A role for Fibroblast Growth Factor Receptor 1 in the pathogenesis of *Neisseria meningitidis*

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Abstract

Neisseria meningitidis (the meningococcus) remains an important cause of human disease, including meningitis and sepsis. Adaptation to the host environment includes many interactions with specific cell surface receptors, resulting in intracellular signalling and cytoskeletal rearrangements that contribute to pathogenesis. Here, we assessed the interactions between meningococci and Fibroblast Growth Factor Receptor 1-IIIc (FGFR1-IIIc): a receptor specific to endothelial cells of the microvasculature, including that of the blood-brain barrier. We show that the meningococcus recruits FGFR1-IIIc onto the surface of human blood microvascular endothelial cells (HBMECs). Furthermore, we demonstrate that expression of FGFR1-IIIc is required for optimal invasion of HBMECs by meningococci. We show that the ability of *N. meningitidis* to interact with the ligand-binding domain of FGFR1-IIIc is shared with the other pathogenic *Neisseria* species, *N. gonorrhoeae*, but not with commensal bacteria including non-pathogenic *Neisseria* species.

1. Introduction

Neisseria meningitidis, or the meningococcus, while normally a harmless commensal of the human oropharynx, can occasionally cause devastating disease including meningitis, sepsis, disseminated intravascular coagulation (DIC) and multiple organ failure [1]. Penetration of the oropharyngeal epithelial mucosa and entry into the bloodstream by meningococci is a crucial step in development of systemic disease, while penetration of the blood-brain barrier (BBB) is a prerequisite for development of meningitis [2, 3]. Attachment to vascular endothelial cells induces membrane protrusions at meningococcal binding site and leads to formation of specific protein complexes known as cortical plaques underneath meningococcal colonies [4-7]. The process by which these steps occur is not fully understood [8-12]. Adhesion to human cells by meningococci is mediated by the type IV pilus, major adhesins including Opa and Opc [13, 14], as well as a number of additional adhesins including the porins PorA [15] and PorB [16], and sialic acid residues on either lipooligosaccharide or the Group B polysaccharide capsule [17]. While a number of host cell receptors interacting with meningococci including alpha actinin [18], integrins [8, 19, 20], carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) [21, 22], CD46 [23, 24], platelet activating factor receptor [25], laminin receptor [15], galectin-3 [26], and receptor tyrosine kinases [27, 28], have been identified, no tissue-specific receptors for this pathogen have been identified.

Fibroblast Growth Factor Receptors (FGFRs) are transmembrane proteins that belong to the Receptor Tyrosine Kinase (RTK) family of signalling molecules [29]. Four members comprise the family and are responsible for recognising the 22 Fibroblast Growth Factors (FGFs) found in humans [30-32]. FGFs are involved in cell differentiation, migration and proliferation during early embryogenesis and play an important role in tissue repair, wound healing [33] and tumour angiogenesis in adulthood [29, 34, 35]. Splicing of FGFR transcripts generates a variety of specific isoforms in different cell types and tissues, recognising specific types of FGF molecules [29, 31, 36, 37].

FGFRs are single transmembrane receptors; the extracellular N-terminal region consists of three IgG-like domains that form a ligand-binding domain with an acidic box which interacts with heparin sulphate proteoglycans (HSPGs) and cell adhesion molecules (CAMs) [38]. This is followed by a transmembrane region and a C-terminal cytoplasmic region containing 7 specific tyrosine residues [39-42]. Binding of FGFs to the receptors leads to dimerization of the receptor and tyrosine autophosphorylation, which in turn activates the receptor, triggering downstream signalling pathways [43-46]. FGFR1 signalling and expression is important in maintaining the integrity and differentiation of endothelial cells forming the microvasculature [34, 47, 48]. Here, we demonstrate specific interaction of FGFRs in human brain microvascular endothelial cells (HBMECs) with meningococci, and show that this interaction influences the ability of *N. meningitidis* to invade these cells.

2. Materials and methods

2.1. Bacterial growth and culture

E. coli JM109 cells (Promega) were grown on Lysogeny Broth agar (Oxoid) supplemented where appropriate with 100 µg/ml ampicillin (Sigma). *N. meningitidis* serogroup B strain MC58 (ATCC® BAA335™) [49] and clinical isolates of *N. meningitidis* from our laboratory collection, *N. gonorrhoeae* strain FA1090 (ATCC 700825)*, N. lactamica* strain ATCC23970, *N. polysaccharea* (a clinical isolate from laboratory stocks), *H. influenzae* Rd KW20 (ATCC 51097) [50], and *S. pneumoniae* T4 (unencapsulated) [51] were routinely cultured on chocolated horse blood agar (Chocolate agar, Oxoid) at 37°C, in an atmosphere of air plus 5% CO_2.

2.2. Cell association assay and cell invasion (Gentamicin protection) assay

To quantify cell association and cell invasion of HBMECs with *N. meningitidis* HBMECs were seeded and grown overnight or for 48 h after siRNA transfection until 100% confluent in 24-well plates. Cells were infected with 1×10^7 CFU bacteria for 4 h in ECM-b media without any supplements. Cell association and invasion were then determined as described previously [52].

2.3. Confocal Immunofluorescent Microscopy

HBMECs were seeded onto fibronectin-coated coverslips (*ca.* 1×10^5 cells) and grown overnight to reach a confluency of 70-80%. Cells were infected for 4 h (MOI 200). Coverslips were washed with PBS and fixed with 4% paraformaldehyde (w/v) in PBS for 5 min. Coverslips were then washed with PBS and blocked in 4% (w/v) BSA/PBS at 4°C overnight. For intracellular staining, cells were permeabilised using 0.1% Saponin, 20 mM glycine in 4% BSA/TBS at 4°C. Subsequent staining procedures were carried out in 4% (w/v) BSA/TBS. Briefly, coverslips incubated with primary antibody for 1 h were washed with PBS followed by one wash with dH_2O. Coverslips were then incubated with secondary antibody for 1 h in the dark followed by washes with PBS-Tween (0.05% v/v; PBS-T), PBS and then dH_2O. Coverslips were then mounted on glass slides with ProLong® Gold and SlowFade® Gold Antifade Reagents with DAPI (Invitrogen). Coverslips were analysed using a Zeiss LSM-700 confocal microscope. Images were processed with ImageJ, Adobe Photoshop and LSM Image Browser software.

2.4. Antibodies and reagents

Antibodies detecting FGFR1: Flg S-16 (goat polyclonal antibody) and Flg C-15 (mouse antibody), phosphorylated FGFR1 (p-Y766, mouse monoclonal antibody), Alpha-actinin (sc-17829) and Rab5 (sc-46692) were purchased from Santa Cruz Biotechnology. Secondary antibodies conjugated to various fluorochromes, and Phalloidin conjugated to fluorochrome 488 were obtained from Life Technologies-Invitrogen. The antibody detecting 37LRP (A-7) was

purchased from Santa Cruz Biotechnology and antibody against 67LR (Mluc-5) was purchased from Thermo Scientific.

