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Short paper

A fusion protein derived from *Moraxella catarrhalis* and *Neisseria meningitidis* aimed for immune modulation of human B cells

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Running title: IgD-binding and immune modulation

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Keywords: B cells, MID, *Neisseria meningitidis*, NhhA, OMV, vaccine

AB.J. supplied reagents; O.M, B.S, B.B and M.M performed experiments; K.R and B.S planned and conceptualized experiments; K.R. and O.M wrote the manuscript.

ABSTRACT

Moraxella IgD-binding protein (MID) is a well characterized trimeric autotransporter that specifically targets the IgD of B cells. We fused the membrane anchor of the meningococcal autotransporter NhhA with the IgD-binding region of MID (aa 962-1200) to create a chimeric protein designated as NID. The aim was to use this specific targeting to provide a better vaccine candidate against meningococci, in particular serogroup B by enhancing the immunogenicity of NhhA. NID was thereafter recombinantly expressed in *E. coli*. The NID-expressing *E. coli* bound to peripheral B lymphocytes that resulted in cellular activation. Furthermore, we also successfully expressed NID on outer membrane vesicles, nanoparticles that are commonly used in meningococcal vaccines. This study thus highlights the applicability of the menigococcal-*Moraxella* fusion protein NID to be used for specific targeting of vaccine components to the IgD B cell receptor.

The outer membrane *Moraxella catarrhalis* IgD binding protein (MID) is a 200-230 kDa trimeric autotransporter that functions as a key adhesin of the respiratory pathogen *M. catarrhalis* (1,2). Intriguingly, MID has been shown to bind specifically to the constant heavy chain domain 1 of immunoglobulin (Ig) D. The region comprising amino acids 962-1200 is the shortest fragment of MID that retains its IgD-binding capacity (3). Using ultracentrifugation and gel electrophoresis we showed that $MID^{962-1200}$ exists as a trimer and that this configuration is crucial for optimal IgD-binding capacity (4). The specific binding of MID to soluble IgD and the IgD B cell receptor (BCR) leads to induction and proliferation of human B lymphocytes, resulting in production of non-specific IgGs, thereby regulating the host–pathogen interaction (2,5).

Release of outer membrane vesicles (OMV) is another mechanism by which Gram-negative bacteria regulate their interaction with the host. OMV are biologically active nanoparticles (20-250 nm) constitutively released by bacteria into the extracellular milleu. They are produced in response to bacterial stress and are known to be important in mediating several key functions like nutrient acquisition, biofilm development, survival and pathogenesis (6). Considering the advanced properties of OMV like the versatility of administration via the mucosal or parenteral route, their intrinsic adjuvant effect and rapid uptake into mammalian cells (7), OMV (and Outer Membrane proteins; OMP) and their possible design are being widely studied for therapeutic use. A particular utility of the OMV has been in vaccine development, and OMV-based vaccines are now available for many pathogens like enteric pathogens, *Bordetella pertussis* and *Neisseria meningitides.*

N. meningitidis is the leading causative of bacterial meningitidis, a deadly illness that comprises the bloodstream and the lining that surrounds the brain and spinal cord. The majority of meningococcal infections are caused by five serogroups: A, B, C, Y, and W135, with serogroup B accounting for about one third of meningococcal infections. Quadrivalent conjugate vaccines against serogroup A, C, Y, and W135 are licensed and in clinical use. In contrast, the capsular polysaccharide of MenB is poorly immunogenic in humans, and consequently unlikely to provide the basis for a protective vaccine (8). As an alternative, OMP have been widely investigated for their vaccine potential, either as undefined complex mixtures, as in OMV preparations, or also as highly characterized individual proteins in the study of vaccines against MenB (8).

