Cooperative role for tetraspanins in adhesin-mediated attachment of bacterial species to human epithelial cells

Running Title: The role of tetraspanins in bacterial adherence

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Abstract

The tetraspanins are a superfamily of transmembrane proteins with diverse functions, and can form extended microdomains within the plasma membrane in conjunction with partner proteins, which probably includes receptors for bacterial adhesins. *Neisseria meningitidis*, the causative agent of meningococcal disease, attaches to host nasopharyngeal epithelial cells via type IV pili and opacity (Opa) proteins. We examined the role of tetraspanin function in *Neisseria meningitidis* adherence to epithelial cells. Tetraspanins CD9, CD63 and CD51 were expressed by HEC-1-B and DETROIT 562 cells. Co-incubation of cells with antibodies against all three tetraspanin molecules used individually or in combination, with recombinant tetraspanin extracellular domains (EC2) or with small interfering RNAs (siRNAs) significantly reduced adherence of *Neisseria meningitidis*. In contrast, recombinant CD81, a different tetraspanin, had no effect on meningococcal adherence. Anti-tetraspanin antibodies reduced the adherence to epithelial cells of *Neisseria meningitidis* strain derivatives expressing Opa and pili significantly more than isogenic strains lacking these determinants. Adherence to epithelial cells of strains of *Staphylococcus aureus*, *Neisseria lactamica*, *Escherichia coli*, and *Streptococcus pneumoniae* was also reduced by pretreatment of cells with tetraspanin antibodies and recombinant proteins. These data suggest that tetraspanins are required for optimal function of epithelial adhesion platforms containing specific receptors for *Neisseria meningitidis* and potentially for multiple species of bacteria.

Introduction

The tetraspanins are a family of mammalian transmembrane proteins comprising 33 members. All share similar structural motifs with four transmembrane domains (TM1-4), a small (EC1) and a large (EC2) extracellular loop (18). The tetraspanins can homo- and heterodimerise whilst also associating with partner proteins, including CD46 (17) and members of the immunoglobulin superfamily (7), to form tetraspanin-enriched microdomains (TEMs) (18). These associations allow the tetraspanins to facilitate many functions through several signalling pathways, including the binding and processing of pathogens. Some viruses have been shown to utilise the tetraspanins for host cell entry, including HIV-1 with CD63 (39) and HCV with CD81 (25). In contrast, the contribution of tetraspanins to bacterial cell attachment and entry is not well defined.

Neisseria meningitidis (Nm), the cause of meningococcal disease, has well-described antigenic and phase-variable adhesins involved in attachment to the epithelial barrier (23). Type IV pili and the opacity proteins (Opa) require host cell membrane proteins for the facilitation of attachment and invasion of host cells. Pilus-mediated attachment may require CD46 on epithelial cells (14, 32). This initial adhesion process places the meningococcus in proximity to the host cell for a secondary stage of attachment with different adhesins such as the opacity proteins Opa and Opc. *Neisseria meningitidis* possesses three to four phase variable *opa* genes (28, 30), which encode proteins with distinguishable characteristics due to variable regions within their three surface-exposed loops (19). Opa proteins are able to bind both the CEACAM (carcinoembryonic antigen-

related cell adhesion molecule) receptors and HSPGs (heparan sulphate proteoglycans) found on human epithelial cells (6, 35). CEACAMs are members of the immunoglobulin superfamily consisting of 12 differentially expressed proteins, where CEACAM1 is the most widely distributed upon host cells.

A small number of studies have suggested a possible role for tetraspanins in bacterial attachment to cells. Uropathogenic *Escherichia coli* interacts with the uroplakins through the use of the FimH adhesin (37) while *Listeria monocytogenes* requires CD81 for entry into epithelial cells (31). A relationship with meningococcal adherence is possible, because tetraspanins associate with members of the immunoglobulin superfamily (16) while CD9 and CD151 associate indirectly with CD46, through integrins (17). Furthermore, intracellular CD63 is depleted after the action of meningococcal IgA protease within lysosomes (2).

Here, we investigate the role of the tetraspanins in meningococcal colonisation of epithelial cells and further study their involvement with multiple bacterial species. We demonstrate that interference with tetraspanin function modifies meningococcal adherence to epithelial cells, likely via an effect on specific receptors, and demonstrate a wider involvement of tetraspanins in the adherence of multiple diverse bacterial species.

Materials and Methods

Strains and bacterial growth conditions

N. meningitidis derivatives tested and their relevant characteristics are shown in Table 1. Strains of *N. lactamica* (NL1009), *S. pneumoniae* (D39), *E. coli (*ATCC 25922; kindly provided by Dr. Mark Thomas, Sheffield Medical School) and *S. aureus* (NCTC 6571) were utilised in this study. Solid cultures were grown on Columbia horse blood or chocolate agar (Oxoid Ltd., Cambridge, UK) aerobically at 37°C overnight in a humidified atmosphere. Liquid cultures were grown in Mueller-Hinton broth (MHB), brain heart infusion (BHI) or tryptone soya broth (TSB; Oxoid) microaerobically at 37°C in a humidified atmosphere with constant agitation. Freshly grown aerobic plates were used to inoculate all liquid cultures.

Cell culture

Maintenance of DETROIT 562 (American Type Culture Collection, or ATCC CCL-138; Manassas, VA, USA), a human pharynx carcinoma cell line, required Eagle's modified essential media (EMEM; Lonza Group Ltd, Basel, Switzerland), additional supplements of 2 mM glutamine, 1 % non-essential amino acids, 1 mM pyruvate (Lonza) and 0.1 % lactalbumin hydrolysate (LH; Sigma-Aldrich Company Ltd, Gillingham, UK) and 10 % (v/v) heat inactivated foetal calf serum (HI-FCS). The human endometrial adenocarcinoma epithelial cell line, HEC-1-B (ATCC HTB-113), was maintained in EMEM (Lonza)

supplemented with 10 % HI-FCS. Cell lines were grown at 37°C, in a humidified atmosphere with 5% (v/v) CO_2 and passaged using trypsin/versine when confluent.

Antibodies and Recombinant GST fusion proteins

Monoclonal antibodies or Fab fragments directed against CD9 (602.29, 602.29 Fab) (1), CD63 (H5C6, H5C6 Fab) (3), CD151 (14A2) (9) and the IgG isotype control (JC1) (24) were purified from hybridoma supernates generated in house using protein G Sepharose (Amersham-Pharmacia, UK). Anti-CD166 mAbs (B-6; MCA1926F) and an anti-CEACAM mAb (D14HD11) were purchased from Santa Cruz Biotechnology, USA, AbD Serotec, UK and Abcam, UK respectively. The mouse anti-meningococcal monoclonal antibody, 2-1-P15 (02/310; National Institute for Biological Standards and Control, NIBSC) was utilised for staining procedures. Recombinant GST fusion proteins were assembled from CD9, CD63 and CD151 tetraspanin EC2 extracellular domains fused with glutathione S-transferase (GST) (12).