2.5. Cloning and expression of FGFR1 IIIc and Fc-stop

HBMECs grown in fibronectin-coated T75 flasks (BD Bioscience) were harvested by trypsin treatment and centrifugation for 5 min at 300 × *g*. Cells were lysed in lysis buffer (SIGMA GenElute™ Mammalian Total RNA Miniprep Kits) supplemented with 1% (v/v) 2-β-Mercaptoethanol and RNA was extracted according to the manufacturer's protocol (Sigma GenElute™ Mammalian Total RNA Miniprep Kit). The concentration of RNA was measured using a Nanodrop spectrophotometer and adjusted to 200 ng μl^{-1}. DNase treatment was performed following the manufacturers protocol (Turbo DNase, Life Technologies). 10 µl of RNA was used for cDNA preparation using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). HBMEC cDNA was then used as template to amplify the extracellular domain of FGFR1 IIIc isoform using primers designed to amplify the extracellular domain of FGFR1 and containing restriction sites for restriction enzymes *NdeI* (Forward; GCGGCTTAATCATATGCAGGGACCCGGATCCATGTGGAGCTGGAAGTGCC) and *NotI* (Reverse; GCGCGATTAAGCGGCCGCttaCAGGGGCGAGGTCA). The amplified PCR product was gel purified, digested and then ligated into *NotI* and *NdeI*-digested pEF-Bos-ss-Fc-FGFR2IIIaTM-ires-TPZ [53-55] using the LigaFast TMRapid DNA Ligation System (Promega). The ligated plasmid was used for transformation of competent *E. coli* JM109 cells (Promega). The resulting plasmid was named pEF-Bos-ss-Fc-extFGFR1IIIc-ires-TPZ. A plasmid expressing LAMR1, which has been described previously [56], was mutated to introduce a stop codon at the junction of the sequence encoding Fc and LAMR1, resulting in a plasmid designated pEF-Bos-ss-Fc-Stop-LRP-ires-TPZ encoding the Fc tag (Fc-stop) only. For expression of Fc-tagged FGFR2 IIIa TM, pEF-Bos-ss-Fc-extFGFR1IIIc-ires-TPZ and Fc-tag, 293T cells were grown overnight to 30% confluence in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with FCS (10%v/v) and transfected by calcium phosphate precipitation. Media was collected 72 h post-transfection and Fc-tagged recombinant proteins were purified on a Protein AIG-Sepharose (Source BioScience LifeSciences) column. Protein concentration in each fraction was quantified using the BCA kit following manufacturer's protocol (Thermo Scientific).

2.6. FGFR1 siRNA transfection in HBMECs

Human FGFR1 siRNA (siGENOME SMART pool) and control scrambled siRNA were obtained from Dharmachon/Thermo Scientific and reconstituted following the manufacturer guidance. FGFR1 siRNA was resuspended in 1 ml of 1 × siRNA buffer to a final concentration of 50 µM (stock). HBMECs were seeded into 24-well plates pre-coated with fibronectin, as previously described, and grown overnight to reach a confluency of 70-80%. Transfection media was prepared by mixing serum- and antibiotic-free media with siRNA from a 50µM stock to a final concentration

of 5μM; in a separate tube Transfection reagent number 1 (Thermo Scientific-Dharmacon) was added to serum and antibiotic-free media. Both tubes were incubated for 5 min at room temperature and then mixed together by pipetting and incubated at room temperature for 20-30 min. Cells were washed with serum and antibiotic-free medium and 240 μl of complete media without antibiotics were added to each well and then the transfection mixture was added drop-wise to each well to a final concentration of 50 nM siRNA/ well. Cells were then incubated for 6 h and then the media was replaced with complete media (Endothelial cell medium (ECM-b) (ScienCell) supplemented with ECGS (containing EGF, VEGF) (ScienCell) (1% v/v) and FBS (5% v/v) and penicillin/streptomycin (ScienCell; 1% v/v). The level of FGFR1 expression was examined at RNA and protein levels at 24, 48 and 72 h post transfection.

2.7. *ELISA*

96-well plates (NUNC Immobilizer Amino) were coated with 100 μl of protein A (Pierce; 1 μg ml^{-1}) in PBS for 1 h, washed once with PBS-T and then 86.5 $\times 10^{-15}$ M of Fc-tagged recombinant proteins added to each well in carbonate buffer (pH 9.6). Plates were incubated for 1 h and then washed three times with PBS-T. Plates were blocked with 1% BSA/PBS (w/v) for 1 h, then washed once with PBS-T. Bacterial cells harvested from overnight plates and resuspended in PBS-T, washed with the same buffer three times and finally resuspended in sodium carbonate buffer (44 mM $NaHCO_3$, 6.0 mM Na_2CO_3; pH: 9.6). The OD_{600} was measured and 20 ng (2 μl of 10 ng μl^{-1}) of digoxigenin (DIG; Roche) was added to 1 ml of bacterial suspension with OD_{600}:1. The bacterial suspensions were incubated for 2 h in the dark at room temperature on the shaker. Bacteria then were washed three times with PBS-T by centrifugation (13000 × *g* for 1 minute) and resuspended in 1% BSA/PBS (w/v). OD_{600} in 1% BSA/PBS was adjusted to 0.02 for ELISA. For each experiment freshly labelled bacterial strains were used. 100 μl of DIG-labelled bacteria were added to each well and plates were incubated at 4°C overnight then washed five times with PBS-T. 100 μl of anti-DIG-alkaline phosphatase antibody (Roche; 0.0002 v/v) in 1% BSA/PBS was then added to each well and incubated for 1h then washed three times with PBS-T. 200 μl of alkaline phosphatase substrate (SIGMA) was added and plates were incubated for 1h. The OD_{405} was measured for each sample and values obtained subtracted from the binding of the same DIG-labelled strain to 1% BSA/PBS.

3. Results

3.1. *N. meningitidis* colonies recruit FGFR1 on the apical surface of Human Brain Microvascular Endothelial Cells (HBMECs).

To study the possible interaction of FGFR1 by meningococcal colonies, HBMECs were infected with *N. meningitidis* for 4 h, fixed, and prepared for subsequent investigation by immunofluorescence microscopy. FGFR1 was recruited by meningococcal colonies on the apical surface of HBMECs (**Figure 1-A**). Previously, we have shown that meningococci interact with both the 67-kDa laminin receptor (67LR) and the 37-kDa laminin receptor precursor (37LRP) isoforms of the laminin receptor [15, 56]. Here, we show that FGFR1 recruitment to meningococcal colonies coincided with recruitment of both isoforms of this receptor (**Figure 1-A**). To address whether FGFR1 recruited by meningococci is activated, HBMECs were labelled with primary antibodies specific for phosphorylated tyrosine 766 (p-Y766), which is a feature of the activated form of FGFR1, and with antibodies specific for the two main isoforms of the laminin receptor, 67LR and 37LRP. In all cases the phosphorylated FGFR1 co-localised with meningococcal cells (**Figure 1-B**). Meningococcal colonies co-localised with FGFR1 and 37LRP, up to 60% of which co-localised with FGFR1. This was significantly higher than co-localisation of the microcolonies with the 67LR isoform of the laminin receptor (**Figure 1-C**).

3.2. Internalized *N. meningitidis* associated with activated FGFR1.

To determine whether internalised bacteria were associated with activated FGFR1, HBMECs were infected for 4 h with *N. meningitidis* and non-internalised bacterial cells were killed by treatment with gentamicin. Immunofluorescent staining for actin (phaloidin) and activated FGFR1 (p-Y766) confirmed that meningococci recruited activated FGFR1 in the cytoplasm of HBMECs, and that the receptor was trafficked inside the cells alongside with meningococcal cells (**Figure 2**). To confirm that the bacterial cells in gentamycin-treated monolayers were internalised, a Z-stack image was constructed. Bacterial cells could be observed beneath the membrane of the endothelial cells (**Figure 2-B and 2C**). To determine whether the internalised bacteria were associated with endosomes we probed infected monolayers for Rab5. Internalised bacteria associated with activated FGFR1 were also co-located with this marker for early endosomes, indicating that the bacteria were endosome-associated (**Figure 2D**).

