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# Vaccination with recombinant adenoviruses expressing Ebola virus glycoprotein elicits protection in the interferon alpha/beta receptor knock-out mouse

Lyn M. O'Brien *, Margaret G. Stokes, Stephen G. Lonsdale, David R. Maslowski, Sophie J. Smither, Mark S. Lever, Thomas R. Laws, Stuart D. Perkins

*Biomedical Sciences Department, Defence Science and Technology Laboratory (Dstl), Porton Down, Salisbury, Wiltshire SP4 0JQ, United Kingdom*

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ABSTRACT

The resistance of adult immunocompetent mice to infection with ebolaviruses has led to the development of alternative small animal models that utilise immunodeficient mice, for example the interferon $\alpha/\beta$ receptor knock-out mouse (IFNR$^{-/-}$). IFNR$^{-/-}$ mice have been shown to be susceptible to infection with ebolaviruses by multiple routes but it is not known if this murine model is suitable for testing therapeutics that rely on the generation of an immune response for efficacy. We have tested recombinant adenovirus vectors for their ability to protect IFNR$^{-/-}$ mice from challenge with Ebola virus and have analysed the humoral response generated after immunisation. The recombinant vaccines elicited good levels of protection in the knock-out mouse and the antibody response in IFNR$^{-/-}$ mice was similar to that observed in vaccinated wild-type mice. These results indicate that the IFNR$^{-/-}$ mouse is a relevant small animal model for studying ebolavirus-specific therapeutics.



## Introduction

The *Ebolavirus* genus is contained within the *Filoviridae* family and consists of enveloped viruses with non-segmented, single-strand, negative-sense RNA genomes (Sanchez et al., 2007). Serological and genetic analysis has identified five distinct species of ebolavirus: *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï Forest ebolavirus*, *Reston ebolavirus* and *Bundibugyo ebolavirus* (Falzarano et al., 2011; Towner et al., 2008). Ebolaviruses can cause outbreaks of severe haemorrhagic fever in humans and non-human primates with up to 90% fatality rates reported in humans (Feldmann et al., 2003). Transmission of ebolaviruses is generally due to direct contact with blood, secretions or infected tissues although there is also evidence for an airborne route of infection (Jaax et al., 1995; Johnson et al., 1995; Roels et al., 1999).

Licensed vaccines and antivirals are currently not available for the treatment of ebolaviruses but there is an urgent requirement for their development due to continual sporadic outbreaks and the potential for use in a bioterrorist attack (Borio et al., 2002). Although non-human primates are believed to be the animal model most representative of human disease (Bente et al., 2009; Bray and Paragas, 2002), ethical, practical and financial considerations have meant that initial efficacy screening has commonly been performed in small animal models. Unlike non-human primates, guinea pigs and adult immunocompetent mice are not susceptible to infection with naturally occurring ebolaviruses and this has led to the development of host-adapted viruses which are able to cause lethal disease in these small animals. Thus, therapeutics for ebolaviruses are often initially tested against adapted virus in a mouse or guinea pig model. They may then progress to testing in non-human primates (Rhesus and Cynomolgus macaques or African green monkeys) if efficacy is observed (Bente et al., 2009). However, positive results obtained with adapted viruses in small animal models have generally not translated into successful outcomes against ebolaviruses in the non-human primate model (Falzarano et al., 2011; Geisbert et al., 2002).

The current and most extensively used murine model employs a mouse-adapted strain of Ebola virus (Bray et al., 1998). Adaptation to mice through sequential passage resulted in a number of nucleotide changes in both coding and non-coding regions of the viral genome (Ebihara et al., 2006). The mutations principally affected the ability of Ebola virus to overcome the type I interferon (IFN) response but other mutations also contributed to the virulent phenotype. Consequently, mouse-adapted Ebola virus does not reflect natural viral properties. Whilst there are some biochemical and pathological similarities between mice infected with the adapted virus and those observed in non-human primates infected with wild-type Ebola virus (EBOV), certain differences are observed,

* Corresponding author.
*E-mail address:* lmobrien@dstl.gov.uk (L.M. O'Brien).

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for example in levels of fibrin deposition (Bray et al., 2001). Additionally, the adapted virus is only lethal to mice when administered by the intraperitoneal route (Bente et al., 2009) whereas non-human primates are susceptible to infection by multiple routes. This small animal model therefore has considerable limitations.

