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## Antibody-Dependent Natural Killer Cell Activation after Ebola Vaccination

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40 word summary:

In this study, we show NK cell degranulation and IFN- $\gamma$  secretion in response to recombinant Ebola virus glycoprotein and post-vaccination sera from healthy volunteers vaccinated with the novel 2-dose heterologous Ad26.ZEBOV, MVA-BN-Filo Ebola vaccine (EBOVAC consortium, EU Innovative Medicines Initiative).

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## Abstract

### Background

Antibody Fc-mediated functions, such as antibody-dependent cellular cytotoxicity, contribute to vaccine-induced protection against viral infections. Fc-mediated function of anti-Ebola glycoprotein antibodies suggest that Fc-dependent activation of effector cells, including NK cells, could play a role in vaccination against Ebola virus disease.

### Methods

We analysed the effect of anti-Ebola glycoprotein antibody in the serum of U.K.-based volunteers vaccinated with the novel 2-dose heterologous Adenovirus type 26.ZEBOV, Modified Vaccinia Ankara-BN-Filo vaccine regimen, on primary human NK cell activation.

### Results

We demonstrate primary human NK cell CD107a and IFN- $\gamma$  expression, combined with downregulation of CD16, in response to recombinant Ebola virus glycoprotein and post-vaccine dose 1 and dose 2 sera. These responses varied significantly with vaccine regimen and NK cell activation was found to correlate with anti-glycoprotein antibody concentration. We also reveal an impact of NK cell differentiation phenotype on antibody-dependent NK cell activation, with highly differentiated CD56<sup>dim</sup>CD57<sup>+</sup> NK cells being the most responsive.

### Conclusions

This study thus highlights the dual importance of vaccine-induced antibody concentration and NK cell differentiation status in promoting Fc-mediated activation of NK cells after vaccination, raising a potential role for antibody-mediated NK cell activation in vaccine-induced immune responses.

**Keywords:** antibody, Ebola, vaccine, natural killer cell.

## Background

Determining correlates of protection for Ebola vaccines has proved difficult and ambiguous [1]. Anti-Ebola antibodies possess strong neutralising capacity [2, 3], moreover, antibodies with limited neutralising activity were protective in animal models and human *in vitro* culture systems suggesting that neutralisation alone presents an incomplete mechanistic picture of *in vivo* protection [3-5]. Ebola-specific antibodies induce antibody-dependent cellular cytotoxicity (ADCC) in human peripheral blood NK cells and NK cell lines *in vitro*; in light of this, Fc-mediated function in anti-Ebola monoclonal antibody (mAb) therapy and vaccine-induced protection is gaining in interest [5-7]. Analysis of the primary response to the candidate Ebola vaccine, rVSV-ZEBOV, revealed a correlation between early NK cell activation and anti-Ebola antibody titre [8]. Also, protection of non-human primates against Ebola virus challenge is associated with a low IgG2:IgG1 antibody isotype ratio, compatible with ADCC as a major mechanism of protection [9]. In murine experimental filovirus vaccines, induction of anti-glycoprotein (GP) antibodies with robust ADCC function was critical for protection [10-12]. Taken together, these studies suggest Fc functions of anti-Ebola antibodies potentially contribute to protection and may be exploited for improving vaccine and therapeutic mAb efficacy and as markers of vaccine-induced immunity.

NK cells, like other innate immune effector cells, express Fc receptors (FcR) on their surface allowing activation of cell-mediated antibody-dependent anti-viral functions [13]. Antibody-dependent phagocytosis (ADP) of virus or virally-infected cells by monocytes, macrophages and neutrophils, and ADCC mediated by NK cells, promote the clearance of infected cells, reducing viral load and dissemination. NK cell ADCC is principally mediated by crosslinking of FcγRIIIa (CD16) by the Fc region of immunoglobulins – subclasses IgG1 and IgG3 in humans – which leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) and downstream pathway activation. Killing proceeds by the release of lytic granules from activated NK cells inducing apoptosis of virally infected cells. Cross-linking of CD16 by antibody induces its cleavage from the NK cell surface [14-16], despite this, NK cells can move

on to kill multiple targets providing effective clearance of infected cells [17]. Fc functions of broadly neutralising antibodies have been shown to be indispensable for protection against influenza virus infection [18, 19], however, the role of Ebola vaccine induced antibody-dependent NK cell functions is unknown.

The novel Ad26.ZEBOV, MVA-BN-Filo 2-dose vaccine regimen has shown promising results in phase 1 and 2 studies; high levels of anti-Ebola GP specific antibody are sustained for at least 360 days with high neutralising activity and a strong correlation between binding and neutralising antibody responses [20-23]. However, vaccine regimens based on Ad26.ZEBOV and MVA-BN-Filo differing in order and interval between dose 1 and dose 2 induce substantially different serum antibody concentrations in U.K. based volunteers at both post-dose 1 and post-dose 2 time points [21]. Therefore, the purpose of this study was to assess the ability of post-Ad26.ZEBOV, MVA-BN-Filo vaccination sera (of differing regimen) to mediate antibody-dependent NK cell function in an *in vitro* ADCC assay targeting immobilised Ebola virus GP (EBOV GP). We observe robust, antibody-dependent activation of NK cells in whole human peripheral blood mononuclear cell (PBMC) preparations cultured with EBOV GP in the presence post-Ad26.ZEBOV, MVA-BN-Filo vaccination serum. NK cell activation varied depending on vaccine regimen and correlated positively with antibody concentration. NK activity also varied between NK cell donors, consistent with differentiation phenotype influencing the potency of antibody-dependent NK cell responses.

## Methods

### Study participants and samples

Eligible, healthy volunteers were recruited to take part in the EBL1001 (EBOVAC consortium) single-centre, randomised, placebo-controlled, observer blind Ebola vaccine trial held in Oxford, U.K. (ClinicalTrials.gov Identifier: NCT02313077). A further 15 volunteers were subsequently recruited for group 5, see Milligan *et al.* for additional methodology [21]. Serum samples from 72 donors (age range 18-50 years) were obtained for this study from non-placebo arms (Table 1).