3.3. FGFR1 expression is required for meningococcal invasion into HBMECs.

To determine whether interaction of meningococci with FGFR1 on the surface of HBMECs was required for internalisation of the bacteria, FGFR1 expression was knocked down in HBMECs using siRNA treatment. Sixty hours post-siRNA treatment, cells were infected with meningococci and cell association and invasion assays were performed. The numbers of meningococcal cells associated with HBMECs was significantly and dramatically reduced in

response to FGFR1 knock-down (**Figure 3-A)**. Treatment with scrambled siRNA under the same conditions did not result in a significant reduction in association of meningococci with HBMEC cells. There was also a significant decrease in the number of internalised meningococcal cells recovered from FGFR1 siRNA-transfected HBMECs (**Figure 3-B**). Again, treatment with a scrambled siRNA did not significantly affect binding of meningococci to the HBMEC cells. This demonstrates that FGFR1 expression plays an important and specific role in meningococcal adhesion to and invasion into HBMECs.

3.4. *N. meningitidis* interacts specifically with FGFR1 IIIc.

To determine which isoforms of FGFR1 are expressed in HBMEC cells we performed RT-PCR using cDNA generated from total RNA extracted from HBMECs. Various combinations of primers specific for exon 7, 8 and 9 for FGFR1, FGFR2 and FGFR3 were used to determine which isoforms of FGFR were expressed. Amplification products across exons 7 and 9 of FGFR1, corresponding to FGFR1 IIIc, but not across exons 7 and 8, corresponding to FGFR1 IIIb were detected, while amplification across exons 7 and 8, corresponding to FGFR3 IIIb, but not across exons 7 and 9, corresponding to FGFR3 IIIc were amplified. No amplicons were detected across either exons 7 and 8 or exons 7 and 9 of FGFR2. Thus, FGFR1 IIIc and FGFR3 IIIb isoforms were both expressed in HBMECs. To determine whether meningococci could interact directly with the extracellular domain of FGFR1 IIIc this protein was cloned and expressed as an Fc-tagged fusion protein. Two other proteins: Fc-FGFR2 IIIa TM, comprising the trans-membrane region of FGFR2 IIIa fused to the immunoglobulin Fc domain, and the Fc portion of immunoglobulin alone (Fc-stop) were used as controls for possible interaction with *N. meningitidis* that was not specific for the FGFR1 IIIc extracellular domain. Both control proteins were derived from clones employing the same vector and purified by the same method as Fc-FGFR1 IIIc. Each of the purified proteins were employed as an immobilised ligand in ELISA experiments to assess the ability of DIG-labelled *N. meningitidis* (MC58) to bind to these ligands. *N. meningitidis* (MC58) bound Fc-FGFR1 to a significantly greater degree than either Fc-FGFR2 IIIa TM or Fc-stop (**Figure 4 A**). This indicates that the observed interaction of Fc-FGFR1 was not due to meningococci binding to the Fc-tag of the expressed extracellular domain of FGFR1 and thus demonstrates a direct interaction between the receptor present on the apical surface of HBMECs and surface structures of *N. meningitidis.*

3.5. Interaction between *N. meningitidis* and FGFR1 is common among clinical isolates of different serogroups and is shared by *N. gonorrhoeae* but not commensal Neisseria species, nor other bacterial pathogens targeting the meninges.

Having shown that Fc-FGFR1 IIIc interacts with meningococci, we sought to establish whether other *Neisseria* species could interact with this receptor. We tested representative strains belonging to several *Neisseria* species, including the human pathogen *N. gonorrhoeae* and the normally non-pathogenic species *N. polysaccharea* and *N. lactamica*, for interaction with Fc-

FGFR1 in ELISA assays. *N. gonorrhoeae* cells bound to wells containing Fc-FGFR1 at similar levels to *N. meningitidis* MC58, and to a significantly higher level than to FGFR2. In both cases *N. gonorrhoeae* bound to Fc-FGFR1 to a significantly higher degree than either of the two commensal species, which did not bind to a significantly higher degree to Fc-FGFR1 than to control wells containing only Fc-FGFR2 TM (**Figure 4 B**). To determine whether binding of *N. meningitidis* to FGFR1 was a phenomenon common to other isolates of *N. meningitidis* we tested a panel of *N. meningitidis* isolates belonging to serogroups A, B, C and X. All strains were able to bind Fc-FGFR1 specifically at levels similar to the serogroup B strain MC58 (**Figure 4 C**). We previously showed that, like the meningococcus, the pathogens *S. pneumoniae* and *H. influenzae*, both of which are known to target the meninges, each targeted the laminin receptor on HBMEC cells as a prerequisite for internalisation [15]. Neither representative *S. pneumoniae* nor *H. influenzae* strains bound significantly to Fc-FGFR1 (**Figure 4 D**), demonstrating that the observed interaction between the pathogenic *Neisseria* species was specific.

4. Discussion

The interactions between meningococci and the BBB are central to the ability of this pathogen to cause meningitis, but they are complex and incompletely understood. Meningococci have been shown to interact with brain microvascular endothelial cells (BMECs) through various molecules of both the bacterium and the endothelial cell and several of these interactions result in disruption of the BBB and favour bacterial entry into the cerebro-spinal fluid. Type IV pili, for example, initiate initial binding to human brain endothelial cells via the pilin molecules PilQ, PilE and PilV, which bind to host laminin receptor [15], CD147 and β-adrenergic receptor [57], respectively. The latter receptor acts as a mechano-sensor, signalling via the β2-adrenoceptor/β-arrestin pathway and recruits Src tyrosine kinase and junctional complex proteins to the site of bacterial attachment [57, 58]. Pilus-mediated meningococcal attachment to BMECs results in recruitment of adherens junction complex components (VE-cadherin, p120-catenin, β-catenin), tight junction components (ZO1, ZO2 and caludin-5), and the Par3/Par6/PKCz polarity complex to the site of bacterial attachment. This results in disruption of adherens junctions and tight junctions and the opening of the paracellular pathway [59]. Meningococci also interact with BMECs indirectly via the major adhesin Opc, which binds fibronectin and thus allows the bacterium to interact with the integrin α-5 β-1-receptor on the endothelial cell surface [60]. Opc/integrin-mediated interaction of endothelial cells with meningococci results in activation and phosphorylation of JNK1 and JNK2, as well as their substrates c-Jun, resulting JNK1 and JNK2-dependent invasion [8]. Meningococci also activate p38 MAPK, but this interaction is independent of the Opc/integrin interaction, and results in the induction of the inflammatory cytokines IL-6 and IL-8 [8]. Attachment of meningococci to endothelial cells also initiates secretion of host matrix metalloproteinase-8, which results in cleavage of the tight junction component occludin, which in turn leads to increased paracellular permeability [61].

A number of receptor tyrosine kinases have been shown to be important for the interaction between BMECs and meningococci. Several activated RTKs including the EebB family receptors epidermal growth factor receptor (EGFR), ErbB2 and ErbB4 were shown to be activated in endothelial cells after interaction with meningococci [27]. A requirement for Focal Adhesion Kinases and activation of Src in the internalization of meningococci via interaction with integrins has also been demonstrated [20]. Considering the role of FGFR1 in maintaining the integrity of the BBB and angiogenesis [62], we examined the possible role of FGFR1 in interactions with meningococci. HBMECs infected with meningococci were shown to recruit FGFR1 to their apical surface. Recently, we showed that meningococci bind to both 37LRP and Galectin-3 on the surface of HBMECs [56]. Here, we showed that FGFR1 recruitment coincided with recruitment of both isoforms (37LRP and 67LR) of the laminin receptor; molecules already implicated in *N. meningitidis*-HBMEC interactions [15]. Ligation of the extracellular domain of FGFRs by their

ligands leads to auto-phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor; these phosphorylated residues subsequently serve as docking sites for a number of adaptor proteins responsible for regulation of various downstream signalling cascades [31]. The FGFR1 molecules recruited by meningococci were shown to be activated and activated receptors and meningococcal cells also co-localised with α-actinin. The microcolonies were also associated with the early endosomal marker Rab5, confirming that the bacteria were entering the endosomal pathway. This is in agreement with previous studies on the trafficking of FGFR1 into early endosomes inside the cytoplasm, and the regulation of its trafficking by Syndecan 4 in a clathrin-independent manner [63]. We also demonstrated by confocal microscopy in HBMECs treated with gentamicin that the receptor is internalised into the cytoplasm along with invading meningococci. To determine whether FGFR1 was required for meningococcal-HBMECs interactions, FGFR1 expression was transiently inhibited by using FGFR1 siRNA transfection. FGFR1 knock-down in HBMECs resulted in a dramatic reduction in both association and internalisation of meningococci into HBMECs. Surprisingly, the residual levels of both cellular association and invasion were very low as we would have expected there to be interaction via the other known receptors for the pathogen. Our data confirm that direct interaction between the extracellular domain of FGFR1 and meningococci is required for consequent activation of the receptor and internalisation of bacteria into the HBMECs.