Numerous studies for the identification of new vaccine candidates against MenB have been pursued in last decade and first ever wide-ranging vaccine 4CMenB (Bexsero®) is now licensed in Europe. 4CMenB consists of three menigococcal proteins (NHBA, NadA, fHbp) with OMV derived from the meningococcal NZ98/254 strain. This vaccine covers, however, only 78% of circulating MenB strains up-to-date and the vaccine coverage may change over time. The wide-ranging coverage of the disease-causing strains in a particular region over time is one of the main effectiveness measurements of a vaccine. In fact, according to a recent study from Spain, the allelic distribution of 4CMenB components changed over a 6 year period time, decreasing the vaccine protection significantly (from 61.8% to below 45%), thus highlighting the need for new vaccine candidates against serogroup B (9) Another MenB vaccine (Trumenba), which is recently approved in the US is composed of two recombinant lipidated factor H binding protein (fHBP) variants from N. meningitidis serogroup B. It is approved for individuals 10 through 25 years of age.

Approval of Trumenba is based on demonstration of immune response, as measured by the serum bactericidal activity against four serogroup B strains representative of prevalent strains in the US. One of them is the same strain that caused a small outbreak in New Jersey and California in 2013 (10). The effectiveness of Trumenba in infancy, one of the two age groups when the disease peaks, is not known, however, and thus more clinical experience is needed.

Autotransporter proteins are one of the most important virulence factors of Gram-negative bacteria and hence investigated as potential vaccine candidates (11). Eight autotransporter proteins have been identified in N. meningitidis; IgA1 protease, NhhA, AutA, AutB, NadA, App, NalP (also known as AspA) and MspA (also known as AusI). The trimeric autotransporter "Neisseria meningitidis hia/hsf homologue A" (NhhA) has been suggested as an ideal candidate in a protein-based vaccine against all serotypes of N. meningitides. NhhA is a strong vaccine candidate because it is surface exposed, has a high level of sequence homology between strains (amino acid identity 85.3% – 99.8%), and is involved in pathogenicity (12). Unlike many other outer membrane proteins of N. meningitidis, there are no obvious sequence features such as short tandem DNA repeats in or upstream of the gene that may mediate phase variable expression of NhhA. Furthermore, this protein is recognized by antisera from patients, implying that it is expressed and immunogenic in vivo (12, 13).

It has previously been demonstrated that antibodies engineered to enhance the delivery of antigen to antigen-presenting cells, such as B lymphocytes make these cells approximately 1000-fold more efficient at presenting antigen and stimulating specific T cells (14). This makes such “designed” antibodies as ideal tools for vaccines. The aim of this study was to enhance the immunogenicity of NhhA by

targeting it to the IgD of B cells by using MID. NhhA with its trimeric configuration would be structurally similar to MID and hence a perfect model to use as the backbone of the fusion protein. NhhA was fused with the IgD-binding region of MID (amino acids 962-1200) to investigate if the resulting chimeric protein could be used to target B cells. We found that the resulting fusion protein designated *Neisseria* IgD-binding protein (NID) was well expressed at the *Escherichia coli* surface and bound to human peripheral blood lymphocytes (PBLs) that resulted in cellular activation. Finally, NID was also highly expressed on the surface of OMV suggesting the chimeric protein as a viable strategy for targeting these vehicles to B cells.

Since recombinant MID has previously been shown to specifically bind to and activate B cells via the IgD BCR (2, 3), we used the specific property of the IgD-binding region of MID to make a delivery vehicle for a possible use in a therapeutical setting. To construct a chimeric NhhA-MID (*i.e.,* NID) molecule that would specifically target B cells, a part of the leader sequence of NhhA corresponding to amino acid region 43-292 was replaced by the IgD-binding region of MID (amino acids 962-1200) (Fig. 1A). Region 1-42 corresponded to the signal peptide (SP) of the protein, which originated from the leader peptide from *N. meningitidis*. The region comprising residues 42-272 was the IgD-binding domain of MID from *M. catarrhalis.* The remaining part of NID was derived from NhhA. $MID^{920-1200}$ together with $NhhA^{293-509}$ formed the passenger domain of NID, which contributed to the specificity. $NhhA^{510-594}$ functioned as the translocator domain and provided the trimerization and translocation of the N-terminal portion of NID to the bacterial surface.

The chimeric protein NID was recombinantly expressed in *E. coli*, and total bacterial cell proteins were analyzed in a Western blot probed with anti-MID

polyclonal antibodies (pAbs). As shown in Fig. 1B (right panel), *E. coli* carrying the plasmid encoding for NID expressed the protein, whereas the control *E. coli* transformed with an empty vector without insert did not.