Active vaccination comprised monovalent Ad26.ZEBOV expressing the GP of the Ebola Zaire virus (Mayinga variant) and multivalent MVA-BN-Filo expressing the GP of the Sudan and Zaire Ebola viruses and Marburg virus together with Tai Forest virus nucleoprotein (Janssen Vaccines and Prevention B.V., The Netherlands and Bavarian Nordic, Denmark). Participants received vaccination schedules as follows; groups 1 and 2 received MVA-BN-Filo on day 1 and Ad26.ZEBOV on either day 29 or 57 respectively, groups 3, 4 and 5 received Ad26.ZEBOV on day 1 and MVA-BN-Filo on days 29, 57 or 15 respectively.

Additional blood samples were obtained from non-vaccinated, non-study volunteers. PBMC were isolated using Histopaque 1077 (Sigma-Aldrich, U.S.A) gradient centrifugation and cryopreserved in liquid nitrogen or used immediately. Ebola GP-specific IgG concentration and Ebola GP-specific pseudovirion virus neutralising antibody titres (psVNA) were determined previously [21] and human cytomegalovirus (HCMV) serostatus was determined by IgG ELISA (Demeditec, Kassel, Germany), 36% HCMV seropositive. The trial protocol and study documents were approved by the National Research Ethics Service (reference number 14/SC/1408) and the LSHTM Research Ethics Committee (reference number 14383).

### ***In vitro* culture assays**

For antibody-dependent NK cell activation assays, 10µg/ml purified EBOV GP, Mayinga variant (Janssen Vaccines and Prevention B.V.) was immobilised on 96-well flat-bottom tissue culture plates overnight at 4°C, washed, blocked with 5% FCS in RPMI 1640 supplemented with 100U/ml penicillin/streptomycin and 20mM L-glutamine (Gibco, ThermoFisher) and washed again. Fresh PBMC from a single individual donor (non-vaccinated) were washed in RPMI 1640 supplemented as above and counted using Countess II FL Automated Cell Counter (Invitrogen, ThermoFisher). PBMC were seeded ( $3 \times 10^5$ /well) onto the antigen-coated plates together with pre or post-vaccination serum at various concentrations (with total serum concentration made up to 5% with FCS) and incubated for 6 hours at 37°C. Alternatively, cryopreserved PBMC from multiple (non-vaccinated) donors were thawed, washed and

seeded onto the antigen-coated plates with pooled pre or post-vaccination serum from group 2 (regimen; MVA-BN-Filo on day 1 and Ad26.ZEBOV on day 57).

Anti-CD107a-FITC (clone H4A3; BD Biosciences) was added to the cultures for the entire culture period and GolgiStop (Monensin; 1/1500 concentration; BD Biosciences) and GolgiPlug (Brefeldin A; 1/1000 final concentration; BD Biosciences) were added for the final 3 hours of culture. Positive control cultures comprised the CD20 expressing human Burkitt's Lymphoma cell line (RAJI; ECACC, Salisbury, U.K.) with monoclonal anti-CD20, Rituximab (Ritxan®; Genentech, San Francisco, USA) at varying concentrations. In all cases, cells were harvested into round-bottom plates by soaking and resuspension in FACS buffer (PBS, 0.5% FCS, 0.05% sodium azide and 2mM EDTA) for staining.

### **Flow cytometry**

Cells were stained in 96-well round-bottom plates as described previously [24]. Briefly, cells were blocked with Fc Receptor (FcR) Blocking Reagent (Miltenyi Biotech, Germany) and stained with fluorophore labelled antibodies for surface markers including viability marker (Fixable Viability Stain 700; BD Biosciences) in FACS buffer. Cells were washed in FACS buffer, fixed and permeabilised using Cytofix/Cytoperm Kit (BD Biosciences). Cells were then stained for intracellular markers with further FcR blocking, washed again and resuspended in FACS buffer and acquired using a BD LSRII flow cytometer using FACSDiva software and analysed using FlowJo V10 (Tree Star, Oregon, U.S.A). FACS gates were set using unstimulated cells or FMO controls, a minimum cut off was determined as the frequency of responding NK cells in the presence of FCS alone [21], samples with less than 100 NK cell events were excluded from the analysis.

Fluorophore-labelled antibodies used were: anti-CD3-V500 (clone UCHT1) (BD Biosciences), anti-CD56-BV605 (clone HCD56) and anti-IFN- $\gamma$ -BV785 (clone 45.B3) (Biolegend, London, U.K.), anti-CD16-APC (clone CB16), anti-CD57-e450 (clone TB01) (eBiosciences) and anti-NKG2C-PE (clone 134591) (R&D systems). Cells were acquired using FACSDiva software, data were analysed using FlowJo V10 (Tree Star, Oregon, U.S.A).



## Statistics

Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad, California, U.S.A.). Functional responses were compared using Wilcoxon signed-rank test or one-way ANOVA Friedman test with Dunn's correction for multiple comparisons. Correlation analysis was performed using linear and nonlinear regression models, the p value of the correlation of the two variables was determined using Pearson correlation analysis. Significance levels are assigned as \*p, 0.05, \*\*p, 0.01, \*\*\*p, 0.001, and \*\*\*\*p, 0.0001 for all tests.

## Results

### **Ad26.ZEBOV, MVA-BN-Filo Ebola vaccine induced antibody-dependent NK cell activation *in vitro*.**

To assess the effect of Ad26.ZEBOV, MVA-BN-Filo induced anti-GP antibody on NK cell activation, whole PBMC from one non-vaccinated donor were cultured with plate bound EBOV GP plus pre or post-vaccination serum. Optimal serum concentrations for CD3<sup>+</sup>CD56<sup>+</sup> NK cell CD107a surface expression (gating strategy shown in Figure 1a and Supplementary Figure 1a) were established (Supplementary figure 1b). NK cell CD107a, CD16 and IFN- $\gamma$  expression was then measured in response to 5% pre (visit 0), post-dose 1 (visit 1) or post-dose 2 (visit 2) serum from each individual study participant (n=72) (gating strategy shown in Figure 1a-c). Initially, data from all five vaccination arms were combined for analysis. Significantly higher frequencies of CD107a<sup>+</sup> NK cells were observed with post-dose 1 serum compared with pre-vaccination serum and was further enhanced with post-dose 2 serum (median 2.39% post-dose 1, 8.24% post-dose 2) (Figure 2a). CD56<sup>dim</sup> NK cell CD16 expression measured by mean fluorescence intensity (MFI) decreased significantly in cells cultured with post-dose 1 serum and there was a further decrease in cells cultured with post-dose 2 serum (median MFI 8990 post-dose 1, 4020 post-dose 2) (Figure 2a). Frequencies of NK cells producing IFN- $\gamma$  in response to post-dose 1 serum were low but significantly higher than in response to pre-vaccination serum, and again, this was strongly increased with post-dose 2 serum (median 0.28% post-dose 1, 1.17% post-dose 2) (Figure 2).