The mechanisms by which recruitment of FGFR1 by meningococcal colonies leads to their internalisation is unknown. Interaction of meningococci with HBMECs has previously been shown to lead to higher levels of activation of ERK 1, 2 due to activation of ErbB2 in these cells [28]. However, levels of ERK 1,2 activation in cells in which FGFR1 was knocked down by siRNA transfection were unaffected, demonstrating that FGFR1 does not regulate the levels of ERK1, 2 activation [64]. Several studies on meningococcal infection of endothelial cells showed that invasion of bacteria requires activation of Src, phosphorylation of cortactin via the Src pathway and activation of focal adhesion kinases (FAKs) [20, 28, 65]. Also, it has been shown that meningococcal cells hijack the β-arrestin/β 2-adrenoreceptor pathway to invade endothelial cells and cross the BBB: inhibition of β-arrestin mediated activation of Src, prevents the invasion of meningococcal cells [58]. Src is required for cortactin phosphorylation by FGF1 which can provide an alternate downstream pathway of FGFR1 from PLCγ and can be involved in cytoskeletal rearrangement [66, 67]. However it was reported that mutation of Y766 in FGFR1 leads to higher level activations of PLCγ which inhibits Src activation [68]. These observations suggest that FGFR1 siRNA transfection of HBMECs may have led to the same effect on inhibition of Src activation which consequently inhibited meningococcal invasion into HBMECs. This effect appears to be specific to the meningococcus as the bacterial pathogens *H. influenzae* and *S. pneumoniae*, which also cross the BBB and can cause meningitis, do not interact with FGFR1 IIIc on the surface of HBMECs. On the other hand, *N. gonorrhoeae*, which

does not usually interact with the BBB is also able to interact with FGFR1 IIIc to the same extent as *N. meningitidis.*

Although the specific role of FGFRs in infectious diseases has not been excessively investigated, an increase in FGF2 expression enhances *Chlamydia trachomatis* binding and internalisation into epithelial cells [69]. *C. trachomatis* facilitates entry by binding directly to FGF2, which results in binding of FGF2-bacteria-heperan sulfate proteoglycan (HSPG) complexes to FGFR and internalisation of the elementary bodies of these bacteria into the cells. More recently, HSPG-associated FGFR1 has been implicated in internalisation of *Rickettsia rickettsii* into cultured human microvascular endothelial cells and inhibition of FGFR1 in a *R. conorii* murine model of endothelial-target spotted fever rickettsiosis; reduced the rickettsial burden in infected mice [70].

Interaction of meningococci and FGFR1 in BMECs occurs within a complex network of interactions. The various pathways are likely to be both synergistic and antagonistic. FGFR1 engagement by basic fibroblast growth factor receptor has recently been shown to protect the integrity of HBMEC monolayers preventing the downregulation of the junction proteins ZO-1, occludin and VE-cadherin in response to oxygen-glucose deprivation and deoxygenation [71]. This is in contrast to the known effects of meningococcal interactions on these molecules. Thus, engagement of FGFR1 may favour a trans-cellular pathway through HBMECs rather than a para-cellular pathway in which the integrity of the monolayer would have to be compromised. Further investigations are required to identify and unravel the role of FGFR1 signalling in meningococcal invasion into HBMECs and other type of endothelial cells forming the microvasculature in other organs.

Declaration of competing interest: none

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Figure Legends

Figure 1. Activated FGFR1 is recruited by meningococcal colonies. (A) HBMECs were infected with meningococcal cells (MOI: 200) for 4 h. *Neisseria meningitidis* colonies were visualized with DAPI (blue), FGFR1 was probed with anti-FGFR1 primary antibody and detected with anti-goat Alexa Fluor 680 antibody (red). 37LRP and 67LR were probed with primary antibodies and detected with anti-mouse Alexa Fluor 488 antibodies (green). FGFR1 was recruited by meningococcal colonies, which coincides with recruitment of 37LRP and, to a less extent, 67LR. Co-localizations area is shown by arrows (images are representative of 10 infected cells). (B) FGFR1 phosphorylated at Tyrosine 766 (p-Y766) was labelled with Alexa Fluor 680 (red) and both isoforms of Laminin receptor (67LR and 37LRP) with Alexa Fluor 488 (green). Recruitment of activated FGFR1 (p-Y766) coincided with recruitment of 37LRP and, to a less extent, 67LR. (C) Levels of co-localization of MC58 with 37LRP, 67LR and p-Y766 (activated FGFR1) were quantified by measuring the percentage of co-localization of each receptor with MC58 in 30 fields. There was a significant difference between recruitment by MC58 of activated FGFR1 or 37LRP with recruitment of 67LR ($p = 0.04$, One Way ANOVA, multiple comparison, Dunn's Test).

Figure 2. Activated FGFR1 is recruited by internalised meningococci within HBMECs. (A) In infected HBMECs α-actinin and actin were labelled with Alexa Fluor 680 (Red) and the activated form of FGFR1 labelled with Alexa Fluor 488 (Green). Bacteria were labelled with DAPI (Blue). Internalized bacteria co-localized with both α-actinin (sc-17829), actin (phaloidin) and activated FGFR1 (p-Y766) (arrowheads). (B) Z-stack image of meningococcal colonies shows that internalized bacteria (co-localizing with α-actinin) recruit activated FGFR1 (p-Y766) inside the cells. (C) Orthogonal view of Z-stack image of meningococcal colonies on the apical surface (arrows) of the cells which only recruited activated FGFR1 (p-Y766, Green) and internalized colonies colocalizing with in α-actinin (pY766). (D) Infected HBMECs probed for activated FGFR1 (p-Y766) and Rab5 (sc-46692) showing bacteria co-localised with both molecules.

Figure 3. FGFR1 down regulation plays an important role in invasion of meningococci into HBMECs. (A) HBMECs were infected with Nm MC58 for 4h (MOI: 10). Cells were washed and lysed in 500 µl of 1% saponin in PBS and 100 µl of homogenized lysates used for serial dilution preparation. 10 µl of each dilution was plated onto chocolate agar and CFUs were calculated for each sample. There was a significant reduction in the number of meningococci associated with HMBECs after FGFR1 knockdown (FGFR1 siRNA; $p=0.0031$, two-tailed unpaired t-test, n=8). Experiments were performed in triplicate wells and means shown represent 8 independent experiments. (B) For invasion assays, gentamicin was added after 4h of infection and plates were incubated for a further 1h. Cells were then washed, lysed, homogenised and dilutions plated onto chocolate blood agar plates. There was a significant difference between the number of internalised meningococci in untreated cells compared to HBMECs transfected with FGFR1 siRNA ($p=0.0003$, two-tailed t-test, n=7). Error bars represent standard deviation of mean.