The use of NID as a targeting molecule would only be possible if the protein is expressed at the bacterial surface. To verify and visualize NID at the surface of *E. coli,* we therefore used Transmission electron microscopy (TEM) and gold-labelled specific anti-MID pAbs (Fig. 1C). Interestingly, TEM revealed that NID on *E. coli* retained a characteristic structure that was similar to native MID at the surface of *M. catarrhalis* (4). Taken together, our results demonstrate that a chimeric NID molecule can be constructed and expressed at the *E. coli* surface. A cartoon with the proposed trimeric structure of NID is shown i Fig. 1D.

The next aim was to analyse if NID when expressed at the bacterial surface would bind IgD as observed with the native MID molecule (1). We chose to analyse *E. coli* transformants expressing NID by flow cytometry. As can be seen in Fig. 2A (right panel), *E. coli*-NID was well expressed at the surface as determined by anti-MID pAbs followed by detection with FITC-conjugated secondary pAbs. Importantly, *E. coli*-NID also bound recombinant soluble IgD when analyzed with specific FITC-conjugated anti-IgD pAbs (Fig. 2B, right panel).

Since MID has been demonstrated to bind the IgD BCR on B cells as well as to stimulate those cells to proliferate (4), we wanted to analyse whether NID had the same functional property. Bacteria carrying NID were labeled with FITC and incubated with human PBLs. A significant shift was observed when NID-expressing *E. coli* bound to B cells in the PBL population as defined with RPE-conjugated anti-CD19 monoclonal antibodies that specifically detected B cells (Fig. 2C). Bacteria with plasmid only (the negative control) did not show any significant shift.

To further evaluate the capacity of NID to activate human B cells, freshly isolated PBLs were incubated with formaldehyde killed NID-expressing *E. coli* and analyzed for [methyl-^{3}H]-thymidine incorporation after 72 h (Fig. 2D). *E. coli*-NID was found to have a stimulatory effect on B cells, whereas the *E. coli*-ctrl (negative control) did not induce any proliferation. The IgD-binding region MID$^{962\text{-}1200}$ could thus be used as a delivery molecule to specifically target B cells that lead to B cell activation .

The IgD binding of MID has been shown to lead to both B cell proliferation and polyclonal B cell activation (3). The biological implication of such a polyclonal B cell activation is still debated. On one hand, the induction of polyclonal activation has been suggested as a strategy used by microorganisms to avoid the host-specific immune response. This could occur as a result of the activation of B cell clones with irrelevant specificities (15). However, based on several emerging reports, polyclonal B cell activation has now also been suggested to play an important role in defence against infections by enhancing natural antibody production, which is a collection of germ-line-encoded antigen-recognition molecules. It has been suggested that after a polyclonal B cell activation, the levels of the natural antibodies can increase early to keep up with multiplication of the microorganisms, thus limiting the initial pathogen distribution and leading to early dissemination (16). In addition to enhancing the immunogenicity by specific targeting, the extreme specificity that MID confers to any such chimeric construct could also thus be utilized in targeting molecules specifically to the IgD BCR, possibly in the production of natural antibodies thereby modulating pathogenesis.

A current focus of research within the field of microbial pathogenesis in general and in the vaccine development in particular is "antigen delivery" by OMV,

nanoparticles that are released at high concentrations by Gram-negative bacteria. These biologically active components are efficiently taken up by mammalian cells, have both an intrinsic adjuvanic and immunogenic property, and can be recombinantly engineered making them at the forefront for usage as vaccine candidates and adjuvants (7). NID was therefore tested to check if the fusion protein could be expressed also on the surface of *E. coli* OMVs. Accordingly, *E. coli* OMVs were isolated from recombinant bacteria and the presence of NID was monitored by flow cytometry using anti-MID pAbs. As can be seen in Fig. 2E, NID was expressed on OMV derived from bacteria containing NID, whereas OMV isolated from native *E. coli* was negative. NID could thus be used to deliver a protein of interest to the bacterial surface, and was also successfully expressed on the surface of OMVs derived from recombinant bacteria. This postulates a possible role of MID as a delivery tool for OMV-containing vaccines.

