalis* Bc5 in each experiment was defined as 100 arbitrary units. Error bars indicate SD.

Fig. 7. The native folding of tetrameric $MID^{962-1200}$ is crucial for efficient activation of PBLs. $MID^{962-1200}$ was treated at room temperature, 37, 60, or 100°C and thereafter

flat-bottomed cell culture plates were coated with the resulting tetramers (native $MID^{962-1200}$) or monomers. After incubation with PBLs for 96 hrs, the [^{3}H]-thymidine uptake was determined. Results are presented as means of three separate experiments with different donors. The highest value in each experiment was defined as 100 arbitrary units. Error bars indicate SD.

Fig. 8. Purified B cells are activated in the presence of $MID^{962-1200}$ and T cell cytokines. B cells were incubated with $MID^{962-1200}$ at 0.5 or 1 μg/ml with or without a supernatant obtained from stimulated T lymphocytes (A). The cytokine-rich supernatant was manufactured by stimulating purified T lymphocytes with PHA and PMA followed by extensive dialysis. $MID^{1000-1200}$ was included as a negative control. $MID^{962-1200}$ at 0.5 μg/ml induced a strong B cell proliferation when incubated with the Th2 cytokine IL-4 (10 ng/ml), whereas a lower activation was observed in the presence of IL-2 (10 units/ml), IL-10 was used at 50 ng/ml (B). Human B lymphocytes were purified by positive selection and incubated with the recombinant proteins for 96 hrs as indicated. Proliferation was analysed by pulsing cells with [^{3}H]-thymidine overnight. Three different donors were analysed at three separate occasions. The highest value in each experiment was defined as 100 arbitrary units. Data are presented as means and error bars show SD.

Fig. 9. $MID^{962-1200}$ -induced B cells mainly produce IL-6. A minor upregulation of IL-1β was also seen. Purified B cells were incubated with (A) or without $MID^{962-1200}$ (B) for 96 hrs. Thereafter, the cytokine contents in the B cell culture supernatants were determined using a human cytokine protein array. The protein microarray cut off

controls are indicated as positive controls. Complete medium was included as a negative control (data not shown).