Figure 4. *N. meningitidis* and *N. gonorrhoeae* interact directly with the extracellular domain of FGFR1. Fc-tagged purified proteins were used as the immobilised ligand in ELISA experiments. Levels of interaction with DIG-labelled bacteria were measured; the values shown are those following subtraction of binding to 1% BSA/PBS. (A) *N. meningitidis* MC58 interacts directly with the extracellular domain of Fc-FGFR1 (two tailed t-test; each experiment was performed in 6 technical replicates and the data shown is derived from 6 independent experiments). (B) Binding of commensal *Neisseria,* but not *N. gonorrhoeae,* to FGFR1 IIIc was significantly lower than that shown by Nm MC58. (C) Clinical isolates of serogroups A, B and C of N*. meningitidis*, display similar binding levels to FGFR1 IIIC, when compared to MC58. (D) The meningeal pathogens *H. influenzae* and *S. pneumoniae* bound FGFR1 IIIc to a negligible degree that was significantly lower than cells of Nm MC58 ($p \leq 0.002$).

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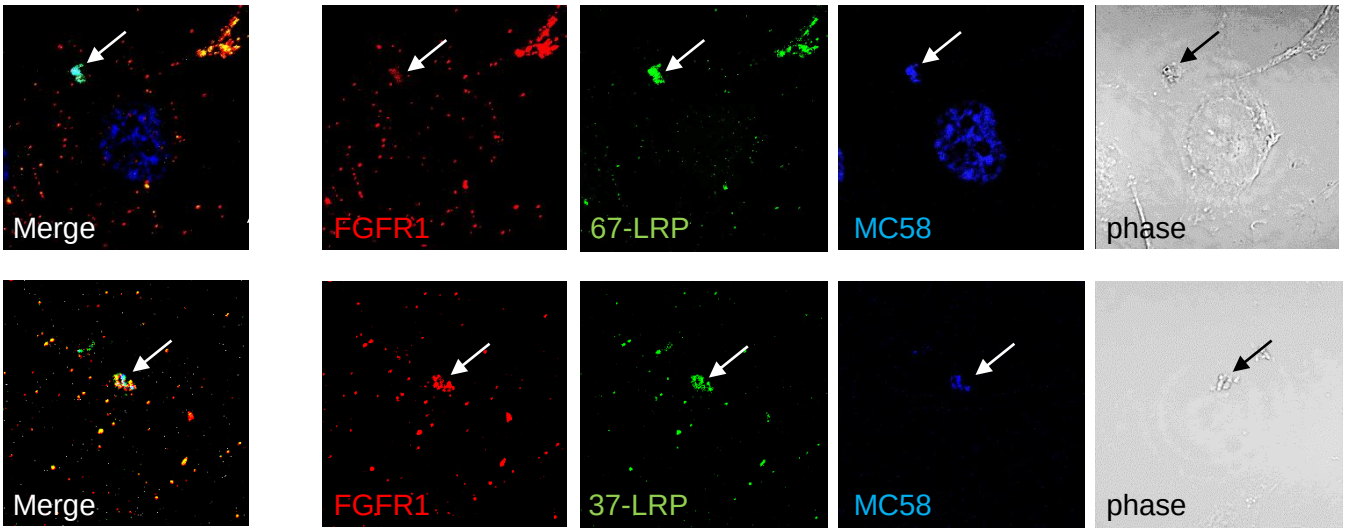
A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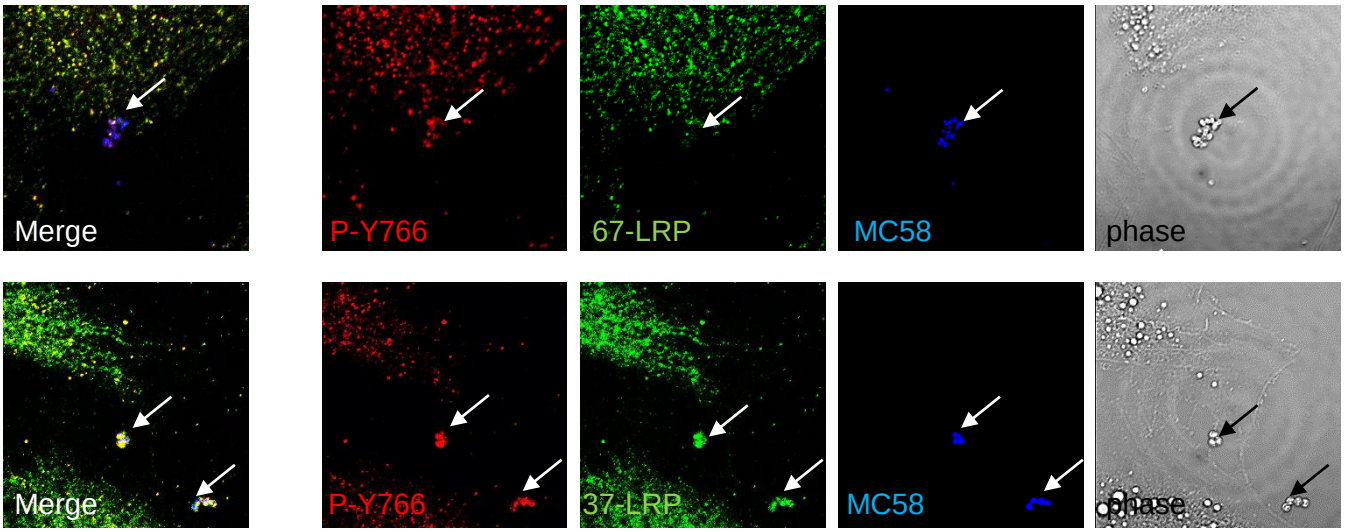
67-LRP
MC58
phase
Merge
FGFR1
37-LRP
MC58
phase