Although the precise mechanisms of ebolavirus disease are not known, the inhibition of type I IFN responses is believed to be crucial. Indeed, ebolaviruses encode two viral proteins, VP24 and VP35, that block IFN responses (Ramanan et al., 2011). Treatment of non-human primates with IFN-α2b prolonged survival and delayed the development of viraemia (Jahrling et al., 1999) and, in humans, it has been shown that fatal infections with EBOV are associated with a lack of an IFN $\alpha$2 response (Wauquier et al., 2010). The resistance of adult immunocompetent mice to ebolavirus infection is believed to be a consequence of the robust innate immune response, particularly the type I IFN response, of these animals (Bray, 2001; Ebihara et al., 2006). A more suitable small animal model of human infection would initiate a less vigorous innate immune response upon administration of ebolavirus. Accordingly, an alternative murine model of ebolavirus infection utilises the IFN $\alpha/\beta$ receptor knock-out (IFNR$^{-/-}$) mouse (Müller et al., 1994). EBOV is able to establish a lethal infection in this model by either the intraperitoneal (Bente et al., 2009; Lever et al., 2012), subcutaneous (Bente et al., 2009) or aerosol route (Lever et al., 2012). The pathology of infection in the IFNR$^{-/-}$ mouse is similar to that observed in non-human primates infected with ebolaviruses by various routes (Lever et al., 2012) and encompasses characteristic pathological features observed in humans (Lever et al., 2012). In addition to susceptibility to infection with EBOV, this mouse strain has been shown to be susceptible to Sudan virus (Bray, 2001) as well as Marburg virus (Bray, 2001; Lever et al., 2012).

As a first step in determining whether the IFNR$^{-/-}$ mouse could be a more appropriate small animal model for ebolavirus infection than the use of adapted virus in adult immunocompetent mice, we investigated the ability of this knock-out mouse strain to respond to adenovirus vectors expressing full-length and truncated forms of EBOV glycoprotein (GP). The GP gene of ebolaviruses has three overlapping open-reading frames which encode three proteins: GP, secreted glycoprotein (sGP) and secondary secreted glycoprotein (ssGP). The transmembrane surface GP, which mediates receptor binding and membrane fusion, is only produced after transcriptional RNA editing of the GP gene and proteolytic processing (Sanchez et al., 1996; Volchkov et al., 1998). The principal product of the GP gene is the non-structural sGP which is expressed from non-edited mRNA (Volchkova et al., 1998). sGP is secreted from infected cells and shares the N-terminal 295 amino acids with GP but differs in the C-terminal by 69 amino acids (Volchkova et al., 1998). Another editing product of the GP gene, ssGP, is also secreted from cells and shares the 295 N-terminal amino acids with sGP but lacks the C-terminal amino acids (Volchkova et al., 1998). During virus replication in vivo, 67% of GP gene-specific mRNAs direct synthesis of sGP, 31% direct expression of GP and 2% direct expression of ssGP (Mehedi et al., 2011). We constructed recombinant human adenovirus type 5 (RAd) expressing GP, sGP or ssGP and tested the ability of each vaccine to protect IFNR$^{-/-}$ mice from EBOV infection. Adenovirus-vectored GP is known to protect non-human primates from ebolavirus infection (Pratt et al., 2010; Sullivan et al., 2006; Swenson et al., 2008) and was chosen for this work as an established efficacious vaccine. However, the protective efficacy of sGP or ssGP has not been investigated. The humoral response of vaccinated IFNR$^{-/-}$ mice was also compared to that generated in mice of the wild-type (WT) parental strain to determine how the absence of an IFN $\alpha/\beta$ receptor affected the antibody response to the three recombinant vaccines.

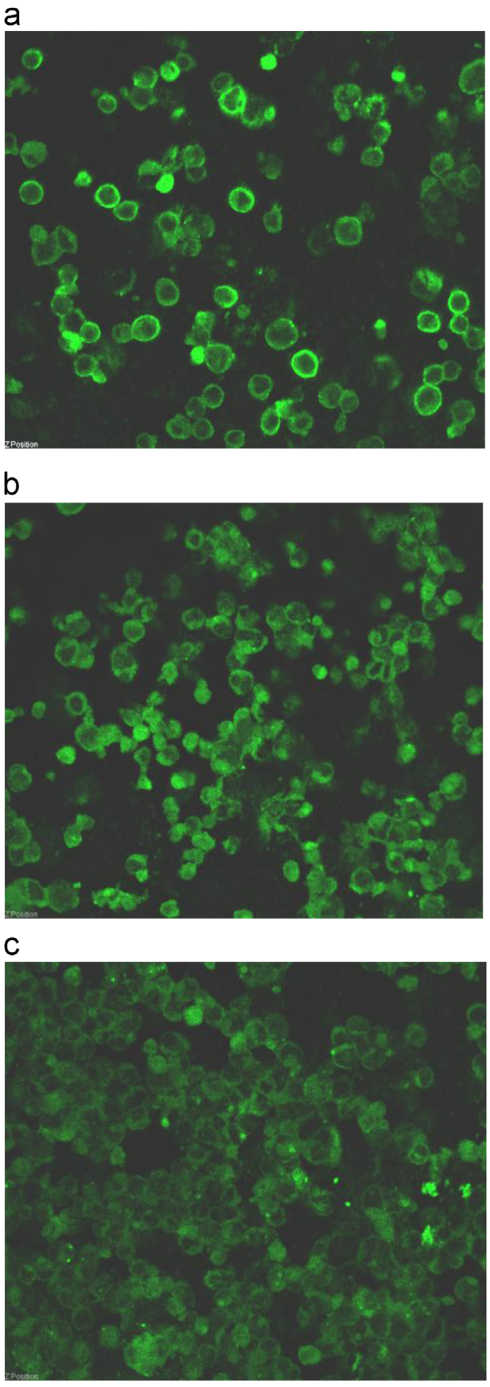


**Fig. 1.** Detection of EBOV antigen expression by immunofluorescence. HEK 293 cells infected with RAd/GP (a), RAd/sGP (b) or RAd/ssGP (c) were fixed in cold acetone and stained with polyclonal anti-EBOV followed by anti-rabbit IgG (whole molecule) conjugated to FITC. Images of representative fields of view under UV illumination were captured using a confocal microscope.