Cell Surface Tetraspanin Expression Analysis

Tetraspanin expression in epithelial cell lines was measured by flow cytometry. Adherent cells were grown to approximately 1 x 10^6, detached by trypsin/versene treatment and transferred to tubes. Cells were fixed with 1 % paraformaldehyde, centrifuged and treated with relevant antibodies (602.29; 20µg ml^{-1}, H5C6; 20µg ml^{-1}, 14A2; 32.5µg ml^{-1}, MCA1926F; 1 µg ml^{-1}) followed by a goat anti-mouse FITC-conjugated antibody if

required (Sigma Aldrich, UK), both at 4°C for 60 min. Labelled cells were analysed with an LSRII (Becton Dickinson, Oxford, UK) and results analysed using BD FACSDiva Software (Becton Dickinson).

Effect of tetraspanin antibodies on bacterial adherence

Inhibition of meningococcal adherence by anti-tetraspanin antibodies was demonstrated using coverslips seeded with approximately 1.5 x 10^5 epithelial cells and blocked by immersion in 5 % bovine serum albumin (BSA). Cells were washed with PBS and treated with anti-tetraspanin and control antibodies (602.29, 602.29 Fab, H5C6, H5C6 Fab, JC1, B-6; 20 μg ml^{-1}, 14A2; 32.5μg ml^{-1};) for 30 min at 37°C. Combination treatment mixed anti-CD9, CD63 and CD151 antibodies (602.29, H5C6; 5.73μg ml^{-1}, 14A2; 8.6μg ml^{-1}). After washing to remove excess antibody, cells were incubated with bacteria for 60 min at a multiplicity of infection (MOI) of 300, except for *N. meningitidis* ¢13 and ¢2 infected cells at an M.O.I. = 30 and *S. aureus* an M.O.I. = 1. Cells were washed and fixed with 2 % paraformaldehyde.

Effect of GST-tetraspanin EC2s on bacterial adherence

Using the adherence assay described above, recombinant GST fusion proteins were added to epithelial cells (CD9:EC2, CD63:EC2, CD81:EC2; 20μg ml^{-1}, CD151:EC2; 32.5μg ml^{-1}). Combination protein treatment mixed CD9, CD63 and CD151 recombinant proteins

(concentrations same as antibodies). A control treatment of free GST was used (GST; 20μg ml^{-1}).

Effect of tetraspanin abatement by siRNA on bacterial attachment

siRNA transfection was carried out as described by Thermo Scientific Dharmacon. HEC-1-B cells seeded at 7.5 x 10^4 were incubated for 48 hours with either media alone or a variety of siRNAs (siGENOME non-targeting siRNA #1, human GAPD control, CD9, CD63, CD151; 40 nM) purchased from Thermo Scientific, USA. Transfection was performed using DharmaFECT 1 purchased from Thermo Scientific. After incubation, transfection efficiency was tested by flow cytometry while the previous adherence assay was executed.

Immunofluorescence microscopy

Fixed coverslips were washed and treated with i) anti-meningococcal antibody or anti-tetraspanin antibody followed by goat anti-mouse FITC conjugated antibody to visualise all external meningococci or tetraspanins and ii) stained with DAPI to visualise DNA and nuclei. Antibodies were incubated at room temperature and diluted in PBS. Vectashield mounting medium with DAPI (Vector Labs, Burlingame, CA, USA) was used to mount coverslips allowing examination on a Leica DMRB fluorescent microscope. 100 cells were counted and the number of bacteria associated, either bound or internalised, were noted.

Tetraspanins were visualised on a bright field and fluorescence Leica DMRB upright microscope.

Statistical Analysis

All data was analysed for normality by skewness using GraphPad Prism 5.01 (GraphPad Software, Inc, La Jolla, CA, USA). Specific statistical considerations and the tests used are described separately for each sub-section. All analyses used GraphPad Prism 5 for Windows Version 5.01. Data is given as mean $\pm$ SD. Significance was established at $p \leq 0.05$.

Results

Tetraspanins are variably expressed on epithelial cells

Expression of tetraspanins by epithelial cells was examined by flow cytometry and immunofluorescence (Fig. 1) and normalised against non-specific isotype control antibody JC1. CD9 was richly expressed on both species of epithelial cell whilst CD63 and CD151 were expressed but at much lower levels of intensity. As expected, the non-tetraspanin epithelial cell molecule CD166 was expressed strongly. CD9 was most intense at intercellular junctions whilst CD63 and CD151 exhibited punctate expression patterns.

Adherence of *N. meningtidis* is reduced after treatment of epithelial cells with anti-tetraspanin antibodies and Fab fragments.

Treatment of epithelial cells with anti-tetraspanin mAbs significantly reduced meningococcal adherence. In DETROIT 562 cells, pre-treatment with anti-CD9 (34.97 $\pm$ 14.56 %) or anti-CD63 (57.28 $\pm$ 12.16 %) antibodies significantly reduced bacterial adherence to epithelial cells (Fig. 2A). Anti-CD151 mAbs had no significant effect on meningococcal adherence (Fig. 2B). A combination of anti-tetraspanin mAb treatments also significantly reduced bacterial adherence (40.5 $\pm$ 3.96 %; Fig. 2B). Treatment of HEC-1-B cells with anti-tetraspanin mAbs also demonstrated significant inhibition of bacterial adhesion (CD9, 45.74 $\pm$ 5.75 %; CD63, 52.34 $\pm$ 11.39 %; CD151, 51.84 $\pm$ 6.1 %; a combination of all three antibodies (56.99 $\pm$ 6.47 %; Fig. 2C). As expected, anti-CD166

mAbs had no significant effect on meningococcal adherence, supporting a specific role for the tetraspanin mAbs. (Fig. 2C). Treatment with varying concentrations of Fab fragments demonstrated a typical dose response with significant reductions in meningococcal adherence (Fig. 2D-F). Untransformed data revealed that single tetraspanin mAb treatments significantly reduced meningococcal adherence (Fig. 3). However, in all experiments with tetraspanin antibodies, a subset of cells were unaffected by tetraspanin treatment; 15-20 % of treated cells were colonised by numbers of bacteria similar to those found on control cells (Fig. 3C) which may be the result of variation in tetraspanin expression during the cell cycle. Despite the reduction in adherence, there was no significant effect on bacterial internalisation of bound bacteria (Fig. 3D).

Recombinant GST:EC2 tetraspanin fusion proteins inhibit meningococcal adherence to epithelial cells

Treatment of HEC-1-B cells with recombinant GST:EC2 fusion proteins significantly reduced meningococcal adherence, particularly CD63 (86.57 $\pm$ 7.55 %) and CD151 (94.16 $\pm$ 1.59 %; Fig. 2G). CD81 EC2 proteins demonstrated no significant reduction in meningococcal association (Fig. 2H). Combination treatment, consisting of CD9, CD63 and CD151 EC2 proteins at the same dose as single recombinant protein treatments, significantly reduced meningococcal adherence (85.54 $\pm$ 4.85 %; Fig. 2G). At the doses used, recombinant protein treatments reduced meningococcal adherence by approximately four fold more than anti-tetraspanin mAb (Fig. 2). We found no evidence of direct bacterial binding to recombinant tetraspanins using a solid-phase assay (Supplementary Fig. 1).

