

RESEARCH ARTICLE

Characterisation of the Immunomodulatory Effects of Meningococcal Opa Proteins on Human Peripheral Blood Mononuclear Cells and CD4⁺ T Cells

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OPEN ACCESS

Citation: Jones C, Sadarangani M, Lewis S, Payne I, Saleem M, Derrick JP, et al. (2016) Characterisation of the Immunomodulatory Effects of Meningococcal Opa Proteins on Human Peripheral Blood Mononuclear Cells and CD4⁺ T Cells. PLoS ONE 11 (4): e0154153. doi:10.1371/journal.pone.0154153

Editor: Daniela Flavia Hozbor, Universidad Nacional de la Plata, ARGENTINA

Received: January 25, 2016

Accepted: April 9, 2016

Published: April 25, 2016

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Data Availability Statement: All relevant data are within the paper.

Funding: This study was funded by Action Medical Research through a Research Training Fellowship awarded to MSad (RTF1263) and Meningitis UK through a project grant awarded to AJP and supported by the Oxford Partnership Comprehensive Biomedical Research Centre with funding from the Department of Health's National Institute of Health Research Biomedical Research Centres funding scheme. The funders had no role in study design,

Abstract

Opa proteins are major surface-expressed proteins located in the *Neisseria meningitidis* outer membrane, and are potential meningococcal vaccine candidates. Although Opa proteins elicit high levels of bactericidal antibodies following immunisation in mice, progress towards human clinical trials has been delayed due to previous findings that Opa inhibits T cell proliferation in some *in vitro* assays. However, results from previous studies are conflicting, with different Opa preparations and culture conditions being used. We investigated the effects of various Opa⁺ and Opa⁻ antigens from *N. meningitidis* strain H44/76 in a range of *in vitro* conditions using peripheral blood mononuclear cells (PBMCs) and purified CD4⁺ T cells, measuring T cell proliferation by CFSE dilution using flow cytometry. Wild type recombinant and liposomal Opa proteins inhibited CD4⁺ T cell proliferation after stimulation with IL-2, anti-CD3 and anti-CD28, and these effects were reduced by mutation of the CEA-CAM1-binding region of Opa. These effects were not observed in culture with *ex vivo* PBMCs. Opa⁺ and Opa⁻ OMVs did not consistently exert a stimulatory or inhibitory effect across different culture conditions. These data do not support a hypothesis that Opa proteins would be inhibitory to T cells if given as a vaccine component, and T cell immune responses to OMV vaccines are unlikely to be significantly affected by the presence of Opa proteins.

Introduction

Neisseria meningitidis causes approximately 500,000 cases of meningitis and septicaemia worldwide annually, with a case-fatality rate of approximately 10% [1]. Most disease is caused by capsular group A, B, C, W, X and Y organisms. Protein-polysaccharide conjugate vaccines

data collection and interpretation, or the decision to submit the work for publication.

Competing Interests: AJP is a Jenner Institute Investigator and James Martin Senior Fellow. AJP was previously named as an inventor on a patent for the use of Opa proteins in meningococcal vaccines ('Compositions comprising Opa protein epitopes'; numbers WO2006067469A3, EP1830878A2, US20100183676, WO2006067469A2). AJP has previously conducted clinical trials of meningococcal vaccines on behalf of the University of Oxford, but he receives no personal payments from them. AJP is chair of the UK Department of Health's Joint Committee on Vaccines and Immunisation and chair of the European Medicine Agency's Scientific Advisory Group on Vaccines. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

are in routine use globally for capsular groups A, C, W and Y, and group B is the major cause of disease in most temperate countries [2–6]. The Opacity-associated (Opa) adhesin proteins are major phase-variable proteins found in the outer membrane of *N. meningitidis*. Each bacterium contains four *opa* genes (*opaA*, *opaB*, *opaD* and *opaJ*), which may encode identical or different Opa proteins, and can therefore express up to four different Opa variants at any one time [7–9]. Opa proteins are critical in meningococcal pathogenesis, mediating bacterial adherence to the nasopharynx and modulating human cellular immunity via interactions with T cells and neutrophils [10, 11]. They are also important during infection with the related species *N. gonorrhoeae*, mediating persistence of bacteria in the genitourinary tract via the avoidance of an effective immune response [12].

N. meningitidis can persist in the human nasopharynx without causing symptoms for several months, and *N. gonorrhoeae* can cause prolonged mucosal infection of the genito-urinary tract. This ability to persist relies on their adaptability to the host and their capacity to evade the immune system. Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are cell surface glycoproteins found on a range of cell types. Binding of these proteins by various ligands can result in up- or down-regulation of intracellular signalling pathways [12]. Opa protein binding to CEACAMs on the surface of host cells confers the ability to associate with human epithelial, endothelial and leucocytic cells encountered during neisserial infection, indicating a direct effect on the immune response [13]. Although Opa proteins are able to bind to a number of different CEACAMs, CEACAM1 has a broad expression distribution in normal tissues and is the only member of the family present on the surface of T cells. The response of T cells, and particularly CD4⁺ T cells, is important during infection with pathogenic *Neisseria* as these cells are involved in directing the magnitude and quality of humoral immune response. Antibodies directed against surface structures of *N. meningitidis* are important in immunity but gonococci do not induce a strong, protective antibody response following infection [14]. T cells are also important in the generation of immunological memory and possibly cell-mediated immunity, which is therefore relevant to vaccine development [15]. The interaction between meningococci and human T cells and the particular role of Opa proteins in this interaction has therefore been the subject of intense, and conflicting, study in the last decades [16–24]. Furthermore, Opa proteins have been suggested as potential meningococcal vaccine candidates as they elicit high levels of bactericidal antibodies in mice [13]. However, sequence variability of some of the surface-exposed loops and uncertainty regarding their immunomodulatory effect on human T cells has delayed further development into clinical trials.

In this study we investigated the effects of recombinant and liposomal Opa proteins, in addition to Opa⁺ and Opa⁻ outer membrane vesicles (OMVs) and bacteria based on isogenic strains, on the immunomodulatory interaction between *N. meningitidis* and human peripheral blood mononuclear cells (PBMCs) and CD4⁺ T cells. In an attempt to clarify the effects of Opa proteins on CD4⁺ T cells, a number of assays were undertaken using different cell culture conditions, and a variety of Opa⁺ and Opa⁻ antigens.

Materials and Methods

Study subjects

Written informed consent was obtained from 46 healthy adult volunteers recruited to the study (aged 18 to 66 years) prior to collection of a single blood sample. Anyone with a history of previous IMD, a known immunodeficiency, or who was enrolled in another study which may affect their immune responses was excluded. The study was approved by the Oxfordshire C Research Ethics Committee (REC No: 07/H0606/84; UKCRN ID 4609).

Isolation of peripheral blood mononuclear cells and purification of CD4⁺ T cells

A maximum of 40 ml of blood was collected from each study participant, and heparinised blood (1000 units/ml heparin) was diluted in an equal volume of culture medium buffer (RPMI-1640 medium, HEPES modification, 25 mM HEPES, 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine [Sigma-Aldrich, Gillingham, UK]). PBMCs were isolated by density gradient centrifugation (Lymphoprep, Axis-Shield, Dundee, UK). Cells were subsequently either labelled with carboxyfluorescein succinimidyl ester (CFSE) prior to culture and stimulation with antigen, or directly processed for purification of CD4⁺ T cells. CD4⁺ T cells were purified from PBMCs by negative selection according to manufacturer's instructions (CD4⁺ T cell isolation kit II, Miltenyi Biotec, Woking, UK). Following separation, cells were labelled with CFSE prior to culture and stimulation with antigen.

Cell labelling with carboxyfluorescein succinimidyl ester (CFSE)

To assess proliferation by flow cytometry, fluorescent intracellular labelling of PBMCs and purified CD4⁺ T cells with CFSE was performed prior to culture and stimulation with antigen [25]. In brief, the cells were resuspended in CFSE buffer (PBS containing 2mM EDTA and 0.5% newborn calf serum [NBCS]), and diluted to a concentration of 1×10^6 cells/ml in CFSE buffer containing 2 µM CFSE. Cells were incubated at 37°C with 5% CO₂ for 10 minutes and washed twice in culture complete medium (containing 10% NBCS). Cell suspensions (2×10^6 cells/ml for PBMCs and 1×10^6 cells/ml for purified CD4⁺ T cells) were prepared with or without IL-2 (final concentration of 1,000 units/ml) (Roche, Welwyn Garden City, UK).