## Results

### *RAd/GP, RAd/sGP and RAd/ssGP express the full-length and truncated variants of GP*

The expression of viral glycoprotein antigen was confirmed by staining cells infected with either RAd/GP, RAd/sGP or RAd/ssGP with rabbit polyclonal anti-EBOV immunoglobulin (Fig. 1). Fluorescence was not observed with cells infected with the empty adenovirus vector, RAd (results not shown).

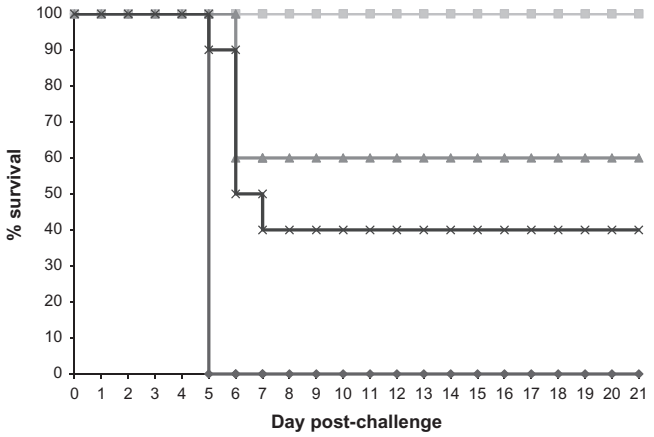


**Fig. 2.** Protection against intraperitoneal EBOV challenge. IFNR$^{-/-}$ mice ($n=10$) were immunised intranasally with $1 \times 10^7$ pfu RAd (♦), RAd/GP (□), RAd/sGP (△) or RAd/ssGP (X) on days 0, 7 and 21 prior to challenge on day 40 with 10 $TCID_{50}$ EBOV. Mice were monitored for 21d and were culled when appropriate.

*Immunisation with recombinant adenoviruses protects against EBOV challenge*

Once antigen expression had been verified, the ability of RAd/GP, RAd/sGP and RAd/ssGP to protect IFNR$^{-/-}$ mice from disease was determined. Mice were immunised by the intranasal route with three doses of RAd, RAd/GP, RAd/sGP or RAd/ssGP on days 0, 7 and 21 (a vaccination schedule shown to be effective previously with other recombinant adenoviruses; Phillpotts et al., 2005). The animals were intraperitoneally challenged on day 40 with approximately 10 $LD_{50}$ EBOV strain E718 (work with EBOV [unpublished] and Marburg virus [Smither et al., 2013] indicates that the $LD_{50}$ in IFNR$^{-/-}$ mice is equivalent to that in common marmosets). Mice vaccinated with RAd did not survive the challenge dose (Fig. 2). In contrast, significant ($P<0.0001$) levels of protection from EBOV disease were observed in mice that had been immunised with RAd/GP, RAd/sGP or RAd/ssGP (100%, 60% and 40% survival rates, respectively; Fig. 2). The protection induced by RAd/GP vaccination was significantly higher than that induced by RAd/sGP ($P<0.05$) or RAd/ssGP ($P<0.005$) vaccination. The levels of protection induced by RAd/sGP and RAd/ssGP were not significantly different ($P>0.05$).

*Immunisation with RAd/GP, RAd/sGP and RAd/ssGP elicits cross-reactive antibody*

In order to test the specificity of sera generated by the three recombinant vaccines, immune sera were utilised in Western blot protocols with recombinant GP or supernates collected from cells infected with RAd/sGP and RAd/ssGP (Fig. 3). Sera harvested from IFNR$^{-/-}$ mice immunised with RAd/GP, RAd/sGP or RAd/ssGP reacted with recombinant GP (approximately 150 kDa) and with proteins present in the supernates of infected cells (approximately 50 kDa). These bands correspond to the size of sGP and ssGP. Mice vaccinated with RAd did not react with either recombinant GP or supernatant proteins (results not shown). Vaccination with any of the three recombinant adenoviruses therefore generated antibody capable of reacting with GP, sGP or ssGP.