The effect of Ad26.ZEBOV, MVA-BN-Filo-induced anti-GP antibody on antibody-dependent NK cell activation was analysed according to NK cell differentiation subset (gating strategy shown in Supplementary Figure 1c, Supplementary Figure 1d shows the NK cell subset distribution for the single donor used in this assay). NK cell CD107a expression was induced equally in less differentiated CD56<sup>bright</sup> and more differentiated CD56<sup>dim</sup> NK cell subsets and in subsets further subdivided into moderately and highly differentiated CD56<sup>dim</sup>CD57<sup>-</sup> and CD56<sup>dim</sup>CD57<sup>+</sup> (NKG2C<sup>-</sup> and NKG2C<sup>+</sup>) cells (Figure 2b). This was consistent with significant CD16 downregulation observed in all NK cell subsets (Figure 2b). Basal CD16 expression increased with increasing differentiation status (CD56<sup>dim</sup>CD57<sup>-</sup> < CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>-</sup> < CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>+</sup>) and CD16 expression was maintained at higher MFI post-dose 2 in the most differentiated subsets (Figure 2b). CD107a was induced within the CD56<sup>bright</sup> NK cell population in response to post-vaccination serum, however the overall contribution of this was less than 14% of the total expression ( $p < 0.0001$ ) (Figure 2c). IFN- $\gamma$  expression in response to post-vaccination serum was attributed to CD56<sup>dim</sup> NK cells with no increase in expression observed within the least differentiated CD56<sup>bright</sup> NK cell subset (Figure 2b).

The most highly differentiated CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>-</sup> and CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell subsets showed the most extensive CD16 downregulation and the highest frequencies of IFN- $\gamma$  producing cells (Figure 2b). 71.2% of all the NK cells producing IFN- $\gamma$  in response to post-dose 2 serum were CD56<sup>dim</sup>CD57<sup>+</sup> (NKG2C<sup>+/-</sup>) NK cells, with 25.5% of IFN- $\gamma$ <sup>+</sup> cells being CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>+</sup> (Figure 2c). Consistent with antibody-dependent activation of more differentiated NK cell subsets, anti-CD20 (Rituximab) and CD20-expressing RAJI cells also preferentially induced NK cell degranulation and IFN- $\gamma$  expression in CD56<sup>dim</sup>CD57<sup>+</sup> (NKG2C<sup>+/-</sup>) (Supplementary Figure 2). These data suggest that EBOV GP-specific antibody induces antibody-dependent NK cell activation, including IFN- $\gamma$  secretion, in more differentiated NK cell subsets.

### **Variation in antibody-dependent NK cell activation by vaccine regimen.**

The Ebola GP-specific IgG concentration in the serum samples of Ad26.ZEBOV, MVA-BN-Filo vaccinated individuals varied depending on the vaccination regimen [21]. We therefore analysed the impact of vaccine regimen on antibody-dependent NK cell activation. There was significant upregulation of CD107a, IFN- $\gamma$  and downregulation of CD16 with post-dose 2 serum in all groups compared with pre-vaccination serum responses, however responses differed significantly between study arms (Figure 3a-c).

Groups 1 and 2 (MVA-BN-Filo followed by Ad26.ZEBOV) resulted in the strongest induction of CD107a and IFN- $\gamma$  expression and the lowest CD16 MFI of all five groups (Figure 3a-c). Serum collected after MVA-BN-Filo (dose 1) did not induce NK cell activation, but significant induction of CD107a, IFN- $\gamma$  and downregulation of CD16 was seen with post-Ad26.ZEBOV (dose 2) serum (when compared with both pre and post-dose 1 vaccination serum) (Figure 3a-c, Supplementary Table 1). By contrast, in groups 3, 4 and 5 (Ad26.ZEBOV followed by MVA-BN-Filo) there was induction of NK cell responses to post-Ad26.ZEBOV (dose 1) serum that were further enhanced by post-MVA-BN-Filo (dose 2) serum (Figure 3a-c, Supplementary Table 1). However, earlier MVA-BN-Filo dose 2 in group 3 (day 29) and group 5 (day 15) did not result in further significant NK activation (by one-way ANOVA, Supplementary Table 1) over that with the first dose of Ad26.ZEBOV (except weak boosting of IFN- $\gamma$  in group 3) and group 5 resulted in the weakest overall response (Figure 3a-c, Supplementary Table 1). This suggests that Ad26.ZEBOV as first dose induces sufficient concentrations of antibody for a robust NK cell response that is further increased by MVA-BN-Filo second dose, whilst MVA-BN-Filo alone does not induce sufficient antibody (or antibody of the correct isotype or subclass) to mediate ADCC.

### **Correlation of NK cell function with anti-GP antibody concentration and pseudovirion virus neutralising antibody titres.**

Variation in NK cell function according to vaccine regimen is consistent with data on the effect of vaccine regimen on anti-GP antibody concentration [21]. Therefore, we next analysed the relationship between individual Ebola GP-specific IgG concentration (determined by Milligan *et al.* previously [21]) and antibody-dependent NK cell activation. With all groups combined, there was a significant positive correlation between post-dose 2 antibody concentration and frequencies of NK cell CD107a and IFN- $\gamma$  and a negative correlation with CD16 MFI (Figure 4a).