B


Merge
P-Y766
67-LRP
MC58
phase
Merge
P-Y766
37-LRP
MC58
phase

C

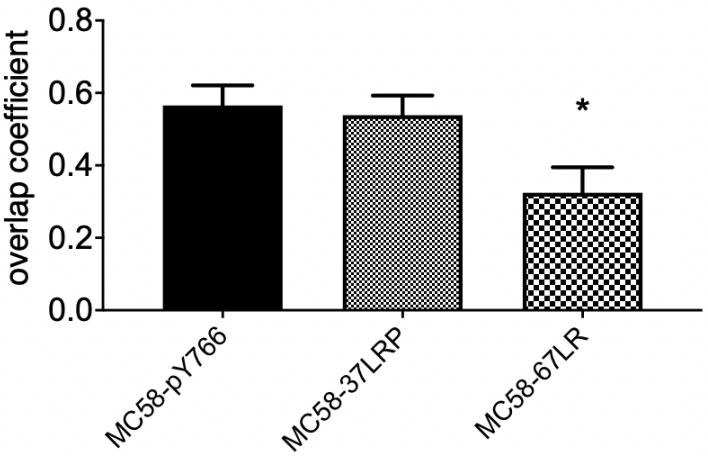
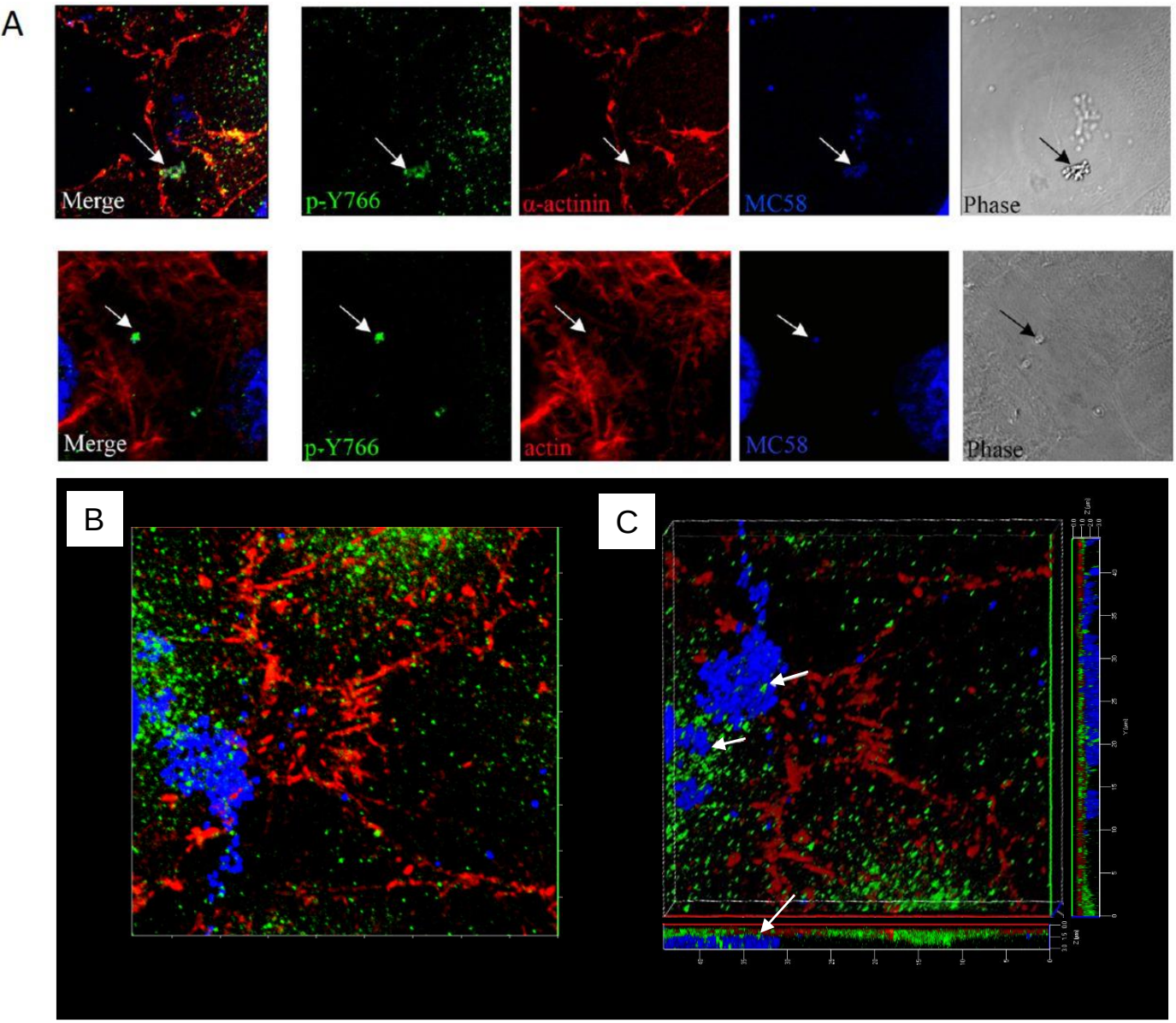

overlap coefficient
0.8
0.6
0.4
0.2
0.0
*
MC58-pY766
MC58-37LRP
MC58-67LR

Figure 1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Merge
p-Y766
α-actinin
MC58
Phase
Merge
p-Y766
actin
MC58
Phase
B
C

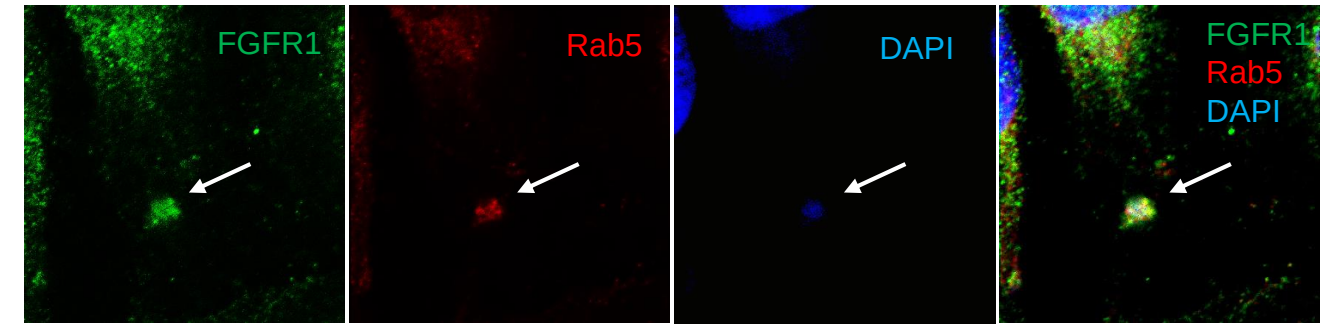
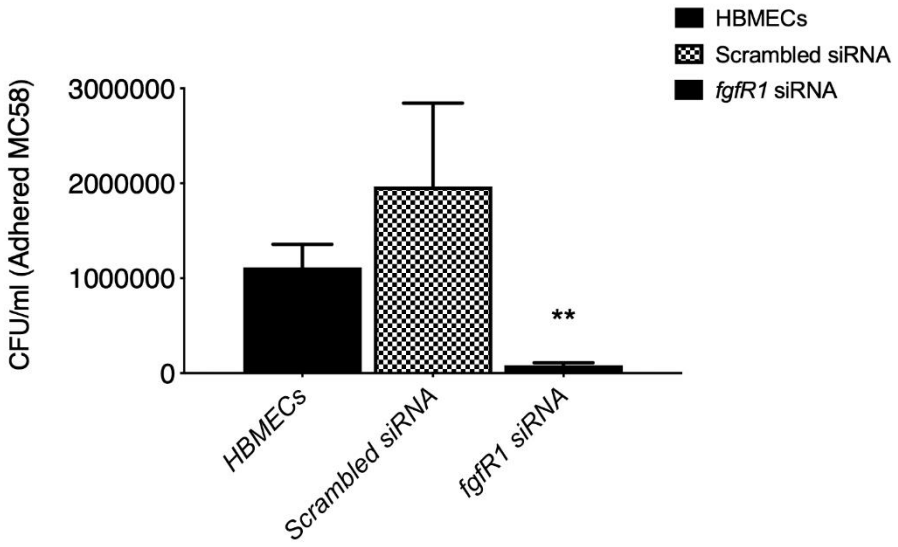
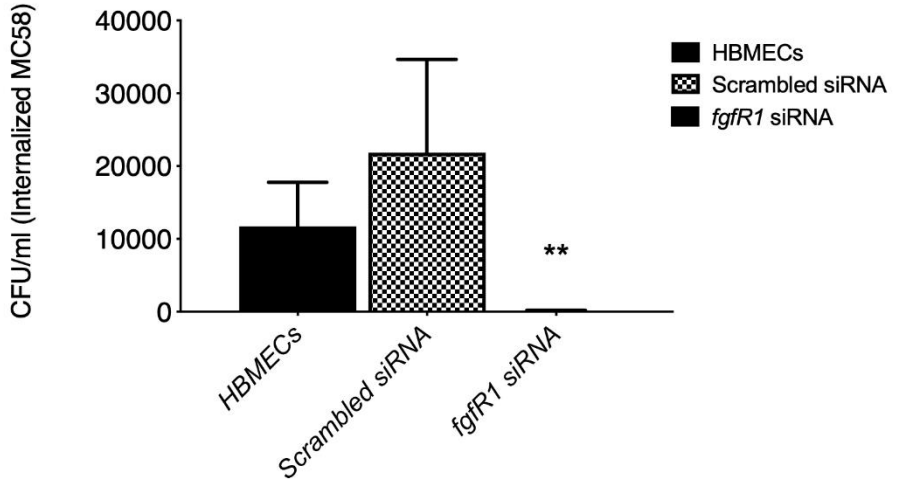