*Characterisation of the B-cell response following vaccination*

It is clear that IFNR$^{-/-}$ mice can respond to immunisation with recombinant adenoviruses by generating a level of immunity capable of overcoming challenge with EBOV. However, it is not known if the lack of an IFN $\alpha/\beta$ receptor has any impact on the immune response compared to that induced in WT mice on administration of the recombinant vaccines. Any differences between the induction of immunity in the IFNR$^{-/-}$ mouse and the WT mouse should be elucidated to ensure that the IFNR$^{-/-}$ mouse remains a suitable candidate for a small animal model of ebolavirus infection. Both humoral and cellular immune responses contribute to the immune response following vaccination (Warfield and Olinger, 2011) but the precise mechanism of protection is currently unknown. However, multiple vaccine studies have shown that the generation of ebolavirus-specific antibodies correlates with protection (Falzarano et al., 2011 and references within; Marzi et al., 2013; Wong et al., 2012) and survival can be reliably predicted when IgG titres reach a certain level in non-human primates vaccinated with a recombinant adenovirus (Sullivan et al., 2009). IFNR$^{-/-}$ and WT mice were therefore vaccinated with RAd/GP, RAd/sGP and RAd/ssGP in order to compare the generation of EBOV-specific antibody responses.

*Isotype profile of immune sera*

Sera were harvested 13 days after the third immunisation of each recombinant adenovirus (day 34). The concentration of EBOV-specific IgG1, IgG2a, IgG2b and IgG3 was then determined by ELISA (Fig. 4). In both IFNR$^{-/-}$ and WT mice, all three vaccines induced a similar response pattern with IgG1 and IgG2a being the principal isotypes. IgG2b and IgG3 were also induced by vaccination, in particular by RAd/GP. Although the isotype profile of IFNR$^{-/-}$ and WT mice appeared very similar, some evidence for modest differences was observed. A small but statistically significant ($P<0.05$) reduction in IgG2a concentration was detected in IFNR$^{-/-}$ mice as compared to WT mice. No significant differences were discerned in the concentrations of IgG1, IgG2b and IgG3 between IFNR$^{-/-}$ and WT mice ($P>0.05$ in all cases). Therefore, the absence of the IFN $\alpha/\beta$ receptor does not greatly affect the class of antibody produced when IFNR$^{-/-}$ mice are immunised with RAd/GP, RAd/sGP and RAd/ssGP. The high levels of both IgG1 and IgG2a, in particular in WT mice, indicate a balanced response to the glycoprotein antigens rather than an immune bias, although the significantly lower levels of IgG2a in IFNR$^{-/-}$ mice may indicate a less balanced immune response that is tending towards a $T_H2$ bias.

*Identification of linear B-cell epitopes within GP, sGP and ssGP*

Synthetic peptide libraries, consisting of 15-mer peptides that overlap by 10 amino acids, were created from the three glycoproteins. Due to the overlapping open-reading frames of the GP gene, the first 57 peptides of each library were identical. Peptides subsequent to the 57th peptide were specific for each library. However, the 58th peptide of the sGP library (designated peptide 59) and of the ssGP library (designated peptide 60) possessed 73% and 67% sequence identity, respectively, to peptide 58 of the GP library. In addition to the shared 57 peptides, the GP library contained 77 peptides (although the two C-terminal peptides were excluded from the analysis because of a failure in quality control) and the sGP library contained 14 peptides. The ssGP library consisted of the shared 57 peptides plus peptide 60 only.

A total of 30 serum samples, harvested on day 34 from groups of five WT and five IFNR$^{-/-}$ mice immunised with each of the three recombinant vaccines, were tested for reactivity to GP-, sGP- or ssGP-derived peptides that had been immobilised on microarrays. Positive responses were defined as normalised average signal intensities three times greater than the average signal intensity of the negative control features within the microarray. Excluding the first 58 peptides of each library, sera were generally specific for peptides derived from the protein used as the immunogen. Sera from one WT mouse immunised with RAd/GP bound to a peptide belonging exclusively to the sGP library, sera from one WT mouse immunised with RAd/sGP bound to five peptides belonging