Tetraspanin abatement by siRNA reduced meningococcal adherence to epithelial cells

Reduction of the tetraspanins by siRNA treatment inhibited meningococcal adherence to epithelial cells (Fig. 4). siRNA treatments demonstrated large reductions in GAPD (-58.82 $\pm$ 2.05 %) and the tetraspanins (CD9; -75.19 $\pm$ 1.50 %, CD63; -87.99 $\pm$ 0.25 %, CD151; -42.61 $\pm$ 12.38 %) (Fig. 4C). Pre-treatment of cells with a variety of siRNAs significantly reduced meningococcal adherence to epithelial cells, however, reduction of the positive control demonstrated no reduction (Fig. 4A, B).

Differential binding of *Neisseria* adhesin variants suggest CEACAM and CD46 require tetraspanins for meningococal adherence

In contrast to wild-type bacteria, no significant reduction in adherence of *pilQ*$^-$ and *pilF*$^-$ mutants to tetraspanin mAb-treated HEC-1-B cells was observed (Fig. 5). Attachment of wild-type piliated bacteria to untreated HEC-1-B cells is approximately 6 fold greater than that of the pil$^-$ mutants. A comparison of the adherence of acapsulate Opa$^+$ and Opa$^-$ variants to HEC-1-B and DETROIT 562 cell lines is shown in Fig. 6. The percentage of HEC-1-B and DETROIT 562 epithelial cells associated with acapsulate bacteria after anti-tetraspanin mAb treatment was significantly reduced compared to the media alone treated cells, this was not observed with the Opa$^-$ variant on HEC-1-B cells (Fig. 6B). Tetraspanin treatment significantly reduced Opa$^-$ variant association with the DETROIT 562 cells although this was reduced from that of the parent strain. In these experiments, the CD151 antibody was not tested.

CEACAM and HSPG blockade demonstrate analogous effects to tetraspanin blockade

Blockade of CEACAM and HSPG demonstrated significant reductions in meningococcal association analogous to tetraspanin blockade (Fig. 6C, D). Pre-treatment with a combination anti-tetraspanin mAb treatment (58.32 $\pm$ 5.64 %) or an anti-CEACAM mAb treatment (64.28 $\pm$ 10.01 %) significantly reduced meningococcal association with epithelial cells. Combination of these treatments also significantly reduced meningococcal association but no additive effect was demonstrated (Fig 6C). Pre-treatment of HEC-1-B cells with heparin (50.49 $\pm$ 6.18 %), anti-tetraspanin mAb, or a combination of the two demonstrated similar effects (Fig. 6D).

Tetraspanins influence epithelial cell adherence of multiple bacterial species

Blockade of the tetraspanins with a combination of anti-tetraspanin mAbs significantly reduced the association of several species (Fig. 7) particularly *N. lactamica* (72.3 $\pm$ 10.29 %; Fig. 7A), *S. pneumoniae* (50.07 $\pm$ 11.83 %; Fig. 7E) and *E. coli* (53.52 $\pm$ 4.83 %; Fig. 7C). The effect on *S. aureus* adhesion was not significant (Fig. 7G). However, HEC-1-B cells treated with a combination of recombinant EC2:GST fusion proteins demonstrated significantly reduced bacterial adherence in all strains tested compared to media alone treated cells (~40-60 %; Fig. 7B, D, F, H)).

Discussion

We have demonstrated that tetraspanins mediate adherence of multiple bacterial species to human epithelial cells, likely due to facilitation of specific receptor-adhesin engagement. By coating plates with recombinant tetraspanin peptides, we found that tetraspanins were not acting as direct receptors for bacterial adherence (Supplementary Fig. 1) suggesting an indirect effect of tetraspanins on adhesin-receptor interactions. The epithelial cell lines exhibited highly variable cell surface expression levels of tetraspanin proteins (Fig. 1). These findings reflect current knowledge of tetraspanin distribution; CD9 is mostly found on the cell surface while its recycling is minimal, whereas CD63 has a high rate of internalisation, being predominantly associated with late endosomal compartments (26), with lower levels of the protein displayed on the cell surface. CD151 is similar to CD63 and has a high rate of internalisation (38), with approximately 50% of the protein presented on the cell surface.

Previous reports have suggested that uropathogenic *Escherichia coli* (UPEC) and *Listeria monocytogenes* use members of the tetraspanin superfamily, the uroplakins and CD81 respectively, for adherence to cells (11, 31, 33, 40). In the current study we have further analysed several bacterial species to determine if they are affected by tetraspanin blockade. Both anti-tetraspanin mAbs and GST:EC2 fusion proteins caused a general reduction of bacterial adherence (Fig. 6). These data suggest that the tetraspanins have a general involvement in bacterial adherence perhaps because of their property of association with partner proteins. We found that GST:EC2 fusion protein treatments were more potent than

antibody treatment suggesting that the GST:EC2 fusion proteins produce a more global, non-specific disruption of tetraspanin function compared to the mAbs.

Anti-tetraspanin Fab fragments are able to reduce meningococcal adherence in an analogous manner to whole antibody (Fig. 2) demonstrating the effects described here are not due to the cross-linking of receptors. We further observe that interference of CD166, a strongly expressed epithelial cell marker, is unable to reduce meningococcal adherence refuting a possible effect by steric hindrance. Tetraspanin abatement by siRNA exhibited comparable reductions in meningococcal adherence demonstrating further evidence that this phenomenon is tetraspanin-specific. The mechanism of action of GST:EC2 fusion proteins is unclear but it is likely the soluble EC2 domains intercalate with endogenous TEMs and alter TEM function, interfering with tetraspanin associations with other proteins resulting in disruption of the TEMs (4). In nature, it is likely that the tetraspanins form an 'adhesion platform' containing the required receptors for meningococcal adherence as has previously been suggested with CD9 and CD81 during HIV infection (10). Redundancy of the tetraspanins is typical within these microdomains as complex interactions within the TEM can allow proximal tetraspanins to interact with the inhibited tetraspanin 'partner' proteins and mediate their functions. However, treatment of cells with a combination of mAbs did not result in an additive effect on meningococcal adherence suggesting that either tetraspanin 'adhesion platforms' do not demonstrate tetraspanin redundancy or further tetraspanin blockade during combination treatment is required.