Cell culture and T cell stimulation

Cells were cultured in flat-bottomed 96-well tissue culture plates (Thermo Scientific Nunc, Loughborough, UK) at a cell density of 2×10^5 or 1×10^5 cells per well for PBMC and CD4⁺ T cells, respectively, in complete culture medium (100 µl per well). To induce the expression of CEACAM1, cells were pre-incubated with IL-2 (1,000 units/ml) for 48 hours at 37°C with 5% CO₂ prior to antigen addition. For *ex vivo* studies, antigens were diluted prior to addition to the culture plate with or without T cell stimulation using purified anti-human CD3 (UCHT1) and anti-human CD28 (CD28.2) antibodies for a further 96 hours (Table 1). Per participant, each antigen and assay controls were tested in triplicate.

In order to comprehensively investigate the effects of Opa proteins on CD4⁺ T cell proliferation, different Opa⁺ or Opa⁻ antigens were tested using a combination of culture conditions. The following culture conditions were tested: (a) *ex vivo* culture of PBMCs with antigen for 5 days; (b) PBMCs cultured for 4 days with antigen and IL-2 (following 48 hours pre-incubation with IL-2); (c) PBMCs cultured for 4 days with antigen, IL-2, anti-CD3 and anti-CD28 (following 48 hours pre-incubation with IL-2); (d) purified CD4⁺ T cells cultured for 4 days with antigen, IL-2, anti-CD3 and anti-CD28, (following 48 hours pre-incubation with IL-2). Priority was given to assays involving *ex vivo* PBMCs, as the convention used to assess T cell stimulation and of most interest to vaccine development. Co-ligation culture conditions including anti-CD3 and anti-CD28 were performed as a priority over those with IL-2 alone when the number of cells recovered from blood was insufficient for all assays.

Antigens for T cell stimulation

An extensive antigen panel consisting of purified recombinant Opa proteins, liposomes, OMVs and killed bacteria from isogenic strains constructed from parent strain H44/76 were studied

Table 1. Antigens used for T cell proliferation assays.

Antigen	Stock concentration	Final concentration in assay
PHA (Sigma-Aldrich)	5 mg/ml	5 µg/ml
Anti-human CD3 + anti-human CD28 antibodies (BioLegend)	1 mg/ml [‡]	1 µg/ml [‡]
Isotype control antibody (DAKO)	15 g/ml	25 µg/ml
Polyclonal anti-CEACAM antibody (DAKO)	2 mg/ml	25 µg/ml
OMVs	Variable	50 µg/ml
Inactivated bacteria [§]	Variable	ratio of 500 bacteria: 1 cell
Recombinant proteins	Variable	1 µg/ml
Liposome proteins	Variable	1 µg/ml

All antigens were diluted in to the required concentration in a buffer containing RPMI-1640 medium, HEPES modification, 25 mM HEPES, 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine supplemented with 10% heat-inactivated newborn calf serum [Sigma-Aldrich, Gillingham, UK].

PHA = phytohaemagglutinin; SEB = Staphylococcal enterotoxin B; OMVs = outer membrane vesicles;

[‡]concentration per antibody;

[§]Bacteria were washed 3 times in complete medium prior to use;

[¶]1 x 10⁸ CFU per well for assays with PBMCs and 5 x 10⁷ CFU per well for assays with purified CD4⁺ T cells.

doi:10.1371/journal.pone.0154153.t001

(Table 1). The isogenic strains used in this study included the wild-type parent strain H44/76, an Opa- strain (M014), a strain expressing OpaD only (M002), and a strain expressing both OpaA and OpaD (M001) [26]. *opa* genes associated with hyperinvasive serogroup B meningococci were cloned, expressed in *Escherichia coli* BL21 (DE3), purified from inclusion bodies using affinity chromatography [13] and incorporated into liposomes [27]. Non-CEACAM binding mutated forms of OpaA and OpaD were constructed by site directed mutagenesis, replacing Gly-172, Ile-174 and Gln-176 with alanine, as previously described [28]. Phytohaemagglutinin (PHA) [29, 30] and Staphylococcal enterotoxin B (SEB) were used as positive controls for the assays. A rabbit polyclonal anti-CEACAM antibody (DAKO, Ely, UK) was used as an inhibitory control for T cell responses while an isotype matched antibody control was used as the comparator [31].

Analysis of cells using flow cytometry

Generations of proliferating CD4⁺ T cells were assessed by flow cytometry [25]. CD4⁺ T cells were positively identified by staining with a mouse anti-human CD4-APC conjugate (BD Pharmingen, Oxford UK) and dead cells were excluded from the analysis using 7-aminoactinomycin D (7-AAD) (Sigma-Aldrich, UK). Flow cytometry was performed using the FACSCalibur (Becton Dickinson, UK). Data from 10,000 CD4^{high} 7-AAD^{low} events were collected for each sample. Data were collected and stored using CellQuestPro for Macintosh (Becton Dickinson). Assay controls for each participant included unstained (minimum auto-fluorescence) and CFSE-stained unstimulated controls (media only control). For analysis, a histogram plot was created and a marker was used to identify the fluorescence peak of the media only control (maximum fluorescence of the undivided cells). The average number of proliferating events in the final culture population for triplicate measurements was determined. Cell proliferation is defined as a percentage of the total number of CD4⁺ T cells analysed.

Bactericidal antibody responses following immunisation of mice

Groups of ten 6–7 week old female BALB/c mice (Charles River, Margate, UK) were immunised subcutaneously with 0.2ml of recombinant Opa protein on days 0, 21 and 35 (5 µg per

dose). The adjuvant used was the oil-in-water emulsion Sigma Adjuvant System (Sigma-Aldrich), which was prepared according to the manufacturer's instructions but in a total volume of 2.4ml of antigen solution per vial, and was reconstituted with the antigen on the day of immunisation. Blood was collected by cardiac puncture on day 42 and serum separated by centrifugation at 16,100 x *g* for 10 minutes. Bactericidal activity in mouse serum pooled within each immunisation group was quantified by SBA [32]. Briefly, pooled murine sera was heated at 56°C for 30 minutes to deactivate endogenous complement and then diluted to give a range from 1:4 to 1:1024. Diluted sera were incubated with mid-log phase meningococci (125 cfu) and baby rabbit complement (lot number 11330, PelFreez Biologicals, Rogers, AR) at a final concentration of 25% (v/v). Reactions were incubated for 60 minutes at 37°C in a humidified 5% CO₂ atmosphere. A sample of each reaction was spread by tilting onto BHI agar plates, which were incubated overnight. The bactericidal antibody titre was reported as the reciprocal of the highest serum dilution at which 50% bacterial survival was observed. Each serum sample was analysed in duplicate against each target strain.

Statistical analysis

CD4⁺ T cell proliferation was calculated for each antigen as the mean of the triplicate wells, by subtracting the mean proliferation of control wells containing media only. Opa⁺ antigens were compared to Opa⁻ antigens in the same way. For wild-type and mutant forms of recombinant or liposomal Opa proteins, the mutated forms were considered Opa⁻ in these analyses. Statistical significance was calculated using a paired two-tailed Student's t-test to compare cells from the same donor, with a *p*-value of <0.01 considered as statistically significant. Analysis was carried out using R (version 3.1.2).