Fig. 1. Nordström *et al.*

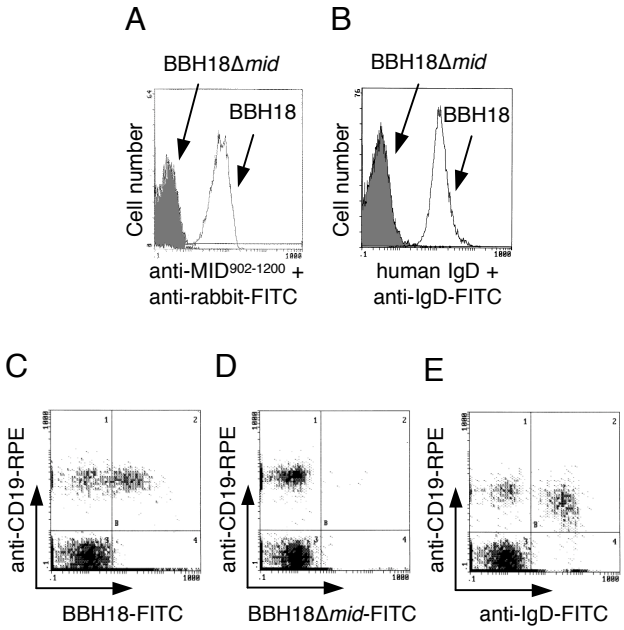


Fig. 2. Nordström *et al.*

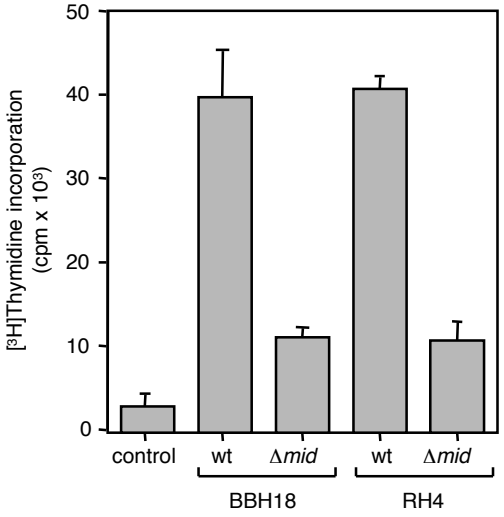
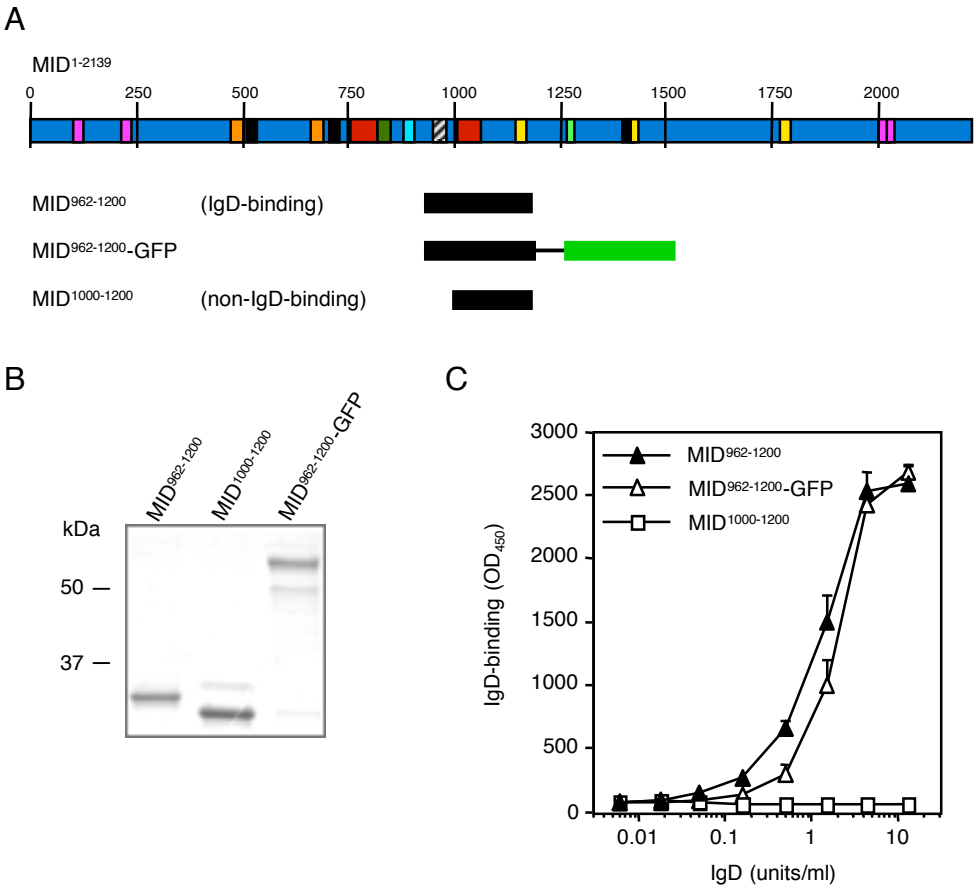