The pharyngeal cell line DETROIT 562 and the endometrial cell line HEC-1-B both support high levels of bacterial adherence and are commonly used in bacterial infection studies. We have demonstrated that type IV pili and Opa variants are relatively less

affected by tetraspanin blockade, suggesting that both type IV pilus and opacity protein receptors are associated with the tetraspanins. The putative pilus receptor, CD46, and the most common Opa receptor, CEACAM, are well characterised and are excellent candidates for tetraspanin partner proteins. Previous reports indicated that CD46 associates with CD9 as well as several integrins (17) and several members of the immunoglobulin superfamily have been reported to associate with the tetraspanins (5, 27), including HB-EGF as a receptor for diphtheria toxin (13) and tetraspanin interactions with B-CAM and EpCAM (15), however, there are currently no reports demonstrating CEACAM association with the tetraspanins. HEC-1-B cells do not express CEACAM (29), yet Opa variant adherence is less affected by tetraspanin blockade than wild-type bacteria. This perplexing data would suggest a secondary Opa receptor is associated with the tetraspanins, perhaps the heparan sulphate proteoglycans (HSPGs) may be a component of the tetraspanin 'adhesion platform'.

In conclusion, we have shown that blockade of tetraspanins CD9, CD63 and CD151, either with antibody or with recombinant peptides, inhibits adherence of *Neisseria meningitidis*. The effect of the tetraspanins was significantly reduced in isogenic strains of *N. meningitidis* lacking the adhesins Opa and pilin, suggesting that the tetraspanins are involved in optimal organisation of receptors for specific meningococcal adhesins. Furthermore, we show that the tetraspanins are generally involved in the attachment of multiple species of bacteria to the surface of epithelial cells demonstrating a wider role in bacterial adherence. Previous reports have also suggested tetraspanin involvement in pathogenesis, whether viral or bacterial (20, 40). However, our study suggests a larger involvement of the tetraspanins not just as receptors but as facilitators of 'adhesion

platforms', allowing bacteria to rapidly associate with cells. These novel findings will prove useful in dissection of a multitude of microbial adhesion cascades and perhaps initiate clinical tetraspanin treatments to reduce colonisation of the epithelial barrier, the first step in bacterial pathogenesis.

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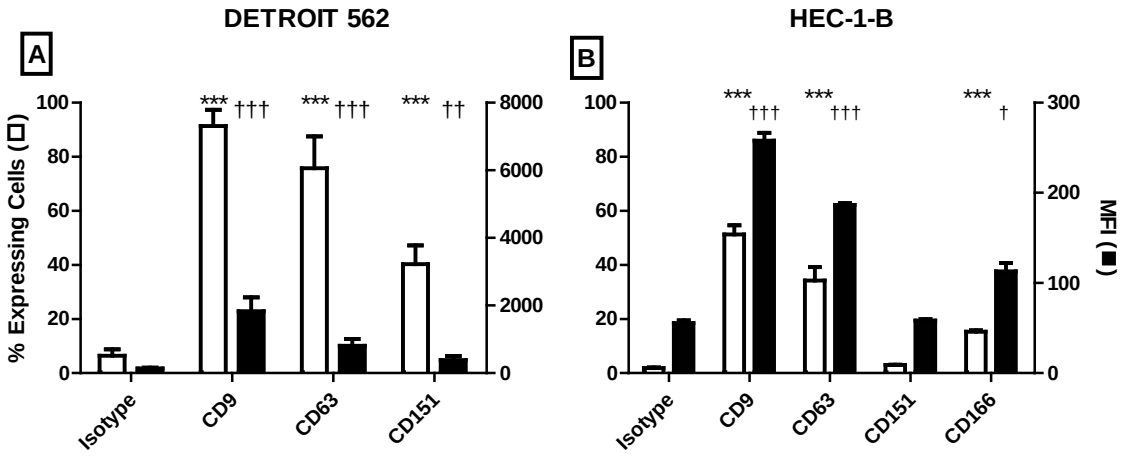
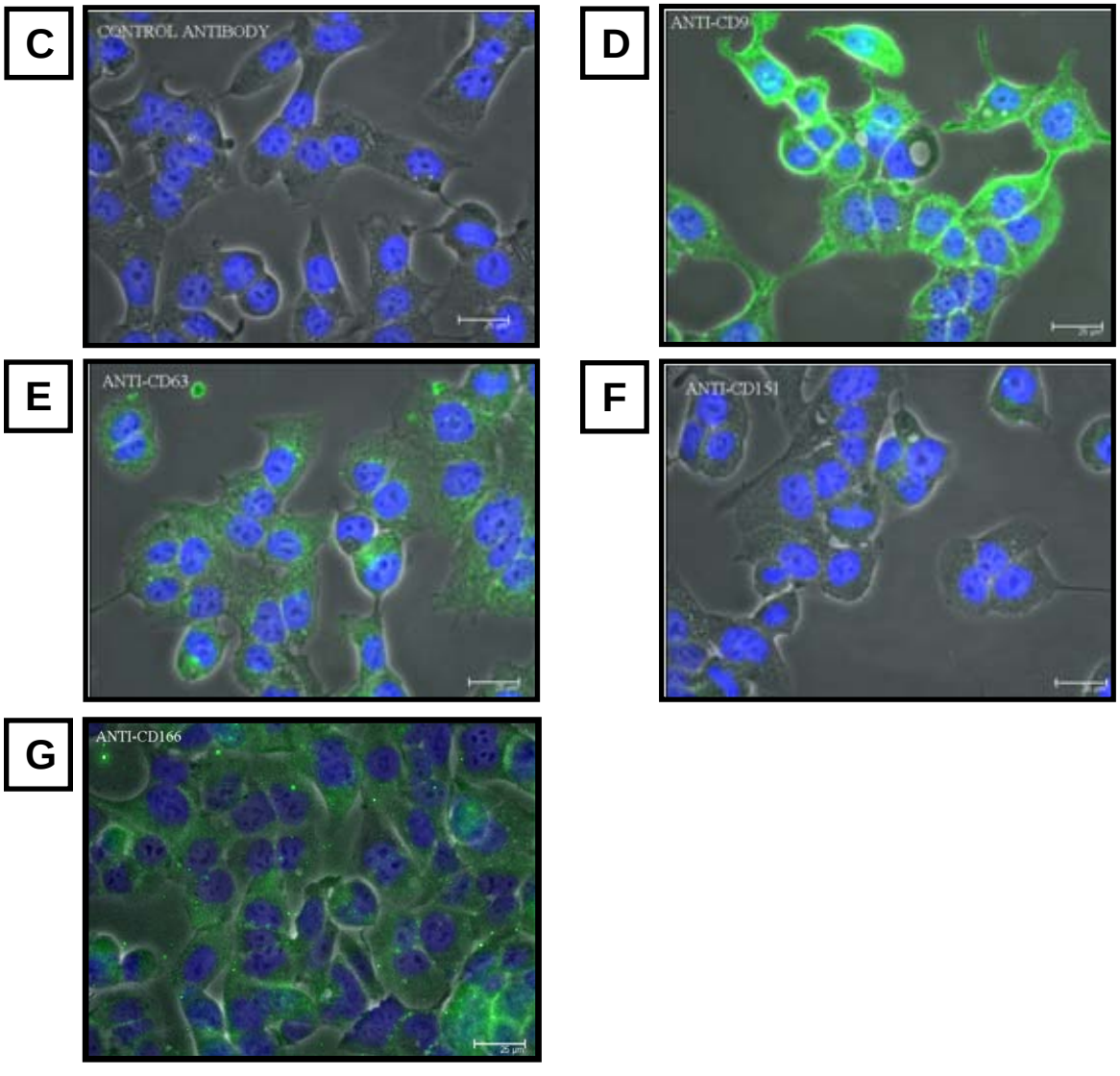
Strain/Derivative	Serotype/ Immunotype	Capsule	Pili	Opa	Opc	Reference
MC58	B:15:P1.7,16:L3	+	+	+	+	(21)
¢13	L8	-	+	+	+	(22)
¢2	L8	-	+	-	+	(36)
C311	B:NT:NT	+	+	+	+	(34)
C311*pilQ* (M1)		+	*pilQ*$^{-}$	+	+	(8)
C311*pilF* (M10)		+	*pilF*$^{-}$	+	+	(8)