It must be noted, however, that whole cell and OMV vaccines have their own limitations. Whole-cell vaccines were phased out as a result of the reactogenicity observed with the whole cell pertussis vaccines (17). In fact, the licensed MenB vaccine that includes an OMV preparation has safety warnings in the product label due to reports on pain and fever reported post vaccination. However, a review of the safety of the vaccine has proven it to be without major complications (18).

Taken together, our study aims at providing a better vaccine candidate against meningococci by enhancing the immunogenicity of NhhA. It also highlights the potential of the conserved outer membrane protein MID as a novel tool for use in the field of biotechnology to specifically target proteins to B cells or as a carrier in the design of OMV vaccines. Since, this work has been done in vitro; more work in

animals needs to be followed to demonstrate the efficacy of such a targeting mechanism and help address potential risks, if any.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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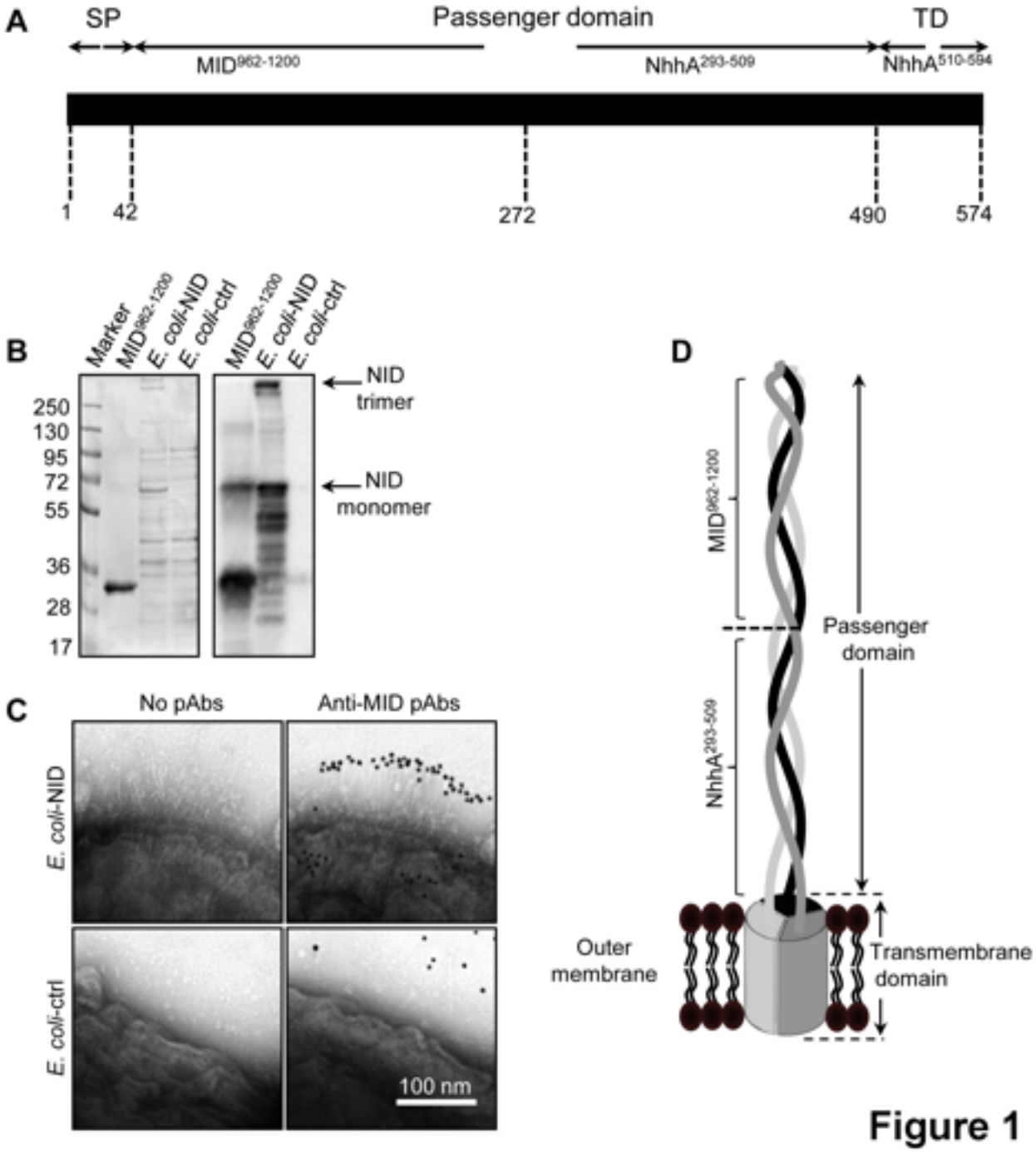
FIGURE LEGENDS