Groups 1 and 2 (MVA-BN-Filo followed by Ad26.ZEBOV) demonstrated the highest median NK cell functional responses post-dose 2 (Wilcoxon paired t test CD107a<sup>+</sup> NK cell frequency; group 1 vs 3;  $p=0.0479$ , group 2 vs 4;  $p=0.0166$ ). However, when analysed according to vaccination group, only groups 3-5 (Ad26.ZEBOV followed by MVA-BN-Filo) demonstrated a significant correlation between post-dose 2 antibody concentration and NK cell function (Table 2). Antibody concentration and NK cell function also correlated significantly after dose 1 when all groups were combined (Supplementary Figure 3a-c), however, this relationship was only significant for group 3 (Ad26.ZEBOV followed by MVA-BN-Filo at day 29) when analysed separately (Table 2). There was no correlation between antibody concentration and antibody-dependent NK cell function at post-dose 1 and post-dose 2 in MVA-BN-Filo followed by Ad26.ZEBOV vaccinated individuals (groups 1 and 2) (Table 2). Therefore, in vaccine regimen inducing the highest post-dose 2 responses (groups 1 and 2), the association between the two variables is lost.

Analysis of antibody-dependent NK cell responses and Ebola GP-specific pseudovirus neutralising antibody titres (psVNA) titres revealed a significant positive correlation across the entire cohort with the frequency of NK cell IFN- $\gamma$  and a negative correlation with CD16 expression, although no association was observed with CD107a expression (Figure 4b). Consistent with the association with anti-GP antibody concentration, we observed the strongest correlations between all NK cell functions and psVNA titres for groups 3 and 4 (Ad26.ZEBOV followed by MVA-BN-Filo) (Table 3).

### Variation in antibody-dependent NK cell function by NK cell donor.

To analyse the effect of donor variation, PBMC from non-vaccinated donors (n=16) were cultured with plate bound EBOV GP and pooled pre or post-dose 2 vaccination serum samples from group 2 (MVA-BN-Filo followed by Ad26.ZEBOV at day 57). NK cell CD107a (14 of 16 responding), CD16 downregulation (16 of 16 donors responding) and IFN- $\gamma$  (13 of 16 donors responding) was induced with pooled post-dose 2 serum compared with pooled pre-vaccination serum (Figure 5a) suggesting the majority of donors respond to Ad26.ZEBOV, MVA-BN-Filo vaccine-induced antibody.

We next analysed NK cell activation in response to pooled post-dose 2 serum according to NK cell differentiation subset. Amongst the individuals tested, frequencies of the most highly differentiated CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells varied widely (with 5 of 16 donors showing frequencies above 10%) with a wide range of subset frequency (Figure 5b). Overall, NK cell CD107a expression was apparent in all NK cell subsets, as was CD16 downregulation (Figure 5c). IFN- $\gamma$  was significantly upregulated with post-dose 2 serum in CD56<sup>dim</sup>CD57<sup>-</sup> and CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>-</sup> subsets with the highest frequency of IFN- $\gamma$  expression observed within the CD56<sup>dim</sup> NK cell subsets (Figure 5c). Almost half (41.0%) of total NK cell IFN- $\gamma$  production was attributed to CD56<sup>dim</sup>CD57<sup>+</sup> NK cells (Figure 5d). These data demonstrate that differences in NK cell differentiation status introduce additional inter-donor variation in NK cell ADCC responses.

### Discussion

We have shown that antibodies to the Ebola virus GP induced by the Ad26.ZEBOV, MVA-BN-Filo vaccine regimen activate robust *in vitro* NK cell degranulation and IFN- $\gamma$  secretion on co-culture with Ebola GP antigen. These NK cell responses are highly variable depending on vaccine regimen and interval and correlate with anti-GP IgG concentration and are markedly enriched in (although not limited to) more differentiated NK cell subsets. Varying NK cell

differentiation status between donors contributes to the heterogeneity of post-vaccination ADCC responses.

The positive correlation between post-dose 2 antibody concentration and NK cell activation demonstrates a good read-out of antibody-dependent effector function. Interestingly, MVA-BN-Filo followed by Ad26.ZEBOV vaccine regimen (groups 1 and 2), giving rise to both the highest numerical geometric mean Ebola GP-specific IgG concentration and median NK cell function did not result in significant correlations between the two factors on an individual level. NK cell CD107a expression did not increase further with the higher antibody concentrations induced by delayed second dose (day 57) compared with earlier second dose (day 29) suggesting sufficient antibody concentrations can be achieved with the early (day 29) dose 2 to obtain optimal responses [21]. NK cell IFN- $\gamma$  expression was higher with a later second dose (day 57) compared with the earlier second dose (day 29) suggesting that increasing levels of anti-GP antibody are associated with stronger NK cell cytokine secretion. The lack of correlation between antibody concentration and NK cell responses after MVA-BN-Filo followed by Ad26.ZEBOV regimen highlights a requirement for a two-dose vaccine regimen or primary vaccination with Ad26.ZEBOV to induce robust NK cell responses. This may additionally reflect effects of antibody affinity maturation and isotype/subclass switching. Of note, the Ad26.ZEBOV followed by MVA-BN-Filo vaccine regimen are being further evaluated in phase 2 and 3 clinical studies.

NK cell activation after Ad26.ZEBOV, MVA-BN-Filo vaccination regimen required relatively high serum concentrations, similar levels of NK cell degranulation occurring with up to 40-fold lower concentrations of post-seasonal influenza vaccination serum [16]. Importantly, only antibodies binding to Ebola virus GP contributed to the response; antibodies specific for the nucleoprotein (contained in MVA-BN-Filo) were not assessed. Significant correlations between NK cell function and psVNA were also observed post-dose 2, most significantly for Ad26.ZEBOV, MVA-BN-Filo vaccine regimen. This is consistent with a previously reported direct temporal and quantitative relationship between specific IgG concentrations and

neutralising activity and with a subset of vaccine induced antibodies having both NK cell activation and virus neutralising properties [20, 22].