1 **Table 1. *N. meningitidis* strains and adhesin variants utilised in this study.**


DETROIT 562
HEC-1-B
A
B
% Expressing Cells (□)
MFI (■)
Isotype
CD9
CD63
CD151
CD166

1
2


C
CONTROL ANTIBODY
D
ANTI-CD9
E
ANTI-CD63
F
ANTI-CD151
G
ANTI-CD166

3

Figure 1. Expression of tetraspanins by epithelial cells. (A): DETROIT 562 cells, (B): HEC-1-B cells. Data was collected by flow cytometry. (A) n = 6 (B) n =3, mean ± SD. * denotes significance in the percentage of expressing cells compared to control cells, † denotes significance in the median fluorescence intensity compared to control cells. ***/††† $p \leq 0.001$, †† $p \leq 0.01$, One-Way ANOVA with Tukey's multiple comparison test. MFI = Median Fluorescence Intensity. (C-F) Visualisation of protein distribution on HEC-1-B cells by fluorescence microscopy. (C) JC1, (D) CD9, (E) CD63, (F) CD151, (G) CD166. Scale bar = 25 μm.

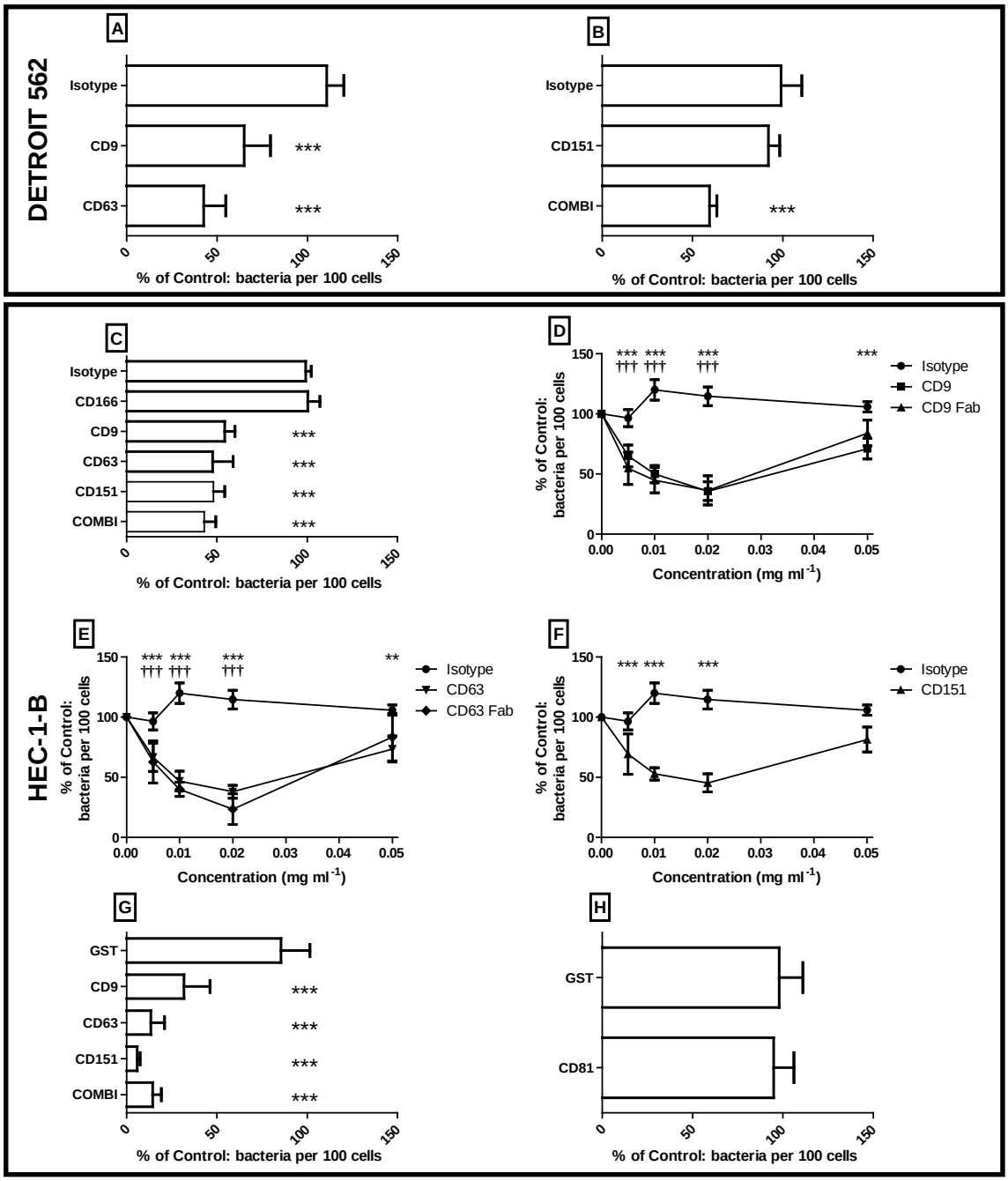


Figure 2. Pre-treatment of tetraspanins with antibodies, Fab fragments or recombinant tetraspanin peptides reduces *N. meningitidis* adherence to epithelial cells. DETROIT 562 (A, B) or HEC-1-B (C-H) cells were immersed in medium alone, isotype control (JC1), anti-CD166 (C) or treated with anti-tetraspanin treatments; 602.29

and 602.29 Fab (anti-CD9), H5C6 and H5C6 Fab (anti-CD63), 14A2 (anti-CD151), or a combination treatment (COMBI) containing all three antibodies for 30 min at 37°C (A-F). Cells were treated with single recombinant GST:EC2 fusion proteins; CD9, CD63, CD81, CD151, a combination treatment containing all three proteins or a GST control (G, H). Samples with treatment were calculated as a percentage of samples with media alone, which was set at 100 %. $n \geq 5$ mean ± SD. * demonstrates significance from media alone cells, † demonstrates significance from ant-CD166 treatment. ** $p \leq 0.01$, ***/††† $p \leq 0.001$, One-Way ANOVA with Tukey's multiple comparison test.