Fig. 1. Construction and expression of the chimeric protein NID. (A) Schematic illustration of the domain organization of NID. SP: Signal peptide, TD: Translocator domain, App: Adhesion and penetration protein, MID: *Moraxella* Immunoglobulin D binding protein, NhhA: *Neisseria* hia/hsf homologue. (B) SDS-PAGE (left panel) and Western blot analysis (right panel) showing expression of NID in *E. coli*. The control *E.coli*-ctrl was transformed with an empty vector. A rabbit serum raised against MID was used for probing in the Western blot (4). (C) NID is located at the surface of *E. coli* as revealed by Transmission electron microscopy (TEM) and gold-labelled anti-MID pAbs (4). (D) Cartoon of the NID structure that demonstrates the trimeric structure of $MID^{962-1200}$ together with $NhhA^{293-509}$.

Fig. 2. Expression of NID at the surface of *E. coli* binds to IgD and activates B cells. (A) NID is expressed at the surface of *E. coli* as revealed by flow cytometry and rabbit anti-MID pAbs and FITC-conjugated secondary anti-rabbit pAbs. The *E. coli*-ctrl (control) was transformed with an empty vector. (B) NID-expressing *E. coli* binds IgD. Soluble recombinant IgD (5) was detected with FITC-conjugated anti-IgD pAbs. (C) Peripheral blood lymphocytes (PBLs) were incubated with FITC-labelled *E. coli*-NID or *E. coli*–ctrl. PBLs were isolated from human blood using a Lymphoprep one-step gradient (Nycomed) and incubated with an RPE-conjugated anti-CD19 monoclonal antibody, washed and further incubated with the FITC-labelled bacteria. In (A) and (B), background fluorescence was determined by incubating bacteria with the secondary detection pAbs only (non-shaded). In (C), dot plots from the flow cytometry are shown. (D) *E. coli*-NID stimulates B cells to proliferate. PBLs were analyzed by measuring [^{3}H]-thymidine uptake at 72 h. PBLs were stimulated with

different concentrations of formaldehyde-killed *E. coli* either containing NID or control bacteria with the empty vector only. (E) *E. coli* OMVs were isolated from the *E. coli*-NID and *E. coli* (control) as described (19). OMVs were probed with anti-MID pAbs and FITC-conjugated secondary anti-rabbit pAbs. The control (ctrl) designates the secondary pAbs only. The y-axis denotes the fold change with the anti-MID pAbs. Data were normalized to the secondary detection-pAbs that were set as 1.0.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SP
Passenger domain
TD
MID962-1200
NhhA293-509
NhhA510-594
1
42
272
490
574
B
Marker
MID962-1200
E. coli-NID
E. coli-ctrl
MID962-1200
E. coli-NID
E. coli-ctrl
NID trimer
NID monomer
250
130
95
72
55
36
28
17
C
No pAbs
Anti-MID pAbs
E. coli-NID
E. coli-ctrl
100 nm
D
MID962-1200
Passenger domain
NhhA293-509
Outer membrane
Transmembrane domain