Our data highlight substantial variation in frequencies of activated NK cells subsets both within a single donor and between donors in response to post-vaccination antibody. Many variables affect NK cell ADCC function including FcR polymorphisms [25], antibody glycosylation [26] and cytokine mediated regulation [27]. Antibody binding epitopes can also affect the ADCC function of vaccine induced antibodies [3] - neutralising antibodies bind Ebola GP core epitopes potentially inhibiting virion cell entry whereas antibodies with Fc function bind epitopes on the exposed upper domains of GP presented on the surface of infected cells [6, 28]. Ebola GP returning to the surface of the infected cell and liberation of soluble GP for immune complex formation could promote NK cell ADCC. However, GP shed from infected cells can also bind anti-GP antibodies and block, rather than facilitate their activity [29].

HCMV has seropositivity rates of up to 60% in adults in developed countries and near universal in developing countries [30]. HCMV infection strongly influences NK cell function in response to viral antigens and promotes accumulation of NK cells expressing NKG2C with a mature (CD56<sup>dim</sup>CD57<sup>+</sup>) and 'adaptive' (FcεRγ) phenotype [31-33] with enhanced IFN-γ secretion in response to antibody-coated targets [34-36]. HCMV serostatus may impact antibody-dependent NK cell activation after Ad26.ZEBOV, MVA-BN-Filo regimen vaccination, therefore, measuring NK cell function may help evaluate vaccine responses across different human populations.

Future use of CD16 expressing or transfected NK cell lines for standardisation of these assays could potentially enable comparison across multiple vaccine studies [37]. However, NK cell tumour lines such as NK-92, are largely derived from pre-NK cells and do not reflect the range of Fc receptor expression, activation requirements or functional differentiation of primary human NK cells; factors important in African settings where NK cells are enriched for highly differentiated subsets. Alternatively, Wines *et al.* have described a system using soluble



dimeric ectodomains of human FcγRIII or FcγRII (CD32) which facilitate evaluation of antibody isotype specificity and binding to low and high affinity variants of these FcR [38].

In summary, Ad26.ZEBOV, MVA-BN-Filo vaccine-induced antibody promotes strong antibody-dependent NK cell activation that correlates with antibody concentration. This study suggests NK cells are potential mediators of immunity after Ebola vaccination where responses are determined by both the level of antibody and effector NK cells differentiation status. Antibody-dependent NK cell function may help define the effector capacity of vaccine-induced antibodies when combined with antibody level or neutralisation assays.

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## **Author contributions**

HRW and MRG designed and performed the experiments, analysed the data, and wrote the manuscript. VB, JNS and KL participated in the analysis of data and advised on the manuscript. MD and GS participated in the conception and design of the work described and advised on the manuscript. AP and EAC were coinvestigators on the above trial and advised on the manuscript. MDS was the Chief Investigator on the phase 1 clinical trial of Ad26.ZEBOV, MVA-BN-Filo and advised on the manuscript. EMR wrote and advised on the manuscript.

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## **Conflict of interest statement**

VB, JNS, KL, MD, GS are employees and potential stockholders of Janssen Pharmaceuticals Inc. AJP chairs the UK Department of Health and Social Care's (DHSC) Joint Committee on Vaccination and Immunisation and the EMA Scientific Advisory Group on vaccines, and he is

a member of WHO's Strategic Advisory Group of Experts. The views expressed in the publication are those of the author(s) and not necessarily those of the DHSC, NIHR or WHO.

MDS acts as an Investigator on behalf of the University of Oxford on clinical research studies funded by vaccine manufacturers including Janssen, Pfizer, GlaxoSmithKline, Novavax, Medimmune and MCM. He receives no personal financial benefit for this work.

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## Tables

Table 1: Vaccination regimen of each trial arm and serum samples used in this study. MVA, MVA-BN-Filo; Ad26, Ad26.ZEBOV.

| Serum samples used in this study: |                  |                    |                       |                       |
|-----------------------------------|------------------|--------------------|-----------------------|-----------------------|
| Group (number of participants)    | Vaccine schedule | Baseline (Visit 0) | Post-dose 1 (Visit 1) | Post-dose 2 (Visit 2) |
| 1 (n=15)                          | MVA, Ad26        | Day 1              | Day 29                | Day 50                |
| 2 (n=15)                          | MVA, Ad26        | Day 1              | Day 57                | Day 78                |
| 3 (n=14)                          | Ad26, MVA        | Day 1              | Day 29                | Day 50                |
| 4 (n=14)                          | Ad26, MVA        | Day 1              | Day 57                | Day 78                |
| 5 (n=14)                          | Ad26, MVA        | Day 1              | Day 15                | Day 36                |

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Table 2: The correlation between NK cell CD107a, CD16 and IFN- $\gamma$  expression in response to plate bound EBOV GP plus post-dose 1 (visit 1) and post-dose 2 (visit 2) serum and anti-GP antibody concentration (determined previously by Milligan *et al.* [21]) according to vaccine regimen group. R<sup>2</sup> determined by linear regression, significance defined as p < 0.05, non-significant correlations shaded in grey.

| Visit | Group      | CD107a (%)               | CD16 (MFI)               | IFN- $\gamma$ (%)        |
|-------|------------|--------------------------|--------------------------|--------------------------|
|       |            | R <sup>2</sup> (p value) | R <sup>2</sup> (p value) | R <sup>2</sup> (p value) |
| 1     | 1 (Day 29) | 0.129 (0.207)            | 0.00292 (0.848)          | 0.0214 (0.603)           |
|       | 2 (Day 57) | 0.00469 (0.816)          | 0.0193 (0.621)           | 0.00638 (0.777)          |
|       | 3 (Day 29) | 0.480 (0.006)            | 0.550 (0.0024)           | 0.553 (0.0023)           |
|       | 4 (Day 57) | 0.0924 (0.291)           | 0.312 (0.0378)           | 0.248 (0.070)            |
|       | 5 (Day 15) | 0.394 (0.0521)           | 0.221 (0.171)            | 0.397 (0.0508)           |
| 2     | 1 (Day 50) | 0.0209 (0.607)           | 0.00775 (0.755)          | 0.00144 (0.893)          |
|       | 2 (Day 78) | 0.0639 (0.363)           | 0.0339 (0.511)           | 0.0895 (0.279)           |
|       | 3 (Day 50) | 0.660 (0.0004)           | 0.554 (0.0023)           | 0.531 (0.0031)           |
|       | 4 (Day 78) | 0.364 (0.0225)           | 0.612 (0.0009)           | 0.327 (0.0326)           |
|       | 5 (Day 36) | 0.859 (0.0001)           | 0.690 (0.0029)           | 0.276 (0.119)            |