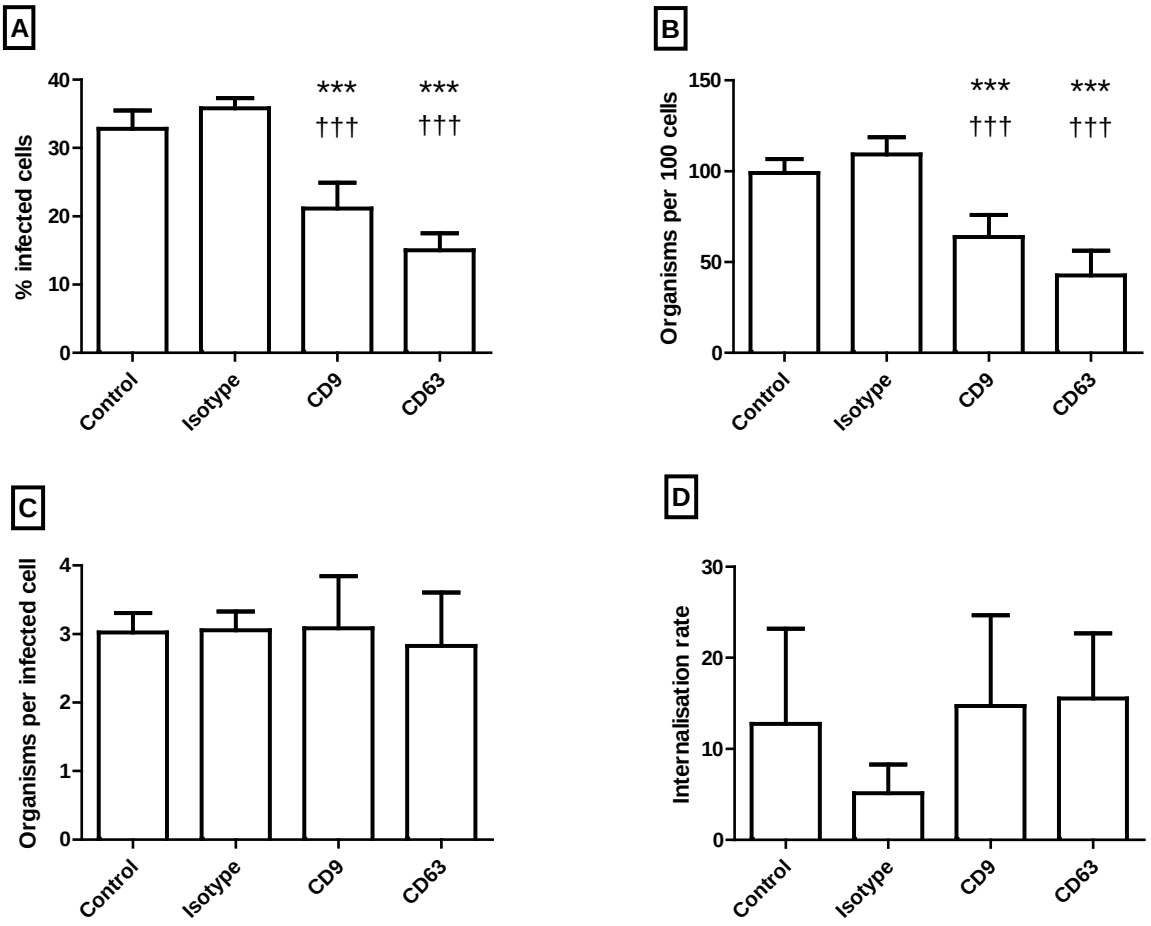


Figure 3. Untransformed data showing blockade of tetraspanins with antibodies reduces *N. meningitidis* adherence to DETROIT 562 epithelial cells. This figure reflects the transformed data demonstrated in Fig. 2A. Method and results collection are as described previously (Fig. 2). Graphs show adherence, by measurement of cells binding bacteria, total number of bacteria binding 100 cells and the average number of bacteria bound to a positive cell (A-C), and internalisation (D). n = 6, mean $\pm$ SD. * show statistical significance against infected control cells, † show statistical significance against isotype control treated cells; *** = $p \leq 0.001$, ††† = $p \leq 0.001$, One-Way ANOVA with Tukey's multiple comparison test.