Fig. 3. Nordström *et al.*



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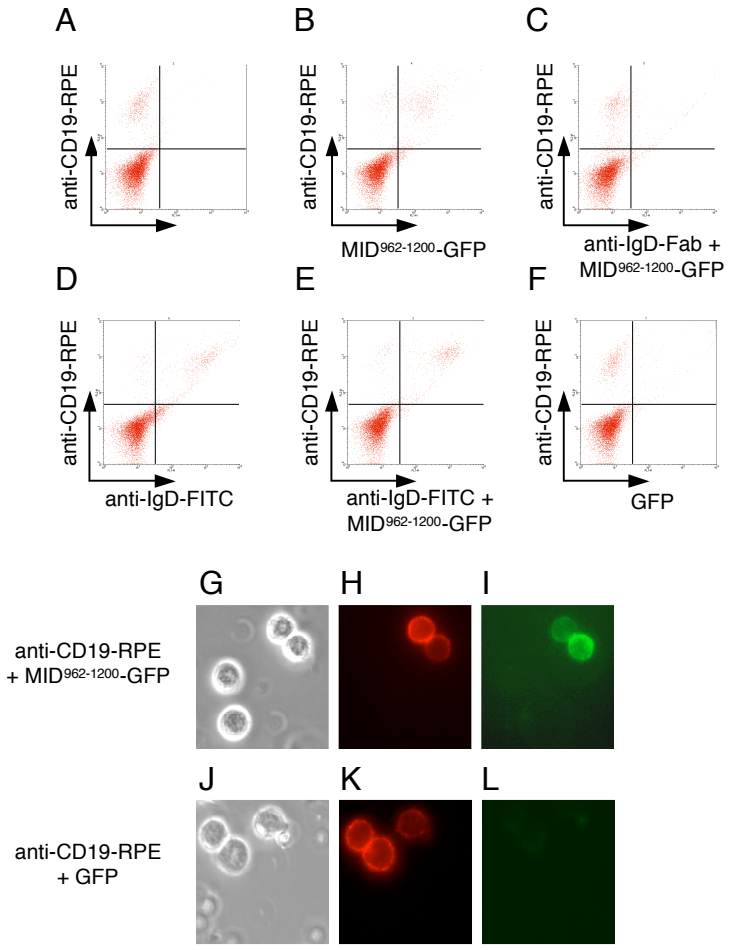


Fig. 5. Nordström *et al.*

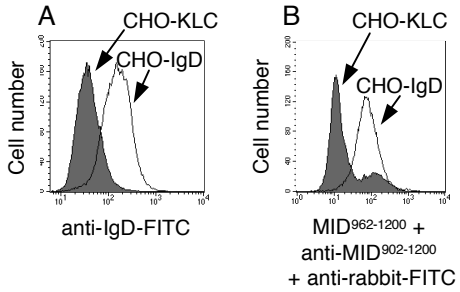


Fig. 6. Nordström *et al.*

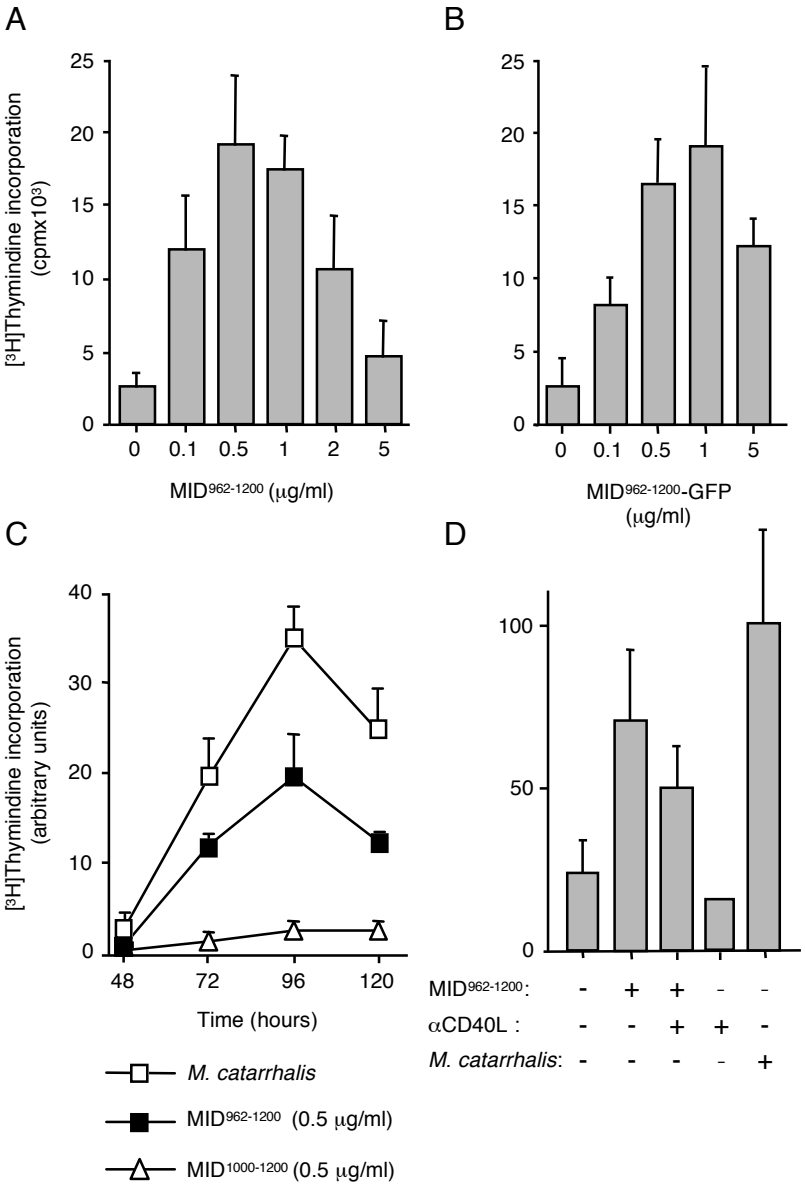
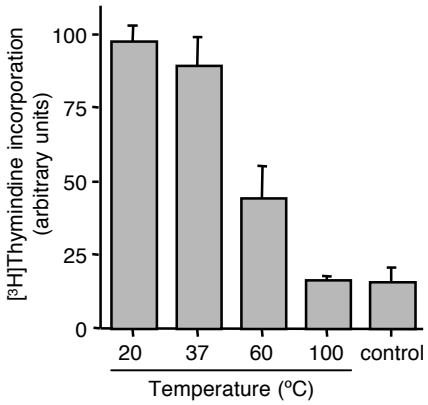