Results

PBMCs ex vivo CD4⁺ T cell proliferation

Proliferative responses to all antigens were low in these conditions, with a maximum amplitude of mean stimulation/inhibition of 1.8%, with the exception of PHA (Table 2, Fig 1). There was statistically significant inhibition of T cell proliferation by the liposomal wild-type OpaD, although the magnitude of this inhibition was low (-1.6%), compared to the stimulatory effect seen with the PHA control (+61.3%). Otherwise there was no significant stimulation or inhibition by any of the Opa⁺ or Opa⁻ antigens. When Opa⁺ antigens were compared to their Opa-counterparts there were no significant differences, with the exception that Opa⁺ bacteria were relatively stimulatory. The magnitudes of these differences were small, +0.5% and +1.3% for OpaA⁺ OpaD⁺ and OpaD⁺ strains respectively. Although culture of PBMCs *ex vivo* without IL-2 and/or stimulation of the T cell receptor (e.g. with PHA or anti-CD3) is a standard method for assessing antigen-specific responses following a vaccination, proliferative responses measured *in vitro* in the absence of such a recent stimulus can be more difficult to detect [33]. Further experiments were therefore performed with additional IL-2 to amplify proliferative responses [34].

PBMCs with IL-2 only

A limited number of samples were tested with IL-2 only (up to 5 participants per antigen condition due to limited blood volumes available), to amplify responses by increasing activation and proliferation of T cells. In these conditions, all recombinant proteins caused inhibition of CD4⁺ T cell proliferation compared to media alone (between -16.3% and -6.5%), in addition to the liposomal wild-type OpaD (-18.0%) (Table 3, Fig 2). Wild-type recombinant Opa proteins

Table 2. CD4⁺ T cell proliferation following incubation of PBMCs ex vivo with different antigens.

Antigen	Mean % CD4 ⁺ T-cell proliferation				
	n	vs. media (95% CI)	p-value	vs. Opa- (95% CI)	p-value
Control antigens					
PHA	33	+61.3 (50.8, 71.8)	<0.0001	-	-
Isotype antibody	33	-0.8 (-2.4, 0.8)	0.3281	-	-
Anti-CEACAM antibody	33	-1.2 (-2.6, 0.2)	0.0979	-	-
SEB	12	+0.1 (-5.4, 5.5)	0.9776	-	-
Recombinant proteins					
Protein buffer (PBS/LDAO)	33	-1.4 (-3.1, 0.3)	0.1037	-	-
rOpaA wt	33	-1.7 (-3.4, 0.1)	0.0638	0.0 (-3.3, 3.2)	0.9804
rOpaD wt	33	-1.3 (-2.8, 0.2)	0.0892	0.0 (-3.4, 3.3)	0.9869
rOpaA mutant	33	-1.6 (-4.9, 1.7)	0.3252	REF	-
rOpaD mutant	33	-1.3 (-4.6, 2.1)	0.4501	REF	-
Liposomal proteins					
Empty liposome	33	-0.1 (-1.7, 1.5)	0.8741	-	-
lOpaA wt	33	-1.1 (-2.8, 0.5)	0.1719	+0.1 (-0.6, 0.7)	0.8583
lOpaD wt	33	-1.6 (-3.1, 0)	0.0468	+0.3 (-0.4, 0.9)	0.3885
lOpaA mutant	33	-1.2 (-2.8, 0.5)	0.153	REF	-
lOpaD mutant	33	-1.8 (-3.7, 0)	0.0543	REF	-
Outer membrane vesicles (OMVs)					
OMV buffer (3% sucrose)	33	-0.9 (-2.1, 0.4)	0.1876	-	-
wt OMV	33	-0.7 (-2.1, 0.7)	0.3308	+0.1 (-0.4, 0.6)	0.6926
Opa- OMV	33	-0.8 (-2.1, 0.5)	0.2213	REF	-
OpaA+ OpaD+ OMV	33	-0.8 (-2.7, 1.2)	0.4234	0.0 (-0.7, 0.8)	0.9393
OpaD+ OMV	33	-1 (-2.7, 0.7)	0.2556	-0.2 (-0.7, 0.4)	0.5561
Inactivated bacteria					
wt H44/76	33	-1.4 (-3.6, 0.9)	0.223	-0.5 (-1.5, 0.6)	0.3757
Opa-	33	-0.9 (-2.9, 1.1)	0.3695	REF	-
OpaA+ OpaD+	33	-0.4 (-2.2, 1.5)	0.6964	+0.5 (0.1, 1.0)	0.0104
OpaD+	30	+0.3 (-1.9, 2.5)	0.7754	+1.3 (0.5, 2.0)	0.0014

Values in bold represent antigens where the % CD4⁺ T cell proliferation was statistically significant (p < 0.01) compared to media alone or compared to the relevant Opa- antigen (REF). CI = confidence interval; PHA = phytohaemagglutinin; CEACAM = carcinoembryonic antigen-related cell adhesion molecule; SEB = Staphylococcal enterotoxin B; PBS = phosphate-buffered saline; LDAO = lauryldimethylamine-oxide; wt = wild-type.

doi:10.1371/journal.pone.0154153.t002

were significantly more inhibitory than the mutated equivalents, with a difference of approximately 9% for OpaA and OpaD. Wild-type liposomal OpaD was also more inhibitory than the mutated form (difference 14.9%). None of the OMVs or inactivated bacteria were significantly stimulatory or inhibitory compared to the media only control, but Opa+ OMVs were slightly more stimulatory than Opa- OMVs. In contrast OpaA+ OpaD+ bacteria were less stimulatory than Opa- bacteria, although this difference was not observed with OpaD+ bacteria. All differences between Opa+ and Opa- OMVs and bacteria were relatively small in magnitude (between 3.0% and 5.6%).

PBMCs and purified CD4⁺ T cells with IL-2, anti-CD3 and anti-CD28

In these culture conditions, CD4⁺ cells are highly stimulated non-specifically, so inhibitory effects would expect to be seen. When purified CD4⁺ T cells were used, there was significant