Table 3: The correlation between NK cell CD107a, CD16 and IFN- $\gamma$  expression in response to plate bound EBOV GP plus post-dose 2 (visit 2) serum and Ebola GP-specific pseudovirion virus neutralising antibody titres (determined previously by Milligan *et al.* [21]) according to vaccine regimen group. R<sup>2</sup> determined by linear regression, significance defined as  $p < 0.05$ , non-significant correlations shaded in grey.

| Visit | Group      | CD107a (%)               | CD16 (MFI)               | IFN- $\gamma$ (%)        |
|-------|------------|--------------------------|--------------------------|--------------------------|
|       |            | R <sup>2</sup> (p value) | R <sup>2</sup> (p value) | R <sup>2</sup> (p value) |
| 2     | 1 (Day 50) | 0.0001 (0.968)           | 0.006 (0.794)            | 0.425 (0.012)            |
|       | 2 (Day 78) | 0.063 (0.363)            | 0.034 (0.511)            | 0.089 (0.278)            |
|       | 3 (Day 50) | 0.446 (0.018)            | 0.331 (0.050)            | 0.352 (0.042)            |
|       | 4 (Day 78) | 0.485 (0.006)            | 0.548 (0.003)            | 0.503 (0.005)            |
|       | 5 (Day 36) | 0.182 (0.291)            | 0.306 (0.155)            | 0.380 (0.103)            |

## Figure Legends

Figure 1: Flow cytometry gating strategy for NK cell CD107a, CD16 and IFN- $\gamma$  expression.

Flow cytometry plots show CD3<sup>+</sup>CD56<sup>+</sup> NK cell (gating strategy shown in Supplementary Figure 1a) CD107a (a), CD16 (b) and IFN- $\gamma$  (c) expression in response to 5% pre-vaccination (visit 0), post-dose 1 (visit 1) and post-dose 2 (visit 2) vaccination serum and plate bound EBOV GP antigen. Whole PBMC from one non-vaccinated single donor were used for NK cell activation assays in Figures 1-4, the NK cell differentiation phenotype of the donor is shown in Supplementary Figure 1d.

Figure 2: Antibody-dependent NK cell responses to plate bound EBOV GP after Ad26.ZEBOV, MVA-BN-Filo vaccination.

The median and 95% confidence interval of NK cell CD107a, CD56<sup>dim</sup>CD16 MFI and IFN- $\gamma$  responses to pre (visit 0), post-dose 1 (visit 1) and post-dose 2 (visit 2) vaccination serum sample are shown (a) – all vaccine arms combined (n=72). NK cell CD107a, CD16 and IFN- $\gamma$  responses were analysed according to NK cell differentiation subset defined by CD56, CD57 and NKG2C expression (gating strategy shown in Supplementary Figure 1c) (b). Each individual serum donor is represented by a dot with a line at the median. The proportion of total NK cell CD107a and IFN- $\gamma$  expression (at post-dose 2) attributed to each subset is shown as a pie graph with each slice representing the median (c). Comparisons across vaccination visits and between subsets were performed using one-way ANOVA with Holm-Sidak's test for multiple comparisons. \*\*\*\*p < 0.0001.

Figure 3: Antibody-dependent NK cell activation varies with vaccine regimen.

NK cell CD107a (a), CD56<sup>dim</sup>CD16 MFI (b) and IFN- $\gamma$  (c) expression was plotted according to vaccine regimen (groups 1-5) for pre (visit 0), post-dose 1 (visit 1) and post-dose 2 (visit 2) vaccination time points, graphs show median only. Comparisons between visits within each

group were performed using one-way ANOVA with Dunn's correction for multiple comparisons and summarised in Supplementary Table 1.

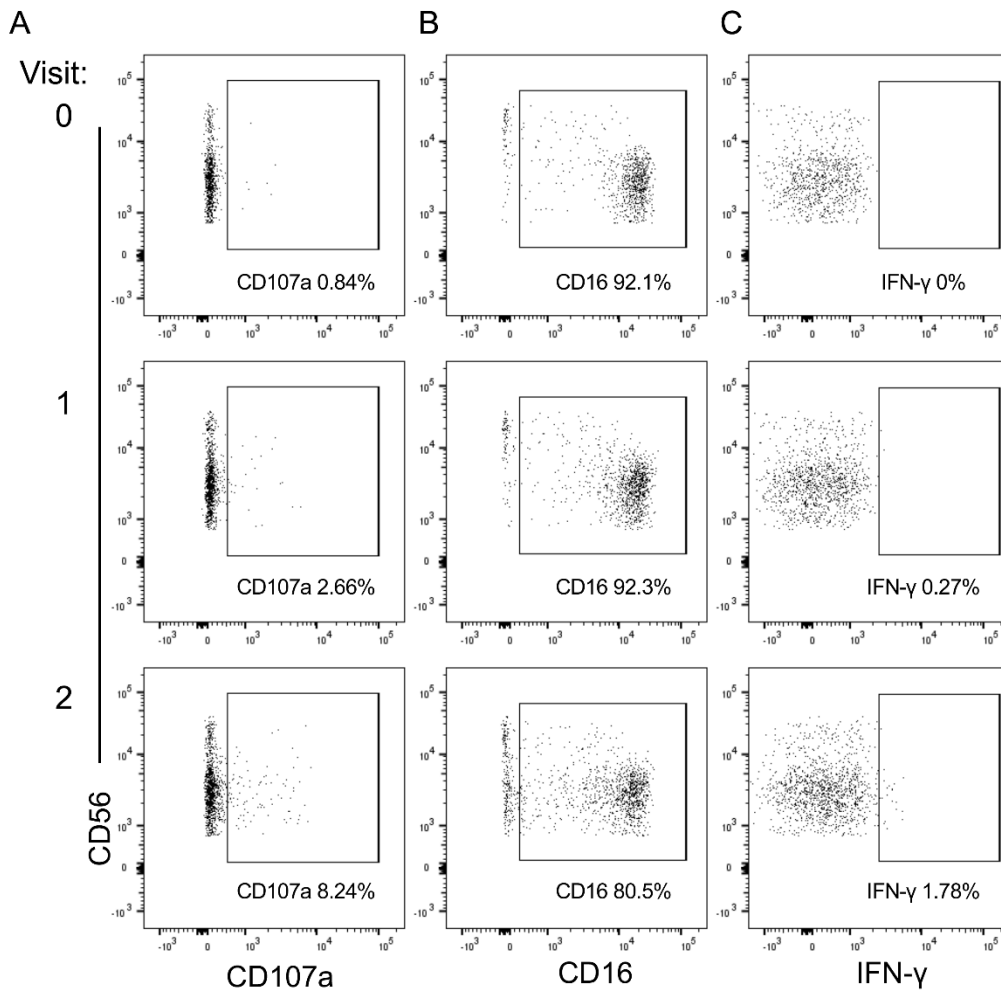
Figure 4: NK cell activation correlates with anti-GP antibody concentration and pseudovirion virus neutralising antibody titres.