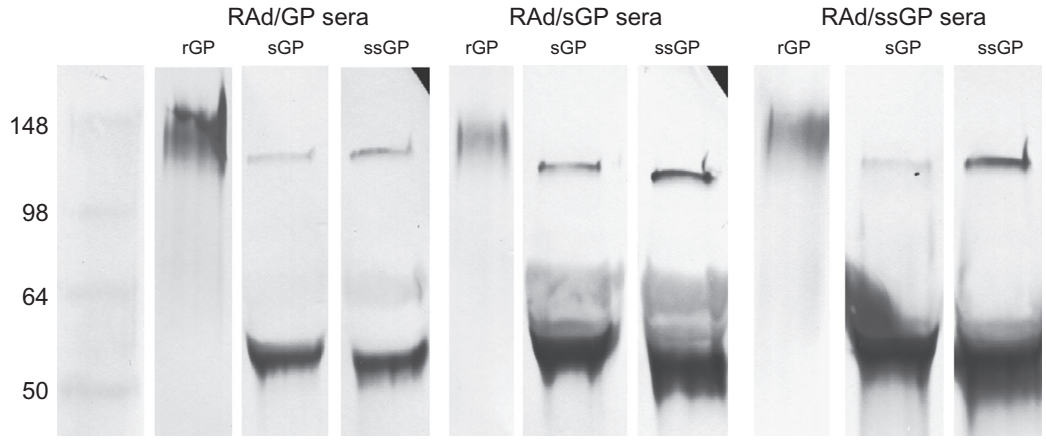


**Fig. 3.** Detection of EBOV GP, sGP and ssGP with polyclonal sera from immunised mice. Recombinant GP (rGP) or proteins present in supernates harvested from HEK 293 cells infected with RAd/sGP (sGP) or RAd/ssGP (ssGP) were resolved on a 4–20% gradient polyacrylamide gel under denaturing and reducing conditions. Membranes were probed with pooled sera from IFNR$^{-/-}$ mice immunised with RAd/GP, RAd/sGP or RAd/ssGP. Scale indicates size in kDa.

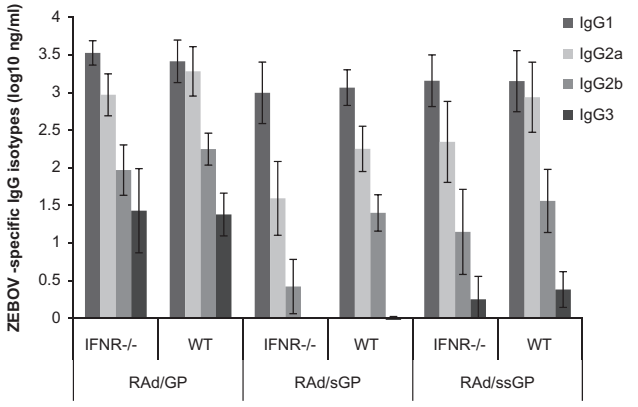


**Fig. 4.** Quantity of EBOV-specific IgG isotypes present in the sera of mice immunised intranasally with recombinant adenoviruses. IFNR$^{-/-}$ and WT mice were immunised with $1 \times 10^7$ pfu RAd/GP, RAd/sGP or RAd/ssGP on days 0, 7 and 21. Sera were collected on day 34 and assayed for anti-EBOV IgG isotypes ($n=4$, 95% confidence intervals are shown).

exclusively to the GP library and sera from two WT mice immunised with RAd/ssGP bound to a total of three peptides belonging exclusively to the GP library. As these responses showed low signal intensities and were only detectable at the lowest serum dilution tested they were excluded from further analysis.

Figs. 5–7 show the number of positive responses to each peptide within each library. In general, the pattern of binding within each library was similar for both WT and IFNR$^{-/-}$ sera. In fact, significant differences between WT and IFNR$^{-/-}$ sera were only observed for peptides 95 ($P<0.05$) and 103 ($P<0.01$) in the GP library (Fig. 5). However, a significantly greater number of positive responses was obtained overall with immune sera from WT mice compared to IFNR$^{-/-}$ mice ($P<0.001$) and signal intensities were usually stronger in samples obtained from WT mice (results not shown). Further interrogation of the data indicated that the difference in the number of positive responses between WT and IFNR$^{-/-}$ sera was only statistically significant in RAd/GP vaccinated mice ($P<0.005$, $P<0.01$ with Bonferroni's correction for multiple tests), although it is conceivable that the difference in this group is more pronounced because it has the greatest number of linear epitopes.

Whilst positive responses to GP and ssGP peptides were dispersed throughout the libraries (Figs. 5 and 7), the positive responses in the sGP library were quite distinct and were restricted principally to peptides 38 and 39 and peptides 57 and 59 (Fig. 6). As sera from RAd/GP- and RAd/ssGP-vaccinated mice also reacted strongly to peptides in these two areas of the libraries' shared region (Figs. 5 and 7, respectively), it is highly likely that they contain the sequences for linear B-cell epitopes. Peptide 38 (LPQAKKDFFSSHPLR), peptide 39 (KDFFSSHPLREPVNA) and peptide 57 (IDTTIGEWAFWETKK) reacted with the majority of serum samples (29/30, 27/30 and 30/30, respectively). As peptide 57 overlaps with peptide 58 (GEWAFWETKKNLTRK) and shares sequence identity with peptides 59 (GEWAFWETKKTSLEK) and 60 (TTIGEWAFWETKKPH), the number of positive responses to these peptides was also high (Figs. 5–7). However, in the majority of cases, the highest signal intensities were obtained with peptides 38 and 57 (results not shown). It can also be hypothesised that linear B-cell epitopes are contained within peptides 9–10, 63, 78, 93–96, 103, 108–113, 117, 121–122 and 140–141 of the GP library (Fig. 5), within peptide 82 of the sGP library (Fig. 6) and within peptides 9–10, 16–21, 26 and 49–50 of the ssGP library (Fig. 7). Thus, immunisation with RAd/GP, RAd/sGP and RAd/ssGP leads to the generation of sera that can recognise shared linear epitopes between the three proteins but that is also capable of recognising distinct linear epitopes, dependent on the protein used as the immunogen.