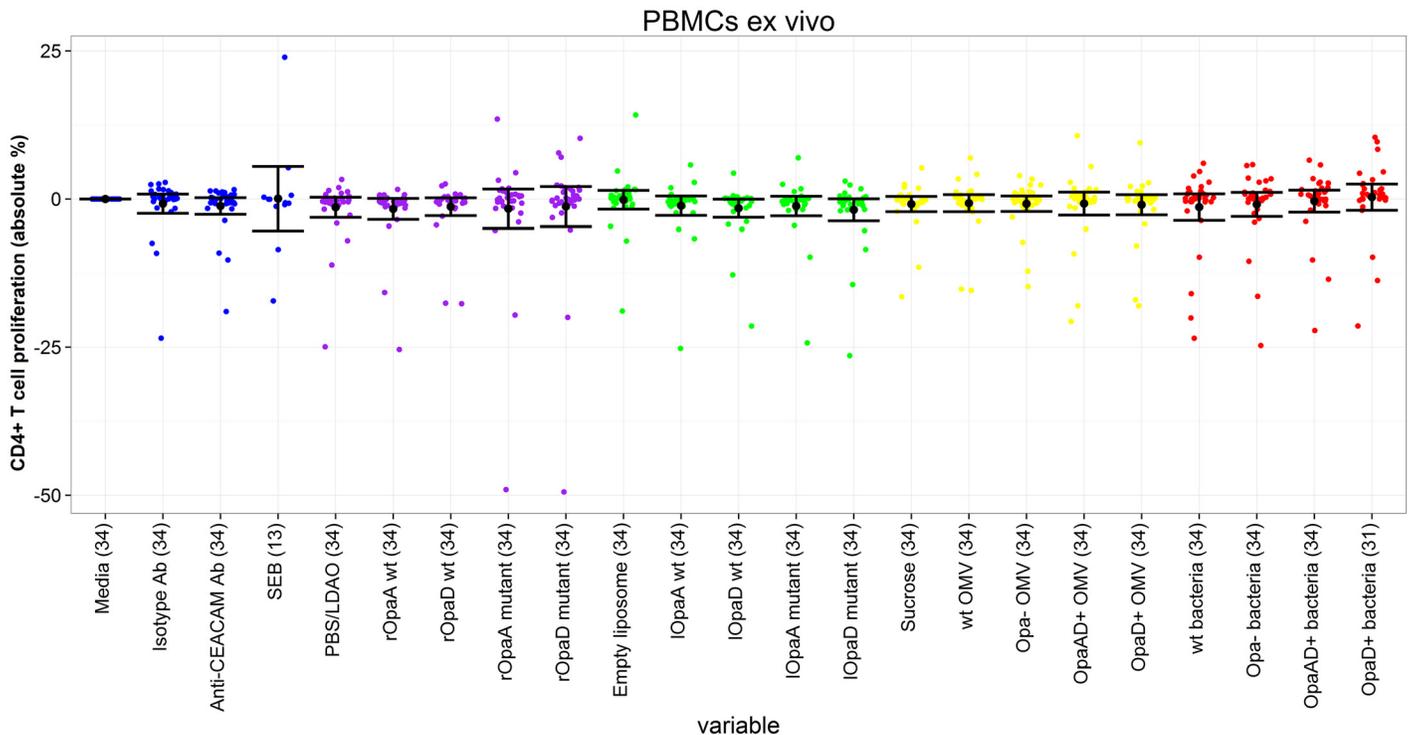


Fig 1. CD4⁺ T cell proliferation following incubation of PBMCs ex vivo with different antigens. Proliferation of each antigen relative to media only control. Each coloured dot represents the value for a single individual (control antigens—blue; recombinant proteins—purple, liposomal proteins—green, outer membrane vesicles—yellow, inactivated bacteria—red). Black dots represent mean value for each antigen, with 95% confidence intervals shown. Number in parentheses after each antigen represents number on individuals in that group. CEACAM = carcinoembryonic antigen-related cell adhesion molecule; SEB = Staphylococcal enterotoxin B; PBS = phosphate-buffered saline; LDAO = lauryldimethylamine-oxide; wt = wild-type. PHA (phytohaemagglutinin) result not shown for clarity, to confine y-axis to relevant range for other antigens.

doi:10.1371/journal.pone.0154153.g001

inhibition of CD4⁺ proliferation by all recombinant proteins and liposomes, with the exception of mutated rOpaA (although the trend was also towards inhibition in this case). Inhibition of proliferation ranged between -9.6% (rOpaD mutant) and -46.1% (IOpaD wild-type) (Tables 4 and 5, Figs 3 and 4). The Opa⁺ recombinant and liposomal proteins were significantly more inhibitory than the Opa⁻ forms (with the exception of liposomal OpaA which also showed the same trend), with differences of between 5.6% and 30.1%. In addition, there was inhibition of CD4⁺ T cell proliferation by all OMVs (reaching statistical significance for all except wild-type), but Opa⁺ OMVs were less inhibitory than Opa⁻ OMVs. All of the bacterial preparations tested were able to provide additional stimulation to CD4⁺ T cells in these conditions, with no significant differences between Opa⁺ and Opa⁻ bacteria.

When mixed PBMCs were used in the same highly stimulated culture conditions, results for the recombinant and liposomal proteins were similar to those found using purified CD4⁺ T cells, with inhibition observed for all preparations, although fewer achieved statistical significance. On comparison of the mutated and wild-type proteins there was only a significant difference for liposomal OpaD, with the wild-type protein again being more inhibitory than the mutated protein (difference 35.6%). All of the bacterial strains tested were able to provide additional stimulation to CD4⁺ T cells, as observed with purified CD4⁺ T cells, and wild-type and OpaD⁺ bacteria were slightly less stimulatory than Opa⁻ bacteria. Most OMVs in these conditions were not significantly stimulatory or inhibitory, although a slight stimulatory effect was observed with Opa⁻ OMVs.

Table 3. CD4⁺ T cell proliferation following incubation of PBMCs with different antigens, plus IL-2.

Antigen	Mean % CD4 ⁺ T-cell proliferation				
	n	vs. media (95% CI)	p-value	vs. Opa- (95% CI)	p-value
Control antigens					
PHA	5	+53.5 (33.8, 73.3)	0.0009	-	-
Isotype antibody	5	+4.1 (-2.6, 10.9)	0.1737	-	-
Anti-CEACAM antibody	5	-12.9 (-23.5, -2.3)	0.0263	-	-
Recombinant proteins					
Protein buffer (PBS/LDAO)	4	-2.5 (-7.6, 2.6)	0.2469	-	-
rOpaA wt	4	-15.4 (-23.1, -7.7)	0.0051	-8.9 (-16.0, -1.8)	0.0258
rOpaD wt	4	-16.3 (-24.0, -8.5)	0.0043	-9.6 (-16.6, -2.6)	0.0187
rOpaA mutant	4	-6.5 (-11.2, -1.9)	0.0176	REF	-
rOpaD mutant	4	-6.7 (-12.1, -1.2)	0.0282	REF	-
Liposomal proteins					
Empty liposome	4	-0.3 (-8.4, 7.8)	0.9250	-	-
lOpaA wt	4	-1.9 (-11.1, 7.2)	0.5872	-1.2 (-3.1, 0.7)	0.1641
lOpaD wt	4	-18.0 (-25.7, -10.3)	0.0029	-14.9 (-24.6, -5.2)	0.0129
lOpaA mutant	4	-0.8 (-10.3, 8.7)	0.8303	REF	-
lOpaD mutant	4	-3.1 (-11.8, 5.7)	0.3845	REF	-
Outer membrane vesicles (OMVs)					
OMV buffer (3% sucrose)	5	-1.3 (-8.0, 5.4)	0.6373	-	-
wt OMV	5	-0.8 (-7.0, 5.4)	0.7571	+1.9 (-0.7, 4.5)	0.1179
Opa- OMV	5	-2.7 (-10.4, 5.0)	0.4073	REF	-
OpaA+ OpaD+ OMV	5	+2.9 (-5.4, 11.2)	0.4150	+5.6 (2.9, 8.3)	0.0030
OpaD+ OMV	5	+1.0 (-7.4, 9.4)	0.7751	+3.7 (1.8, 5.6)	0.0040
Inactivated bacteria					
wt H44/76	4	+2.0 (-9.9, 13.9)	0.6700	-2.3 (-7.5, 2.8)	0.2783
Opa-	4	+4.3 (-8.9, 17.5)	0.4178	REF	-
OpaA+ OpaD+	4	+1.3 (-11.5, 14.2)	0.7869	-3.0 (-5.6, -0.3)	0.0351
OpaD+	1	+9.1 (-23.7, 41.8)	0.1765	-5.1 (-51.4, 41.2)	0.3949

Values in bold represent antigens where the % CD4⁺ T cell proliferation was statistically significant (p < 0.01) compared to media alone or compared to the relevant Opa- antigen (REF). CI = confidence interval; PHA = phytohaemagglutinin; CEACAM = carcinoembryonic antigen-related cell adhesion molecule; PBS = phosphate-buffered saline; LDAO = lauryldimethylamine-oxide; wt = wild-type.

doi:10.1371/journal.pone.0154153.t003

A comparison of results from this study and previous studies of Opa-mediated effects on CD4⁺ T cells is summarised in [Table 6](#).

Bactericidal antibody responses following immunisation of mice with recombinant Opa proteins

The immunogenicity of recombinant forms of wild-type and mutated OpaA and OpaD from H44/76 were tested using Opa+ and Opa- strains as target strains in the serum bactericidal antibody (SBA) assay. Data using wild type proteins has been reported previously, and are included here for comparison to the mutated forms [26]. The recombinant proteins resulted in titres of 1:256 when the target strain expressed the same Opa variant used for immunisation ([Table 7](#)), and <1:4 otherwise. Introduction of mutations in the hypervariable HV2 regions led to complete abrogation of SBA activity for OpaA, but had no effect on the SBA titre for OpaD against either OpaD+ target strain.