D
FGFR1
Rab5
DAPI
FGFR1
Rab5
DAPI

Figure 2

A


HBMECs
Scrambled siRNA
fgfR1 siRNA
CFU/ml (Adhered MC58)
3000000
2000000
1000000
0
**
HBMECs
Scrambled siRNA
fgfR1 siRNA

B


HBMECs
Scrambled siRNA
fgfR1 siRNA
CFU/ml (Internalized MC58)
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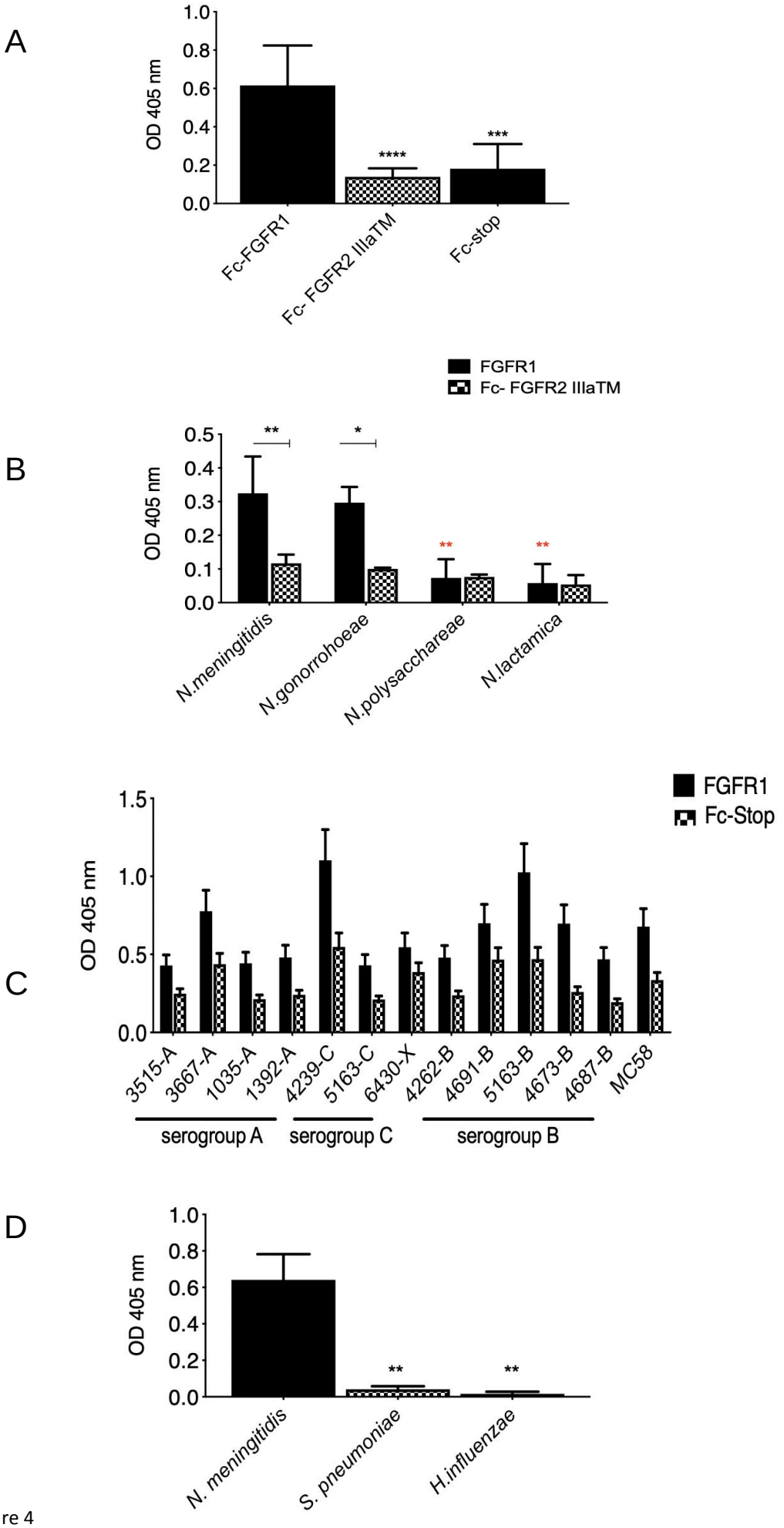
30000
20000
10000
0
**
HBMECs
Scrambled siRNA
fgfR1 siRNA

Figure 3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OD 405 nm
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0.8
0.6
0.4
0.2
0.0
****
***
Fc-FGFR1
Fc- FGFR2 IIIaTM
Fc-stop
FGFR1
Fc- FGFR2 IIIaTM
B
OD 405 nm
0.5
0.4
0.3
0.2
0.1
0.0
**
*
**
**
N.meningitidis
N.gonorrohoeae
N.polysacchareae
N.lactamica
C
FGFR1
Fc-Stop
OD 405 nm
1.5
1.0
0.5
0.0
3515-A
3667-A
1035-A
1392-A
4239-C
5163-C
6430-X
4262-B
4691-B
5163-B
4673-B
4687-B
MC58
serogroup A
serogroup C
serogroup B
D
OD 405 nm
1.0
0.8
0.6
0.4
0.2
0.0
**
**
N. meningitidis
S. pneumoniae
H.influenzae