Tables 1–3 show the frequency of positive responses generated with each serum sample. The results have been recorded so that responses to the peptides shared between the three libraries and responses to those parts of the libraries that are specific to each protein can be distinguished. As the 58th peptide of each library shows high sequence homology to the last peptide in the shared region, it has been included in these results. When sera harvested from mice immunised with RAd/GP were tested, many more peptides in the GP-specific area of the library were recognised than in the shared region ($P<0.001$; Table 1). In contrast, RAd/sGP sera preferentially bound to peptides in the shared region rather than peptides in the specific area of the library ($P<0.05$; Table 2). Sera obtained from mice immunised with RAd/ssGP bound to many peptides in the shared region (Table 3).

## Discussion

The development of antiviral treatments and vaccines for the prevention of disease caused by ebolaviruses has been the focus of research for a number of years. Thus far, limited progress has been made in the development of effective antiviral drugs (de Wit et al., 2011). Potential therapeutics include small molecule inhibitors (Aman et al., 2009; Wolf et al., 2010), monoclonal antibodies, tissue factor inhibitors, phosphorodiamidate morpholino oligomers (Bausch

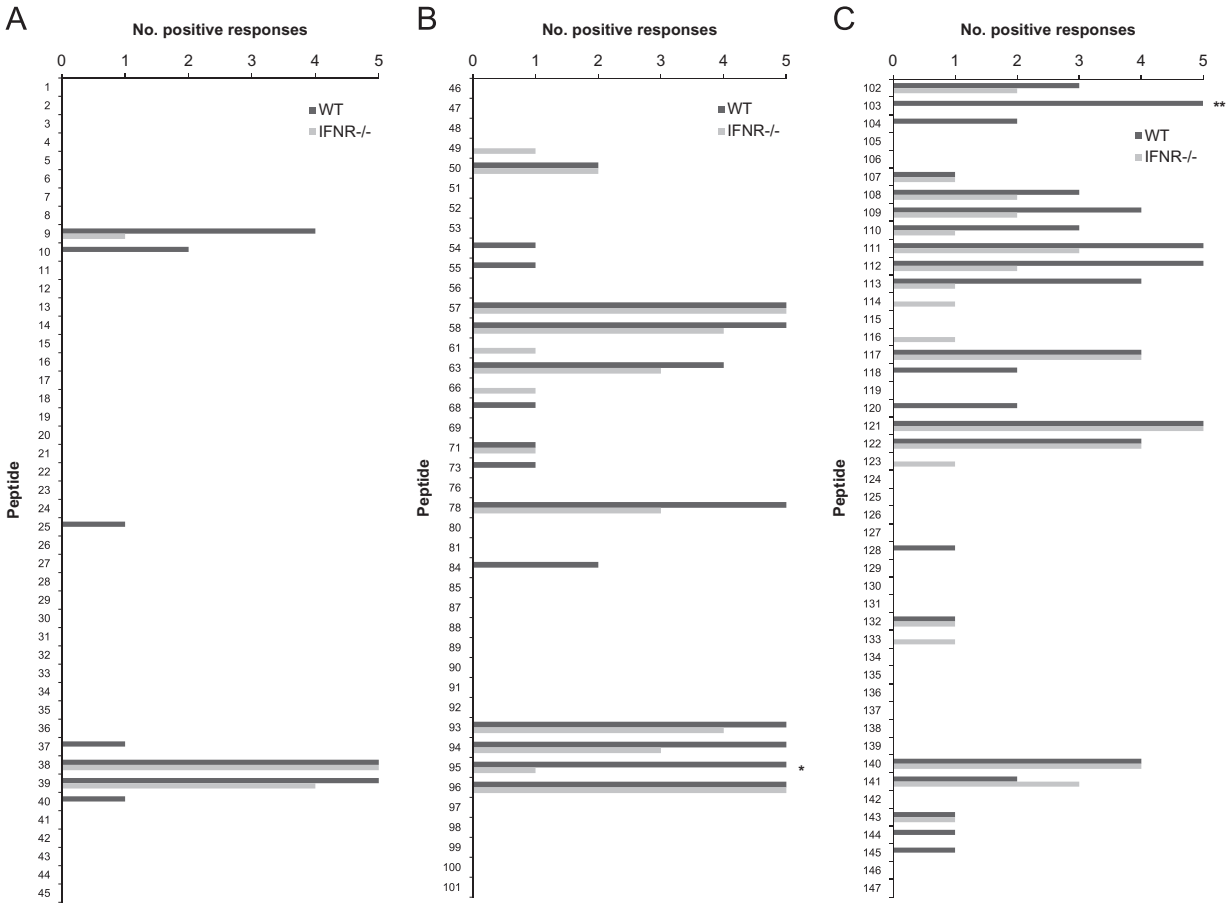


**Fig. 5.** Reactivity of polyclonal sera to EBOV GP peptides. Groups of five IFNR$^{-/-}$ and five WT mice were immunised with RAd/GP on days 0, 7 and 21. Sera were harvested on day 34 and incubated with peptides derived from GP. The figures (A)–(C) show the number of mice reacting to each peptide. Significant differences between WT and IFNR$^{-/-}$ sera are indicated ($^{*}P < 0.05$; $^{**}P < 0.01$).