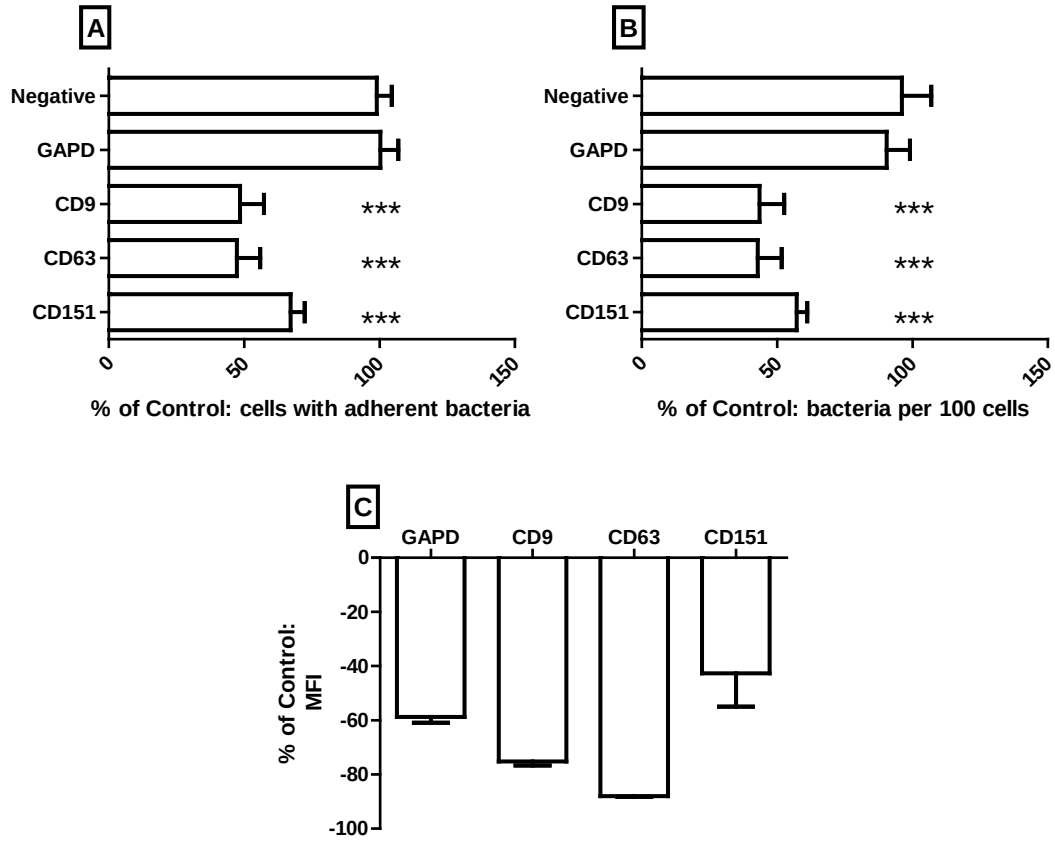


Figure 4. siRNA abatement of tetraspanins reduces meningococcal adherence to HEC-1-B epithelial cells. HEC-1-B cells were incubated in either medium alone or a variety of siRNAs (non-targeting siRNA, GAPD, CD9, CD63, CD151; 40 nM) for 48 hours. (A-B) Cells were infected for 60 minutes with MC58 and adherence was measured by fluorescence microscopy. Samples with treatment were calculated as a percentage of samples with media alone, which was set at 100 %. n = 6, mean ± SD. *** $p \leq 0.001$, One-Way ANOVA with Tukey's multiple comparison test. (C) Relative expression respective proteins on treated cells was measured by flow cytometry. n = 3, mean ± SD.

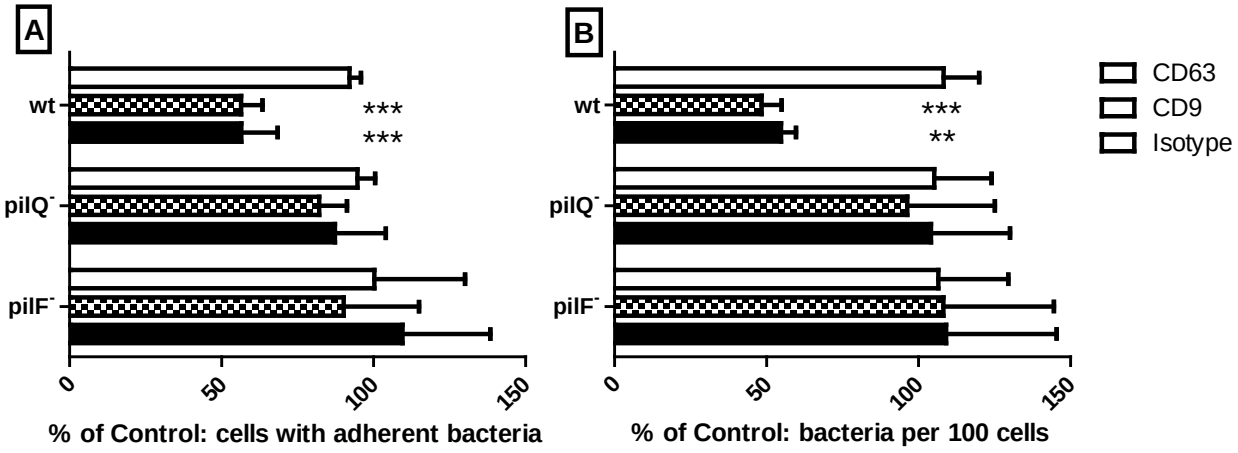


Figure 5. Type IV Pili involvement in tetraspanin-mediated adherence to HEC-1-B epithelial cells. Strains that lack *pilQ* and *pilF* are unable to express type IV pili. Cells treated with no antibody, isotype control or a combination anti-tetraspanin treatment were infected with bacteria separately (M.O.I. = 300), adhesion is measured using microscopy. Samples with antibody were calculated as a percentage of samples with media alone, which was set at 100 %. (A) Change in the number of infected cells. (B) Change in organisms per 100 cells. n = 6, mean ± SD. ** $p \leq 0.01$, *** $p \leq 0.001$, One-Way ANOVA with Tukey's Multiple Comparison Test.

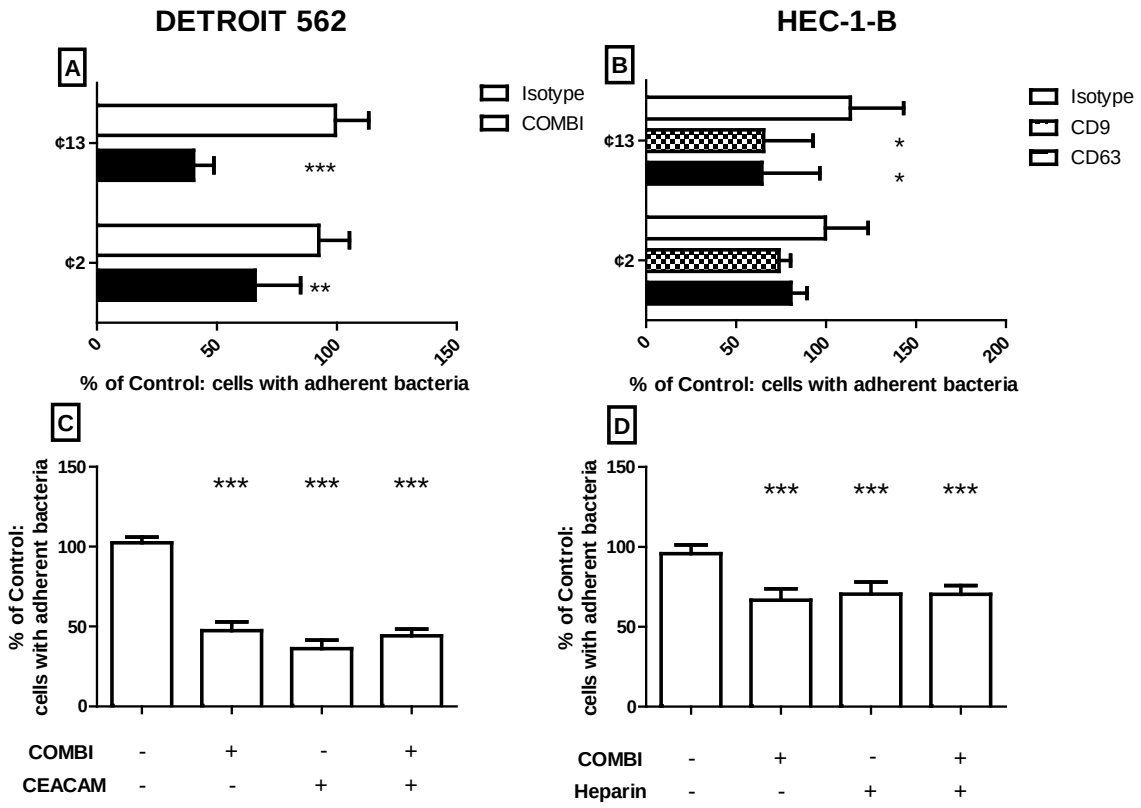


Figure 6. Opa receptor involvement in tetraspanin mediated adherence to epithelial cells. ¢13 and ¢2, derivatives of the parent strain MC58, do not express capsule, and the latter also lacks Opa proteins. DETROIT 562 (A) and HEC-1-B (B) cells were treated with media alone, isotype control and combination or singular anti-tetraspanin treatments. Cells were infected with the MC58 derivatives for 60 min (M.O.I.=30). DETROIT 562 (C) and HEC-1-B (D) cells were treated with media alone, isotype control, combination anti-tetraspanin treatment, anti-CEACAM treatment or a combination of the two. Cells were infected with MC58 for 60 min (M.O.I.=300). Adhesion was measured by fluorescent microscopy. Samples with antibody were calculated as a percentage of samples with media alone, which was set at 100 %. n = 6, mean ± SD. * $p \leq 0.05$, *** $p \leq 0.001$, (A-D) One-Way ANOVA with Tukey's multiple comparison test.