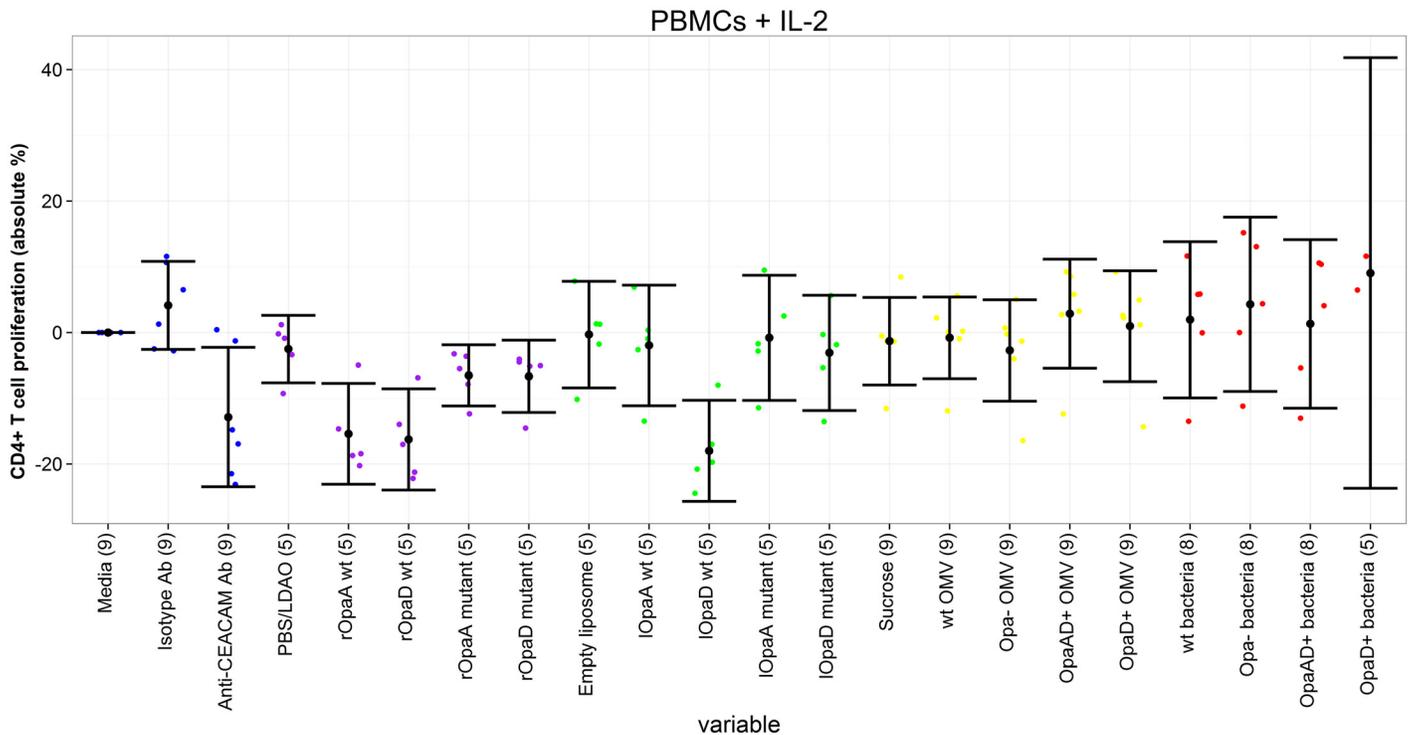


Fig 2. CD4+ T cell proliferation following incubation of PBMCs with different antigens, plus IL-2. Proliferation of each antigen relative to media only control. Each coloured dot represents the value for a single individual (control antigens—blue; recombinant proteins—purple, liposomal proteins—green, outer membrane vesicles—yellow, inactivated bacteria—red). Black dots represent mean value for each antigen, with 95% confidence intervals shown. Number in parentheses after each antigen represents number on individuals in that group. CEACAM = carcinoembryonic antigen-related cell adhesion molecule; SEB = Staphylococcal enterotoxin B; PBS = phosphate-buffered saline; LDAO = lauryldimethylamine-oxide; wt = wild-type. PHA (phytohaemagglutinin) result not shown for clarity, to confine y-axis to relevant range for other antigens.

doi:10.1371/journal.pone.0154153.g002

Discussion

In this study, a range of Opa+ and Opa- antigens was used in culture with both PBMCs and purified CD4+ T cells to demonstrate that recombinant and, to a lesser extent, liposomal OpaA and OpaD proteins from *N. meningitidis* strain H44/76 inhibited CD4+ T cells *in vitro* when cells were pre-stimulated with IL-2, with or without anti-CD3 and anti-CD28 antibodies. When the HV2 region of these Opa proteins was mutated to abrogate CEACAM1 binding, these inhibitory effects were usually reduced, although inhibition still occurred. These same mutations had variable effects on immunogenicity of these proteins when used for immunisation in mice—bactericidal antibodies were still induced with OpaD, but not for OpaA. It is not possible to directly contrast the T cell studies with mouse immunisations because of the different CEACAM repertoire found in humans and mice. In contrast, OMVs and inactivated bacteria based on Opa+ and Opa- strains did not have the same inhibitory effects on CD4+ T cells. All bacterial strains were typically stimulatory and there were no consistent differences between Opa+ and Opa- bacteria or OMVs. It is likely, therefore, that other proteins contained within these preparations are immunodominant with respect to these *in vitro* interactions.

This study was the first to study the culture of freshly isolated PBMCs *ex vivo* to assess Opa-mediated interactions, and under these conditions none of the Opa+ or Opa- antigens exerted a major stimulatory or inhibitory effect on CD4+ T cells. This is a standard method of assessing *in vitro* T cell responses to vaccine candidates and the assessment of antigen-specific proliferation [35] following vaccination. In this study, T cell responses were assessed in healthy adults

Table 4. CD4⁺ T cell proliferation following incubation of PBMCs with different antigens, plus IL-2, anti-CD3 and anti-CD28.

Antigen	Mean % CD4 ⁺ T-cell proliferation				
	n	vs. media (95% CI)	p-value	vs. Opa- (95% CI)	p-value
Control antigens					
PHA	21	+10.0 (4.6, 15.4)	0.0009	-	-
Isotype antibody	21	-2.9 (-4.8, -0.9)	0.0057	-	-
Anti-CEACAM antibody	21	-24.5 (-28.0, -21.0)	<0.0001	-	-
SEB	3	+5.3 (0.9, 9.6)	0.0308	-	-
Recombinant proteins					
Protein buffer (PBS/LDAO)	21	-1.7 (-4.0, 0.5)	0.1272	-	-
rOpaA wt	21	-25.0 (-35.8, -14.1)	0.0001	-3.0 (-9.7, 3.6)	0.3552
rOpaD wt	21	-16.2 (-26.6, -5.7)	0.0041	-1.0 (-4.6, 2.6)	0.5634
rOpaA mutant	21	-21.9 (-33.4, -10.5)	0.0007	REF	-
rOpaD mutant	21	-15.2 (-24.8, -5.6)	0.0035	REF	-
Liposomal proteins					
Empty liposome	12	+2.0 (0.5, 3.4)	0.0106	-	-
lOpaA wt	12	-6.2 (-18.2, 5.9)	0.2848	-3.5 (-13.6, 6.5)	0.4584
lOpaD wt	12	-42.3 (-52.4, -32.2)	<0.0001	-35.6 (-48.4, -22.8)	0.0001
lOpaA mutant	12	-2.7 (-6.0, 0.6)	0.1042	REF	-
lOpaD mutant	12	-6.7 (-17.6, 4.2)	0.2064	REF	-
Outer membrane vesicles (OMVs)					
OMV buffer (3% sucrose)	21	+1.6 (0.0, 3.2)	0.0546	-	-
wt OMV	21	-1.7 (-4.3, 0.9)	0.1805	-3.5 (-6.3, -0.8)	0.0146
Opa- OMV	21	+1.8 (0.1, 3.5)	0.0353	REF	-
OpaA+ OpaD+ OMV	21	-0.4 (-2.7, 1.8)	0.6900	-2.2 (-4.5, 0.0)	0.0513
OpaD+ OMV	21	+0.6 (-1.5, 2.7)	0.5541	-1.2 (-2.7, 0.3)	0.1108
Inactivated bacteria					
wt H44/76	20	+7.1 (3.6, 10.7)	0.0005	-2.1 (-3.4, -0.8)	0.0036
Opa-	20	+9.3 (4.8, 13.7)	0.0003	REF	-
OpaA+ OpaD+	20	+8.9 (4.6, 13.2)	0.0003	-0.4 (-1.7, 1.0)	0.5831
OpaD+	17	+7.6 (3.2, 12.1)	0.0020	-2.8 (-4.9, 0.7)	0.0115

Values in bold represent antigens where the % CD4⁺ T cell proliferation was statistically significant (p < 0.01) compared to media alone or compared to the relevant Opa- antigen (REF). CI = confidence interval; PHA = phytohaemagglutinin; CEACAM = carcinoembryonic antigen-related cell adhesion molecule; SEB = Staphylococcal enterotoxin B; PBS = phosphate-buffered saline; LDAO = lauryldimethylamine-oxide; wt = wild-type.

doi:10.1371/journal.pone.0154153.t004

without recent meningococcal vaccination or infection, which may explain the relative lack of proliferative responses observed in the PBMC *ex vivo* conditions. This study, therefore, does not support the argument that Opa proteins would have an inhibitory effect *in vivo* if given as a vaccine to healthy adults. Immune responses to previous OMV vaccines have included the induction of anti-Opa antibodies, which also suggests that Opa does not have a significant immunosuppressive effect when included within OMVs.