Figure 4

Additional images for review

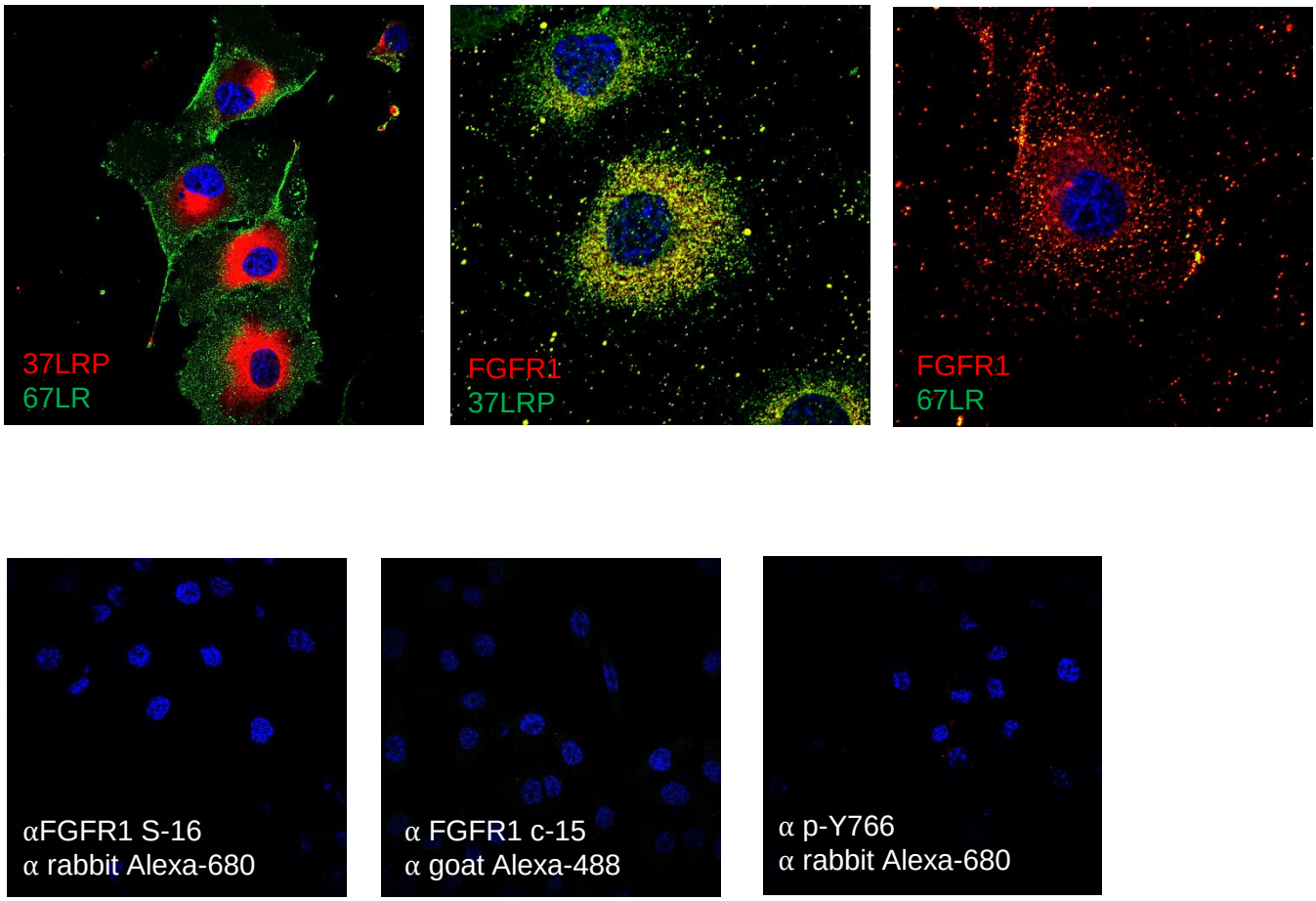
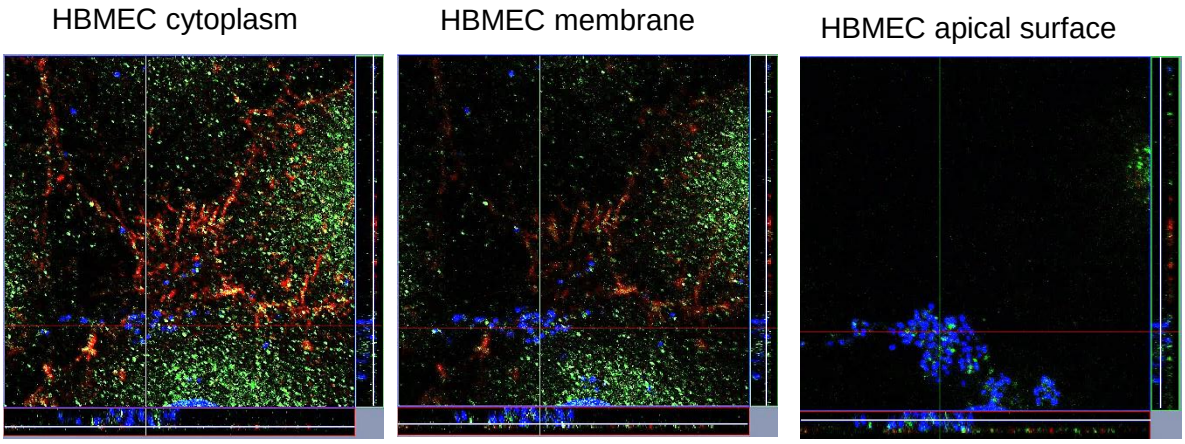


Figure R1. Images of uninfected HBMECs, and the localization patterns of 37LRP and 67LR in regards to FGFR1. We found no cross reactivity of secondary antibodies against 37LRP and 67LR with FGFR1 primary antibodies (S-16 or C-15).



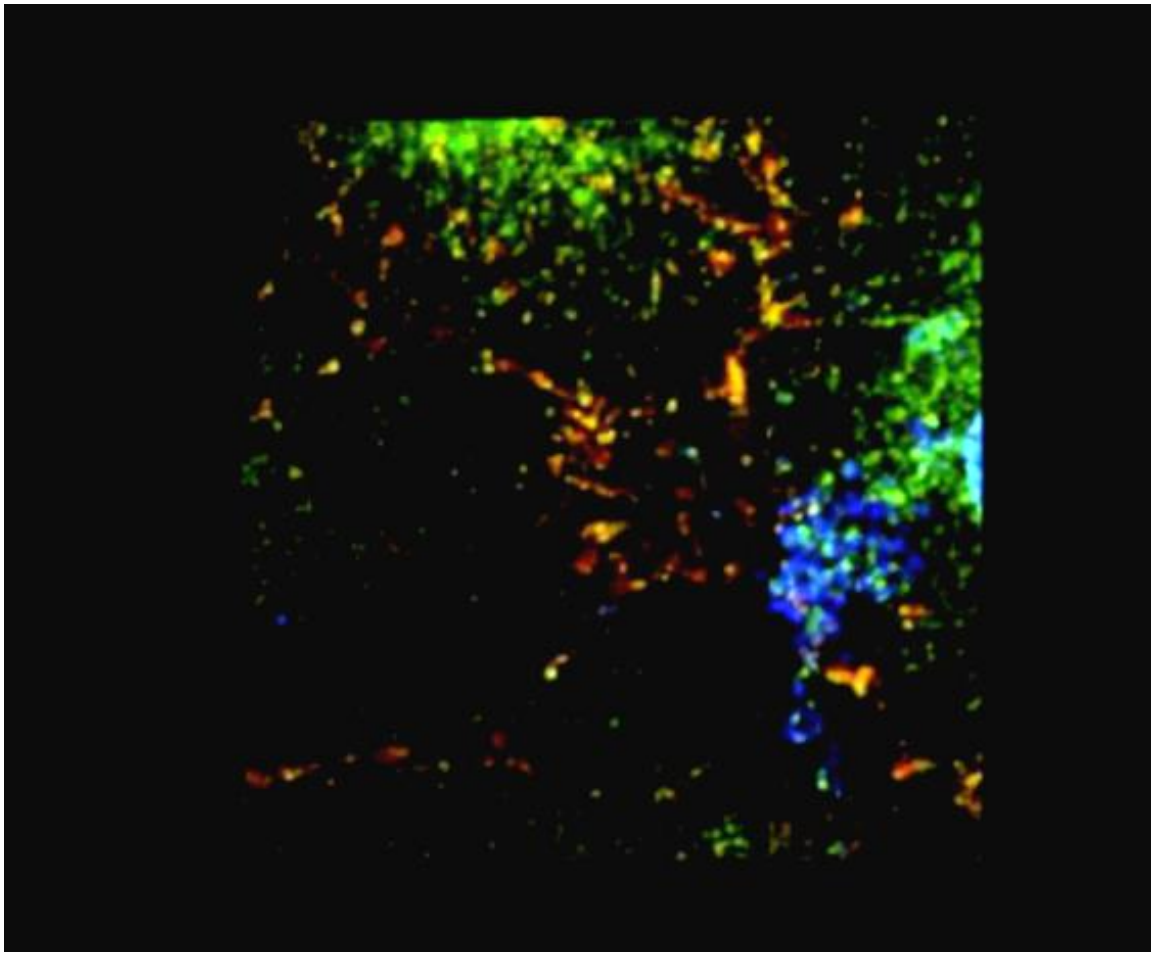


Figure R2. MC58 cells recruit activated FGFR1 (p-Y766 in green) on apical surface of the cells and internalised bacterial cell are co-localized with α-actinin in cytoplasm.

Competing interests
The authors declare that they have no competing interests.

Author Statement

Sheyda Azimi undertook the majority of the practical work under the day-to-day guidance of Lee Wheldon. The programme of work was directed by Karl Wooldridge, Neil Oldfield and Lee Wheldon. The paper was written by Sheyda Azimi, Lee Wheldon and Karl Wooldridge. All authors regularly reviewed the work and commented on draft manuscripts.