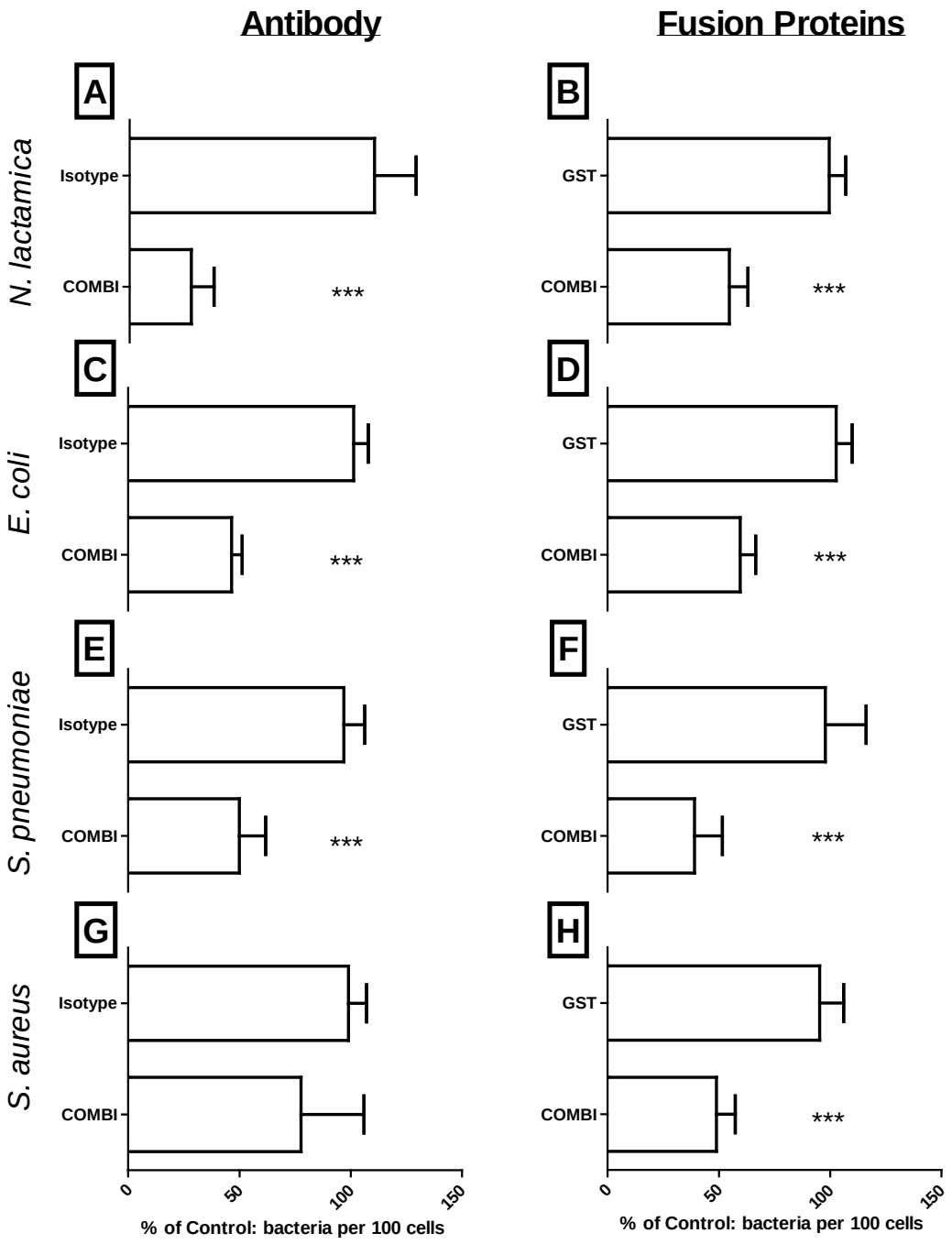
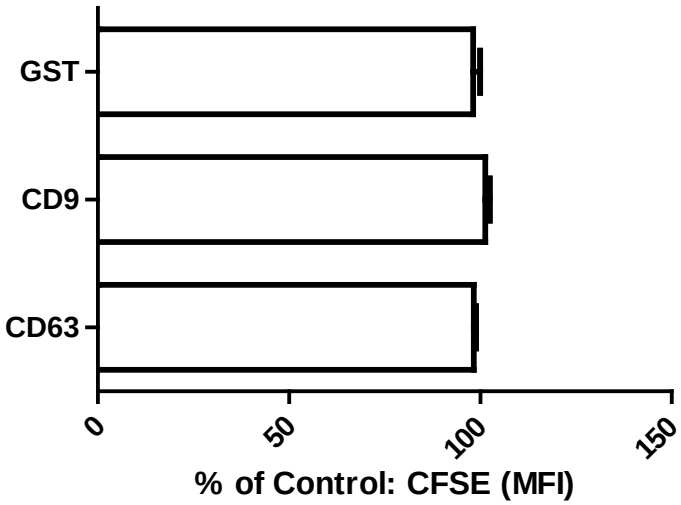


Figure 7. Blockade of tetraspanins affects many bacterial adhesion cascades. Cells were treated with antibody (A, C, E, G), or with recombinant GST:EC2 tetraspanin protein (B, D, F, H). Cells were infected with *N. lactamica* (A, B), E. coli (C, D), *S. pneumoniae* (E, F) or *S. aureus* (G, H). Coverslips were stained with DAPI and results

collected by fluorescence microscopy. Treated samples were calculated as a percentage of samples with media alone, which was set at 100 %. n = 6, mean ± SD, *** $p \leq 0.001$, One-Way ANOVA with Tukey's multiple comparison test.



Supplementary Figure 1. Tetraspanin EC2 domains do not act as receptors for *Neisseria meningitidis*. Recombinant tetraspanin EC2 domains (CD9, CD63) were immobilised on 96 well plates. Wells were infected with CFSE labelled MC58 (M.O.I. = 30) for 60 minutes. Data was analysed with a fusion plate reader. Treated samples were calculated as a percentage of untreated samples, which was set at 100 %. n = 3, mean ± SD. One-Way ANOVA with Tukey's multiple comparison test.