et al., 2007 and references within) and short interfering RNA (Geisbert et al., 2010). Classical vaccine approaches, such as inactivated virus with different adjuvants, were generally not successful in protecting non-human primates from a lethal challenge with ebolaviruses (de Wit et al., 2011). This led to the development of recombinant ebolavirus vaccines which are based on delivery platforms such as DNA, viral vectors and virus-like particles (Falzarano et al., 2011; de Wit et al., 2011). There are advantages to using viral vectors such as human adenovirus type 5 (Croyle et al., 2008; Patel et al., 2007), human parainfluenza virus type 3 (Bukreyev et al., 2007) and Newcastle disease virus (DiNapoli et al., 2011) that have a natural tropism for the respiratory tract as administration of these recombinant vaccines generates a systemic immune response as well as a mucosal response, thereby providing protection against direct contact and the airborne route of infection. In addition to the evidence to suggest that there may be natural aerosol transmission of ebolaviruses (Jaax et al., 1995; Johnson et al., 1995; Roels et al., 1999), the most likely route of infection during a bioterrorism event would be by the airborne route.

It has been reported that a number of vaccines (including RNA replicon particles expressing ebolavirus GP plus nucleoprotein and recombinant vaccinia virus expressing ebolavirus GP) that had successfully protected mice or guinea pigs from lethal ebolavirus infection with adapted virus were subsequently shown to be ineffective when administered to non-human primates (Geisbert et al., 2002). Progress in the development of efficacious therapeutics for ebolaviruses may therefore have been hindered by the limitations of the current small animal models that utilise adapted virus. The ability of recombinant adenovirus vaccines to induce protective levels of immunity in the IFNR$^{-/-}$ mouse indicates that this knock-out mouse has the potential to be a more suitable small animal model for the study of medical countermeasures to ebolaviruses. The ability to test therapeutics against naturally occurring viral species has more relevance for non-human primate models of disease and it may therefore accurately predict the outcome of experiments in these non-human primate models. However, the IFNR$^{-/-}$ mouse would not be an appropriate model for testing antiviral drugs that exert their activity through IFN-α or -β signalling.

Recombinant adenoviruses are attractive vaccine delivery platforms because they are able to induce high levels of specific antibody and strong CD4$^{+}$ and CD8$^{+}$ T-cell responses to the transgene. Adenovirus-vectored GP has already been shown to be a successful vaccine in non-human primates (Pratt et al., 2010; Sullivan et al., 2006; Swenson et al., 2008) and has been tested in a phase I clinical trial where it was shown to be safe, well tolerated and immunogenic in the presence or absence of pre-existing adenovirus immunity (Ledgerwood et al., 2011). The utility of an adenovirus-based vaccine expressing GP, as well as vaccines expressing sGP and ssGP, has now been demonstrated in the IFNR$^{-/-}$ mouse. The reason(s) for the different levels of protection with each vaccine was not definitively ascertained in this study. IFNR$^{-/-}$ mice immunised with RAd/sGP and RAd/ssGP exhibited much lower levels of IgG3 compared to those mice immunised with RAd/GP. This may have abrogated Fc-mediated effector functions such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Sera from IFNR$^{-/-}$ mice immunised with RAd/GP showed a bias in the antibody response to peptides within GP, generally reacting with a higher frequency