Fig. 7. Nordström *et al.*



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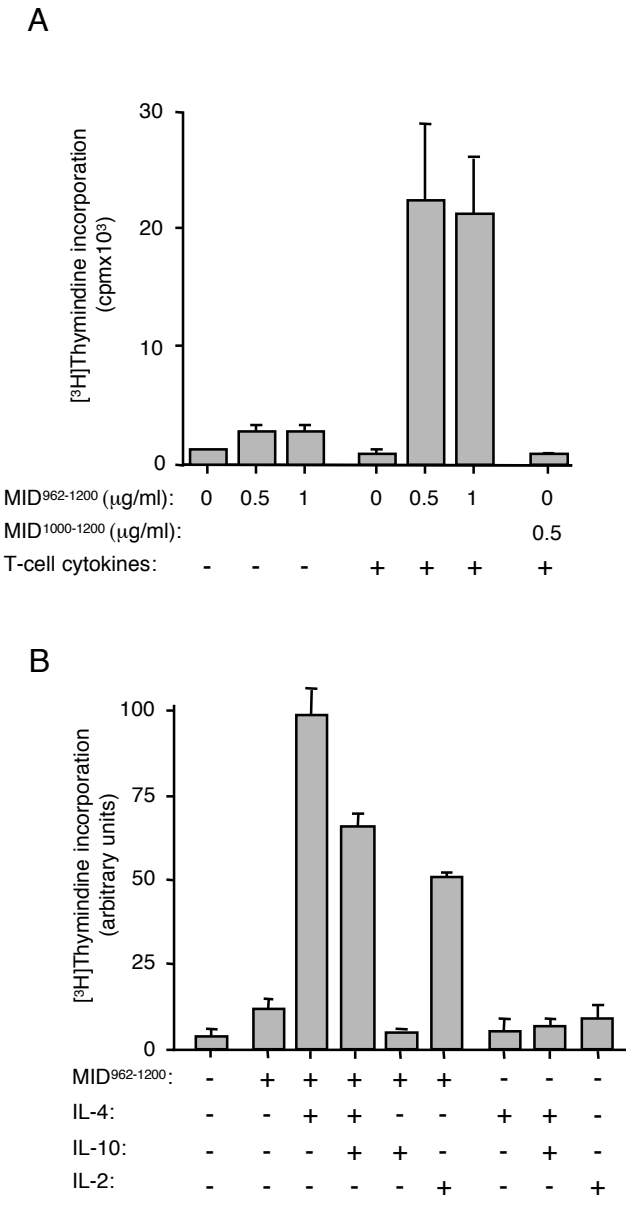

Fig. 9. Nordström *et al.*