Previously published data regarding the effects of meningococcal Opa proteins on CD4⁺ T cell proliferation have been conflicting, creating a significant hurdle in the development of any Opa-containing vaccines (Table 6). Studies comparing the meningococcal proteins PorA, PorB, Opa and Opc showed that Opa induced the strongest T cell proliferative responses [16, 17] and whole bacteria and OMVs have been shown to stimulate CD4⁺ T cell proliferation, independent of their Opa phenotype [18]. Several Opa-containing OMV vaccines are

Table 5. CD4⁺ T cell proliferation following incubation of purified CD4⁺ T cells with different antigens, plus IL-2, anti-CD3 and anti-CD28.

Antigen	Mean % CD4 ⁺ T-cell proliferation				
	n	vs. media (95% CI)	p-value	vs. Opa- (95% CI)	p-value
Control antigens					
PHA	9	+26.6 (16.3, 37.0)	0.0002	-	-
Isotype antibody	9	-1.7 (-5.9, 2.5)	0.3891	-	-
Anti-CEACAM antibody	9	-17.9 (-25.8, -9.9)	0.0006	-	-
Recombinant proteins					
Protein buffer (PBS/LDAO)	7	-3.1 (-7.1, 1.0)	0.1148	-	-
rOpaA wt	7	-24.5 (-31.3, -17.6)	0.0001	-20.3 (-27.7, -12.8)	0.0004
rOpaD wt	7	-25.5 (-35.6, -15.4)	0.0006	-15.9 (-28.1, -3.6)	0.0183
rOpaA mutant	7	-4.2 (-10.9, 2.5)	0.1818	REF	-
rOpaD mutant	7	-9.6 (-13.4, -5.8)	0.0005	REF	-
Liposomal proteins					
Empty liposome	5	+1.1 (-5.8, 8.1)	0.6903	-	-
lOpaA wt	5	-15.5 (-20.3, -10.8)	0.0004	-5.6 (-11.2, 0.0)	0.0512
lOpaD wt	5	-46.1 (-63.1, -29.1)	0.0009	-30.1 (-51.6, -8.6)	0.0155
lOpaA mutant	5	-10.0 (-16.8, -3.1)	0.0132	REF	-
lOpaD mutant	5	-15.9 (-20.9, -10.9)	0.0004	REF	-
Outer membrane vesicles (OMVs)					
OMV buffer (3% sucrose)	8	-0.1 (-3.9, 3.6)	0.9296	-	-
wt OMV	7	-3.7 (-8.3, 0.9)	0.0979	+11.0 (4.7, 17.3)	0.0046
Opa- OMV	9	-13.9 (-20.3, -7.6)	0.0008	REF	-
OpaA+ OpaD+ OMV	8	-6.5 (-13.0, 0.0)	0.0497	+8.1 (2.7, 13.5)	0.0087
OpaD+ OMV	9	-8.4 (-13.9, -3.0)	0.0067	+5.5 (1.5, 9.5)	0.0120
Inactivated bacteria					
wt H44/76	4	+15.3 (5.2, 25.4)	0.0139	-1.4 (-4.7, 1.8)	0.2909
Opa-	6	+15.3 (9.8, 20.8)	0.0005	REF	-
OpaA+ OpaD+	6	+14.4 (7.1, 21.7)	0.0029	-0.9 (-7.4, 5.5)	0.7349
OpaD+	6	+16.3 (5.9, 26.7)	0.0085	+1.0 (-6.8, 8.7)	0.7701

Values in bold represent antigens where the % CD4⁺ T cell proliferation was statistically significant (p < 0.01) compared to media alone or compared to the relevant Opa- antigen (REF). CI = confidence interval; PHA = phytohaemagglutinin; CEACAM = carcinoembryonic antigen-related cell adhesion molecule; PBS = phosphate-buffered saline; LDAO = lauryldimethylamine-oxide; wt = wild-type.

doi:10.1371/journal.pone.0154153.t005

immunogenic in humans [19–23]. In other studies, when CD4⁺ T cells were exposed to OMVs from Opa-expressing meningococci or to gonococci expressing CEACAM-binding Opa variants, their activation and proliferation in response to a variety of stimuli were effectively halted [24, 31].

There may be a number of reasons to explain the differences between various studies. Different studies have used different Opa variants and Opa proteins from H44/76 have not previously been shown to have an inhibitory effect. The proposed mechanism of inhibition is via an Opa-CEACAM1 interaction, and both Opa proteins used in this study bind to CEACAM1 [28]. When OMVs and bacteria are used, there may be variation of Opa expression levels relative to other outer membrane proteins, resulting in different effects. Further major differences between studies are the culture conditions and methods of assessment of proliferation. PBMCs and purified CD4⁺ T cells have been used, with most studies including assays with IL-2 pre-incubation and/or addition of anti-CD3 and anti-CD28. Differences in duration of culture may