Figure 1

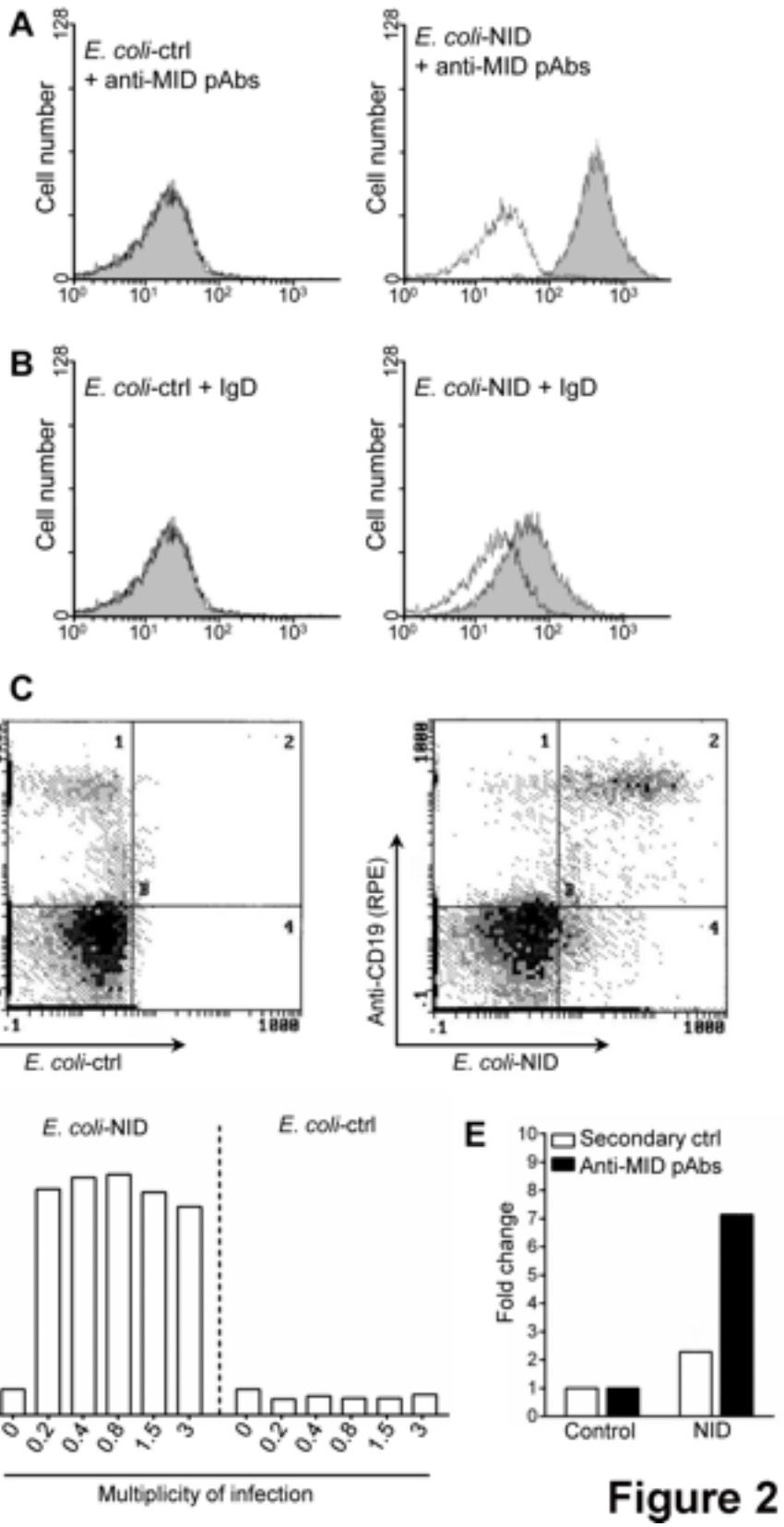
A
E. coli-ctrl
+ anti-MID pAbs
E. coli-NID
+ anti-MID pAbs
Cell number
B
E. coli-ctrl + IgD
E. coli-NID + IgD
C
Anti-CD19 (RPE)
E. coli-ctrl
E. coli-NID
D
E. coli-NID
E. coli-ctrl
[H3]-Thymidine incorporation (cpm)
Multiplicity of infection
E
Secondary ctrl
Anti-MID pAbs
Fold change
Control
NID

Figure 2