Post-dose 2 anti-GP antibody concentration (a) or Ebola GP-specific pseudovirion virus neutralising antibody titres (psVNA) (b), (determined previously by Milligan *et al.* [21]) was correlated with post-dose 2 NK cell CD107a, CD56<sup>dim</sup>CD16 MFI and IFN- $\gamma$  expression, all vaccination groups combined. A two-phase nonlinear regression model was fitted in prism, R<sup>2</sup> goodness-of-fit analysis is shown, p values were determined by Pearson correlation, significance was defined as  $p < 0.05$ .

Figure 5: NK cell activation varies with NK cell donor.

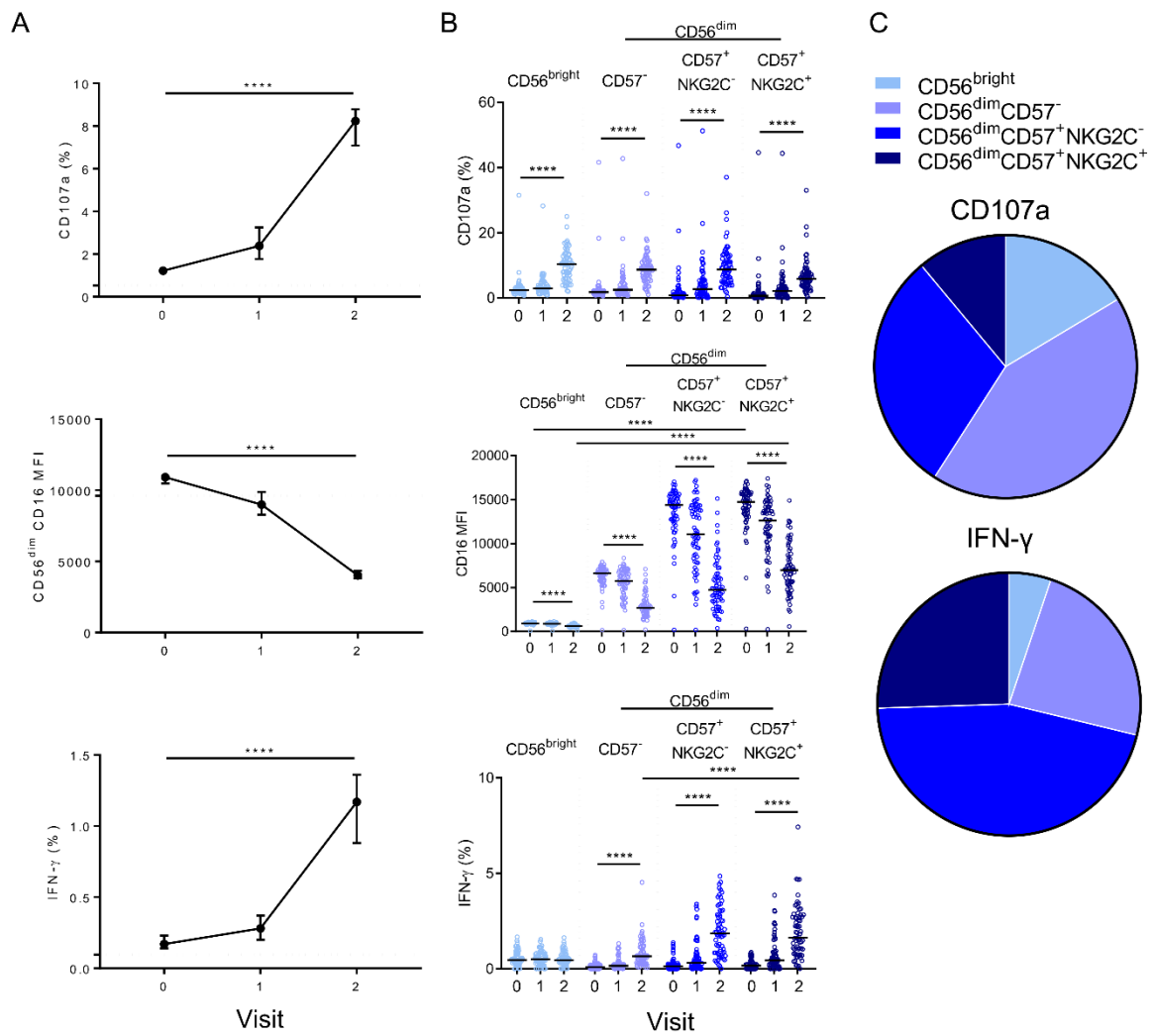
NK cell CD107a, CD56<sup>dim</sup>CD16 MFI and IFN- $\gamma$  expression (multiple non-vaccinated donors; n=16) in response to 5% pooled pre and post-dose 2 vaccination serum (group 2) and plate bound EBOV GP (a). The NK cell subset frequency distribution is shown for each donor (b). The NK cell CD107a, CD16 and IFN- $\gamma$  responses were also analysed according to NK cell differentiation subset (c) and the proportion of total NK cell IFN- $\gamma$  expression (at post-dose 2) attributed to each subset is shown as a pie graph with each slice representing the median (d). Graphs show before and after plots with a line connecting each donor or one dot per donor with a line representing the median. Comparisons between pre and post-vaccination serum responses were performed using Wilcoxon signed-rank test and between subsets using one-way ANOVA with Dunn's correction for multiple comparisons. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .