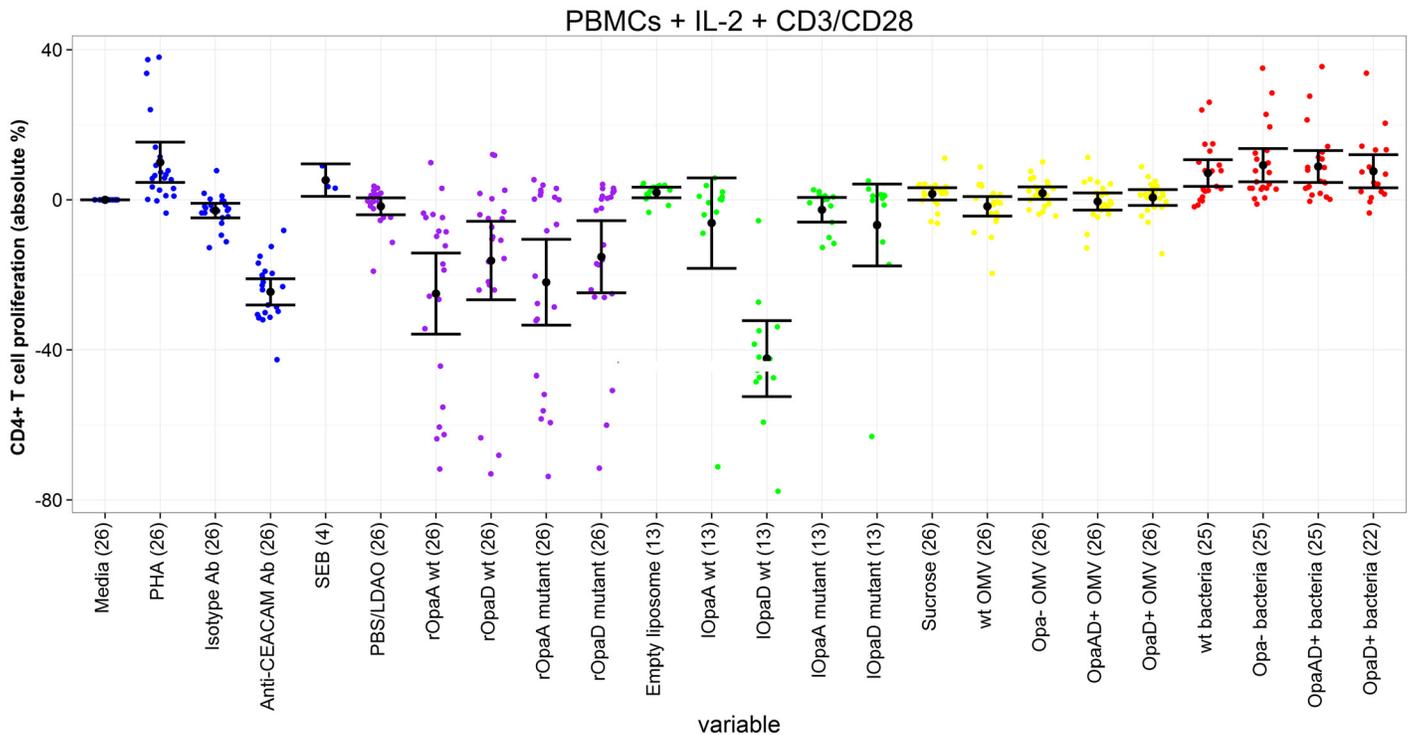


Fig 3. CD4⁺ T cell proliferation following incubation of PBMCs with different antigens, plus IL-2, anti-CD3 and anti-CD28. Proliferation of each antigen relative to media only control. Each coloured dot represents the value for a single individual (control antigens—blue; recombinant proteins—purple, liposomal proteins—green, outer membrane vesicles—yellow, inactivated bacteria—red). Black dots represent mean value for each antigen, with 95% confidence intervals shown. Number in parentheses after each antigen represents number on individuals in that group. PHA = phytohaemagglutinin; CEACAM = carcinoembryonic antigen-related cell adhesion molecule; SEB = Staphylococcal enterotoxin B; PBS = phosphate-buffered saline; LDAO = lauryldimethylamine-oxide; wt = wild-type.

doi:10.1371/journal.pone.0154153.g003

also affect CEACAM1 expression on the T cell surface. While these conditions are useful in exploring specific interactions, they are unlikely to represent what occurs *in vivo*, and should be interpreted with caution when predicting the potential effects of Opa proteins during meningococcal infection or following immunisation in humans. IL-2 is a T cell growth factor and increases CEACAM1 expression, potentially enhancing any CEACAM1-mediated Opa-induced effects [31], supported by the inhibition of proliferation observed with the anti-CEACAM antibody in these conditions. IL-2 is produced *in vivo* during T cell responses, and it is likely that the PBMC *ex vivo* assay represents more physiological levels of IL-2, compared to those assays when exogenous IL-2 was added. Anti-CD3 and anti-CD28 antibodies provide signals for T cell activation, as well as further increasing CEACAM1 expression on the T cell surface [31]. Incubation of cells with IL-2, anti-CD3 and anti-CD28 therefore tends to lead to inhibitory rather than stimulatory effects. The greater baseline stimulation of cells where anti-CD3 and anti-CD28 have been added is also the most likely explanation for the greater amplitude of inhibition observed in these conditions, compared to with IL-2 alone. The addition of PHA in the presence of anti-CD3 and anti-CD28 resulted in less additional proliferation than when they were absent, confirming these cells were already highly stimulated. Methods of measuring CD4⁺ T cell proliferation have varied between each of the published studies. A flow cytometry approach based on CFSE dilution was adopted in this study to ensure that dead cells were excluded from the analysis, but this is more difficult to achieve when using other methods described.

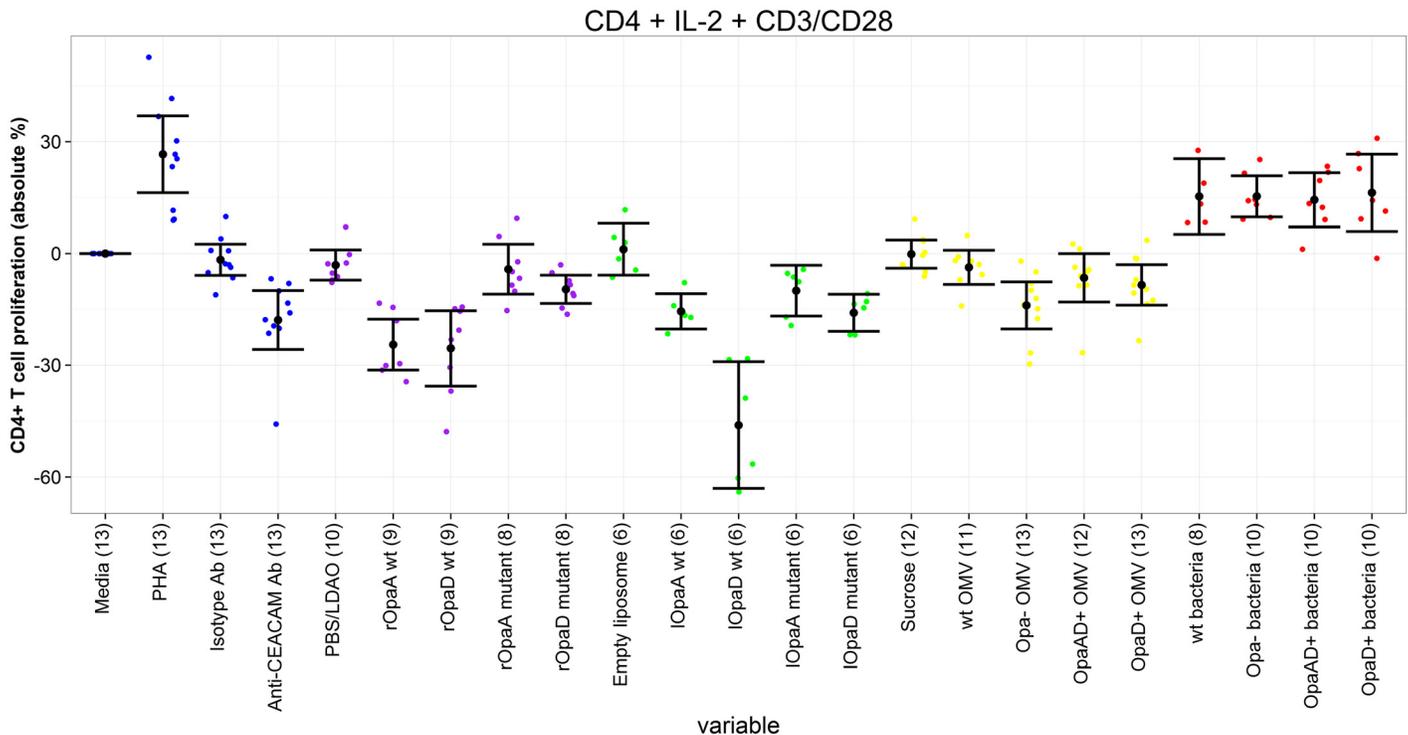


Fig 4. CD4⁺ T cell proliferation following incubation of purified CD4⁺ T cells with different antigens, plus IL-2, anti-CD3 and anti-CD28. Proliferation of each antigen relative to media only control. Each coloured dot represents the value for a single individual (control antigens—blue; recombinant proteins—purple, liposomal proteins—green, outer membrane vesicles—yellow, inactivated bacteria—red). Black dots represent mean value for each antigen, with 95% confidence intervals shown. Number in parentheses after each antigen represents number on individuals in that group. PHA = phytohaemagglutinin; CEACAM = carcinoembryonic antigen-related cell adhesion molecule; SEB = Staphylococcal enterotoxin B; PBS = phosphate-buffered saline; LDAO = lauryldimethylamine-oxide; wt = wild-type.

doi:10.1371/journal.pone.0154153.g004

Our data support the conclusion that isolated Opa proteins have a different effect to Opa expressed on the bacterial surface with other proteins. Considering that *Neisseria* have the capacity to randomly and reversibly switch many surface antigens ‘on’ and ‘off’ by a process of PV, it is also possible that the differential expression of uncharacterised factors other than Opa may influence T cell responses during infection. However, we used OMVs and bacteria from isogenic strains, constructed from the same parent strain, to reduce this risk. We have previously shown that expression of the major proteins in these strains is the same [26]. The results from these experiments do not support a proposed hypothesis that Opa proteins would inhibit CD4⁺ T cell proliferation when part of whole bacteria (as would be the case during infection) or contained within an OMV vaccine. Furthermore, there was no consistent effect between Opa⁺ and Opa⁻ OMVs or bacteria, suggesting that the effects of other proteins within these preparations was immunodominant over any Opa effect. This is further supported by a recent study showing no difference in SBA titres following immunisation of CEACAM1-transgenic mice with Opa⁺ and Opa⁻ OMVs [36]. However, our study and the mouse immunisation study used strain H44/76 and it may be that different Opa proteins have different effects, highlighted by the differences observed between liposomal and recombinant OpaA and OpaD.

One major advantage of this study was the inclusion of a large number of healthy adult subjects. Most previous studies have not stated sample size and the use of buffy coats from the National Blood Service in one study [18] would make it impossible to have specific inclusion and exclusion criteria. Comparison of Opa⁺ and Opa⁻ strains to determine Opa-dependent

Table 6. Studies of the effects of Opa proteins on CD4⁺ T cell proliferation.

Subjects	Antigens	Culture conditions	Method of assessment	Results	Ref
18 healthy adults (29–55 years)	Purified NM Opa proteins (3 variants)	PBMCs <i>ex vivo</i>	[³ H]thymidine incorporation	Opa induced proliferation in most donors, greater than Opc or PorA	[16]
≥4 individuals per experiment, no further details	Live NG expressing different Opa variants (strain MS11)	Purified CD4 ⁺ T cells* +/- IL-2 (48 hours pre-incubation) +/- anti-CD3 +/- anti-CD28	Lymphocyte density, assessed by direct counting	HSPG-binding Opa ⁺ bacteria induced greater proliferation than CEACAM-binding Opa ⁺ bacteria	[31]
Not stated	OMVs from Opa ⁺ NM (strain K454) and Opa ⁻ NL (strain Y92 1009)	Purified CD4 ⁺ T cells + IL-2 (48 hours pre-incubation) + anti-CD3 + anti-CD28	Cell culture density, assessed by direct counting	NL Opa ⁻ OMVs induced greater proliferation than NM Opa ⁺ OMVs	[24]
	OMVs from NG expressing different Opa variants (strain MS11)	Jurkat CD4 ⁺ T cells† +/- IL-2 (48h) and/or anti-CD3		Opa ⁻ or HSPG-binding Opa ⁺ OMVs induced greater proliferation than CEACAM-binding Opa ⁺ OMVs	
Healthy adults and buffy coats, number not stated	Live Opa ⁺ or Opa ⁻ NM (strain C751 or H44/76)	Purified CD4 ⁺ T cells‡ +/- IL-2 (48 hours pre-incubation) +/- anti-CD3	[³ H]thymidine incorporation	No difference in proliferation between isogenic Opa ⁺ and Opa ⁻ strains	[18]
	Live Opa ⁺ or Opa ⁻ NM (strain C751) or heat-killed Opa ⁺ or Opa ⁻ NM (strain H44/76) or Opa ⁺ or Opa ⁻ NM OMVs (strain H44/76)	Purified CD4 ⁺ T cells + IL-2 (96 hours pre-incubation) +/- anti-CD3 and anti-CD28	Flow cytometric analysis of CFSE dilution	No difference in proliferation between isogenic Opa ⁺ and Opa ⁻ strains	
46 healthy adults (18–66 years)	Wild-type and mutant Opa proteins, presented as recombinant proteins and liposome	PBMCs +/- IL-2 (48 hours) +/- anti-CD3 and anti-CD28 or purified CD4 ⁺ T cells + anti-CD3 and anti-CD28	Flow cytometric analysis of CFSE dilution	Proteins and liposomes generally inhibitory in most conditions, with wt variants more inhibitory than mutants	this study
	Opa ⁺ or Opa ⁻ NM OMVs or ethanol-fixed Opa ⁺ or Opa ⁻ NM (strain H44/76)			OMVs all generally inhibitory only when using purified CD4 ⁺ T cells. Bacteria all stimulatory in the presence of anti-CD3 and anti-CD28. No consistent difference between Opa ⁺ and Opa ⁻ OMVs or bacteria	

NM = *N. meningitidis*, NG = *N. gonorrhoeae*, NL = *N. lactamica*; OMV = outer membrane vesicle; HSPG = heparin sulphate proteoglycan; CEACAM = carcinoembryonic antigen-related cell adhesion molecule;

*conditions included CD4⁺ T cells alone, with IL-2 or anti-CD3 only, with IL-2 and anti-CD3, with anti-CD3 and anti-CD28, and with IL-2, anti-CD3 and anti-CD28;

†conditions included CD4⁺ T cells alone, with IL-2 or anti-CD3 only, and with IL-2 and anti-CD3;

‡conditions included CD4⁺ T cells alone, with anti-CD3 only, and with IL-2 and anti-CD3.

doi:10.1371/journal.pone.0154153.t006

Table 7. Serum bactericidal antibody titres of pooled murine sera against 4 target strains, following immunisation with wild-type and mutant recombinant OpaA and OpaD proteins.

Recombinant protein used for immunisation	Target strain in SBA assay (Opa phenotype)			
	H44/76 (Opa-)	M014 (Opa-)	M002 (OpaD+)	M001 (OpaA+ OpaD+)
OpaA wt	<1:4	<1:4	<1:4	1:256
OpaD wt	<1:4	<1:4	1:256	1:256
OpaA mutant	<1:4	<1:4	<1:4	<1:4
OpaD mutant	<1:4	<1:4	1:256	1:256

Titres represent highest dilution at which there was 50% bacterial survival. wt = wild-type.

doi:10.1371/journal.pone.0154153.t007

effects is only valid when comparing otherwise isogenic strains, as used in this study. Although most previous studies have also used isogenic strains, one study compared Opa+ OMVs from *N. meningitidis* with Opa- OMVs from *N. lactamica* where many other surface proteins would also be different and may exert an immunomodulatory effect.

This is the first study of the effects of Opa proteins on CD4⁺ T cells to include a range of culture conditions, including the use of PBMCs and purified CD4⁺ T cells, using the same set of antigens with the same measure of proliferation. Different types of Opa+ and Opa- antigens were included to detect a consistent effect of Opa on CD4⁺ T cells. The lack of inhibitory responses observed with PBMCs *ex vivo* suggest that Opa proteins would not cause immunosuppressive effects if included as a vaccine component. Opa proteins inhibited CD4⁺ T cell proliferation after stimulation with IL-2, anti-CD3 and anti-CD28, possibly due to upregulation of CEACAM1 on the T cell surface and/or greater baseline stimulation, which would tend to highlight any inhibitory effects. Future studies would be required to dissect which CD4⁺ subpopulation is responsible for the effects observed. Opa proteins alone inhibited T cell responses in pre-stimulated *in vitro* conditions—an effect which was reduced by mutation of the CEACAM1-binding regions. However, when included with other antigens in the form of OMVs or killed bacteria, no Opa-dependent inhibition was observed, providing reassurance that current or future meningococcal vaccines containing OMVs would not be negatively affected by Opa protein expression.

Acknowledgments

CJ, MSad, SL and AJP conceived and designed the study. CJ, MSad, SL, IP, MSal, JPD performed the work described. MSad conducted data analysis. CJ and MSad drafted the original manuscript and all authors approved the final version. AJP is a Jenner Institute Investigator and James Martin Senior Fellow. AJP was previously named as an inventor on a patent for the use of Opa proteins in meningococcal vaccines. AJP has previously conducted clinical trials of meningococcal vaccines on behalf of the University of Oxford, but he receives no personal payments from them. AJP is chair of the UK Department of Health's Joint Committee on Vaccines and Immunisation and chair of the European Medicine Agency's Scientific Advisory Group on Vaccines. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health. The authors would like to thank Peter van der Ley, Scott Gray-Owen, Martin Maiden, Ian Feavers and Gunnstein Norheim for their advice and thoughtful insights during the study.

Author Contributions

Conceived and designed the experiments: CJ M. Sadarangani SL AJP. Performed the experiments: CJ M. Sadarangani SL IP M. Saleem JPD. Analyzed the data: M. Sadarangani. Wrote the paper: CJ M. Sadarangani.

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