Figure 1



ACCEPT

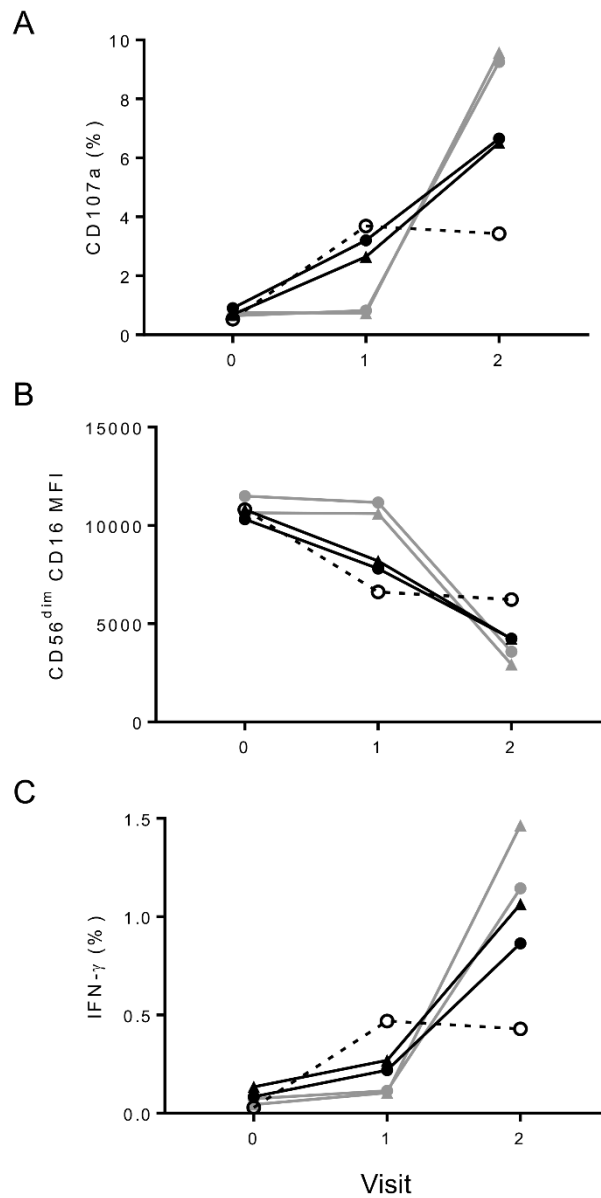
**Figure 2**



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Figure 3

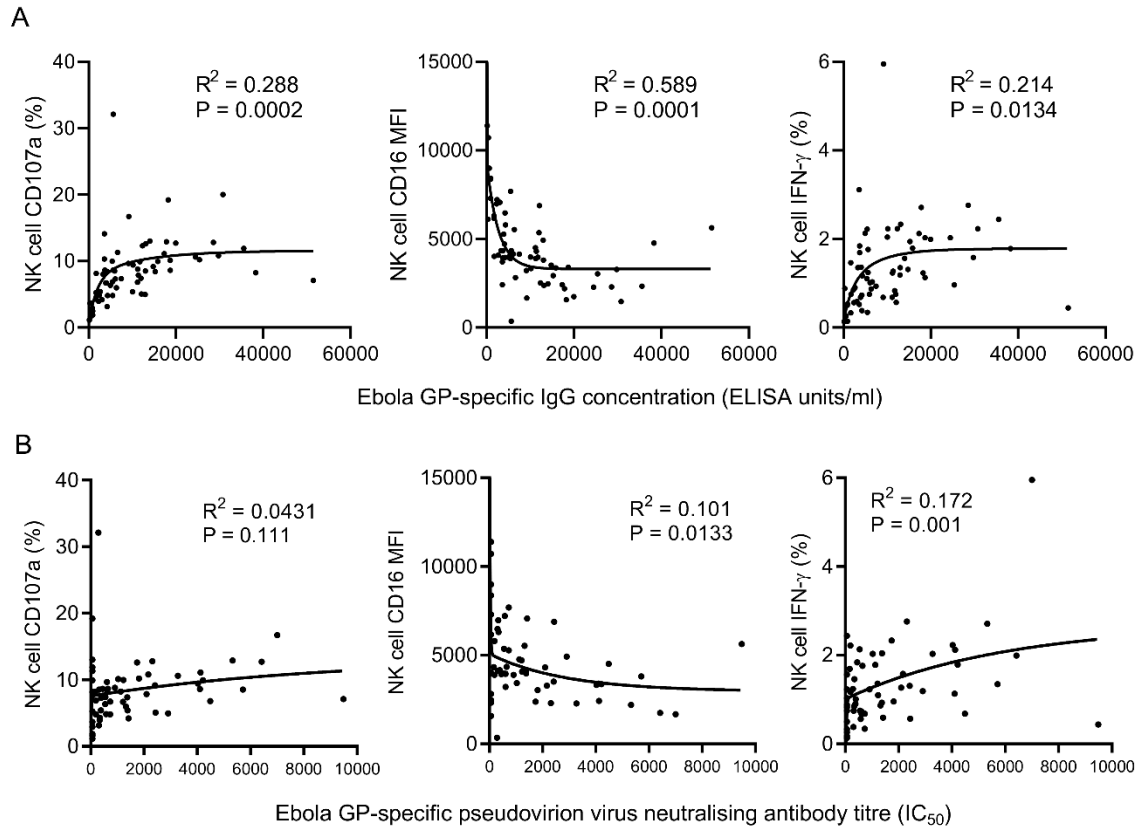
- Group 1: MVA, Ad26; dose 2 d29
- ▲ Group 2: MVA, Ad26; dose 2 d57
- Group 3: Ad26, MVA; dose 2 d29
- ▲ Group 4: Ad26, MVA; dose 2 d57
- Group 5: Ad26, MVA; dose 2 d15



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**Figure 4**



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**Figure 5**