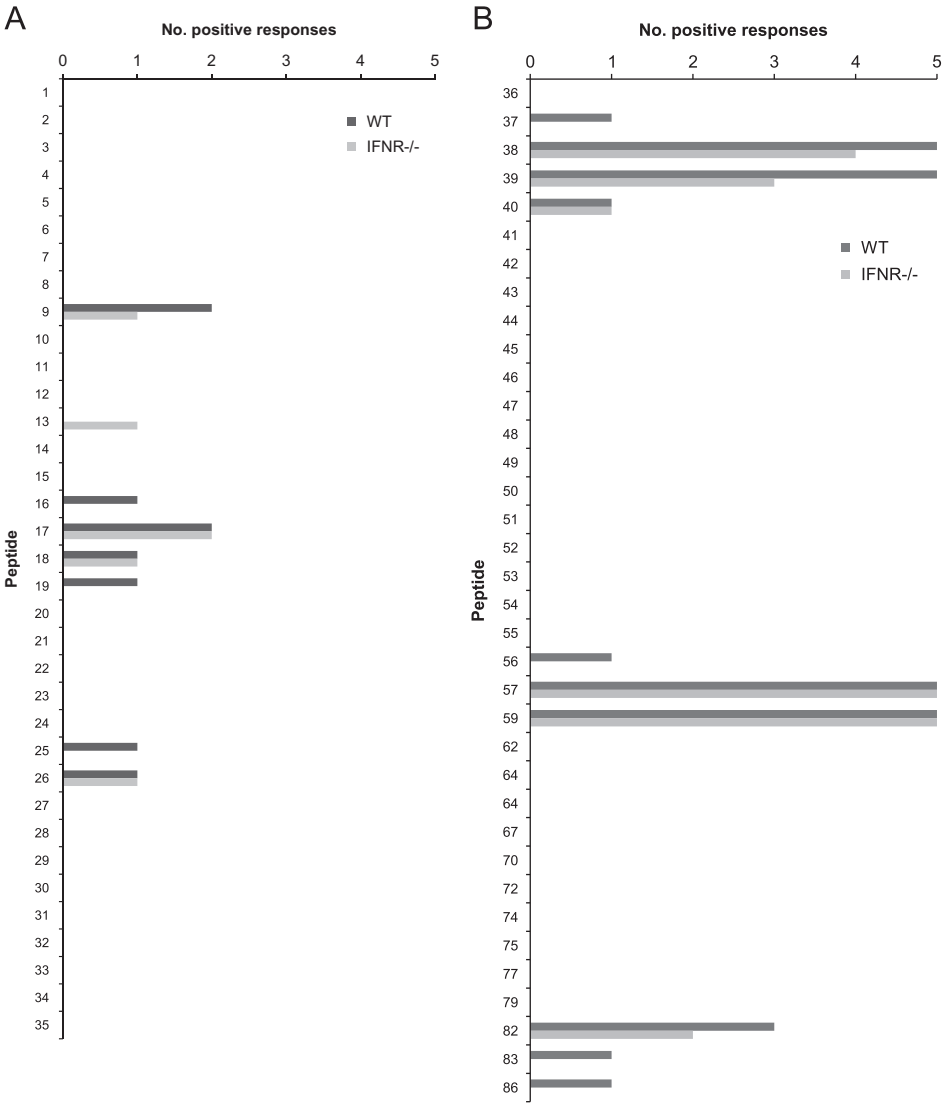


**Fig. 6.** Reactivity of polyclonal sera to EBOV sGP peptides. Groups of five IFNR$^{-/-}$ and five WT mice were immunised with RAd/sGP on days 0, 7 and 21. Sera were harvested on day 34 and incubated with peptides derived from sGP. The figures (A) and (B) show the number of mice reacting to each peptide.

to peptides in the GP-specific region. In contrast, RAd/sGP sera reacted with low frequency to peptides in both the shared region of the GP proteins and the sGP-specific region whilst RAd/ssGP sera could in the main only react with peptides in the shared region. The presence of antibodies that recognised peptides within the specific region of GP may therefore also have contributed to the superior protection elicited by the RAd/GP vaccine in IFNR$^{-/-}$ mice.

Fundamentally, the humoral immunity generated by vaccination with RAd/GP, RAd/sGP and RAd/ssGP was the same in IFNR$^{-/-}$ mice as WT mice and the absence of the IFN $\alpha/\beta$ receptor did not have a considerable impact on the antibody response. However, there were two areas of humoral immunity where the absence of the IFN $\alpha/\beta$ receptor did have a statistically significant effect. First, the IFNR$^{-/-}$ mice produced marginally less IgG2a in response to vaccination. This is consistent with reports that IFN-α promotes a bias towards a $T_H1$ immune response (Huber and Farrar, 2011 and references within) but as the reduction in IgG2a was so small it can be hypothesised that other factors contribute to immune biasing. Second, there was a significant decrease in the number of positive responses to peptides by IFNR$^{-/-}$ sera. The reason for this is unclear but there was little evidence to suggest that, compared to WT mice, IFNR$^{-/-}$ mice have an altered preference for linear B-cell epitopes. It is possible that IFNR$^{-/-}$ mice possess less B-cell diversity or that fewer B-cells in IFNR$^{-/-}$ mice are being activated upon vaccination. We feel that the latter possibility is more likely as lower concentrations of antibody were produced. In summary, the data suggest that signalling through the IFN $\alpha/\beta$ receptor plays a small role in the instigation of the humoral immune response to these recombinant vaccines.

A number of recent studies have demonstrated the essential role of antibodies in the immunity of non-human primates to ebolavirus infection. Passive immunisation with virus-specific antibodies protected non-human primates from ebolavirus infection (Dye et al., 2012; Marzi et al., 2012; Olinger et al., 2012; Qiu et al., 2012) and survival of vaccinated non-human primates correlated with the production of GP-specific antibodies (Marzi et al., 2013; Wong et al., 2012). However, it has previously been shown that protection of wild-type mice against infection with host-adapted virus requires activation of both humoral and cellular immune responses (Warfield et al., 2005). In this study we did not examine T-cell responses and so cannot make any conclusions as to how the absence of the IFN $\alpha/\beta$ receptor affects the cellular immune response or the role of T-cells in vaccine-mediated protection against ebolavirus in this mouse model. For

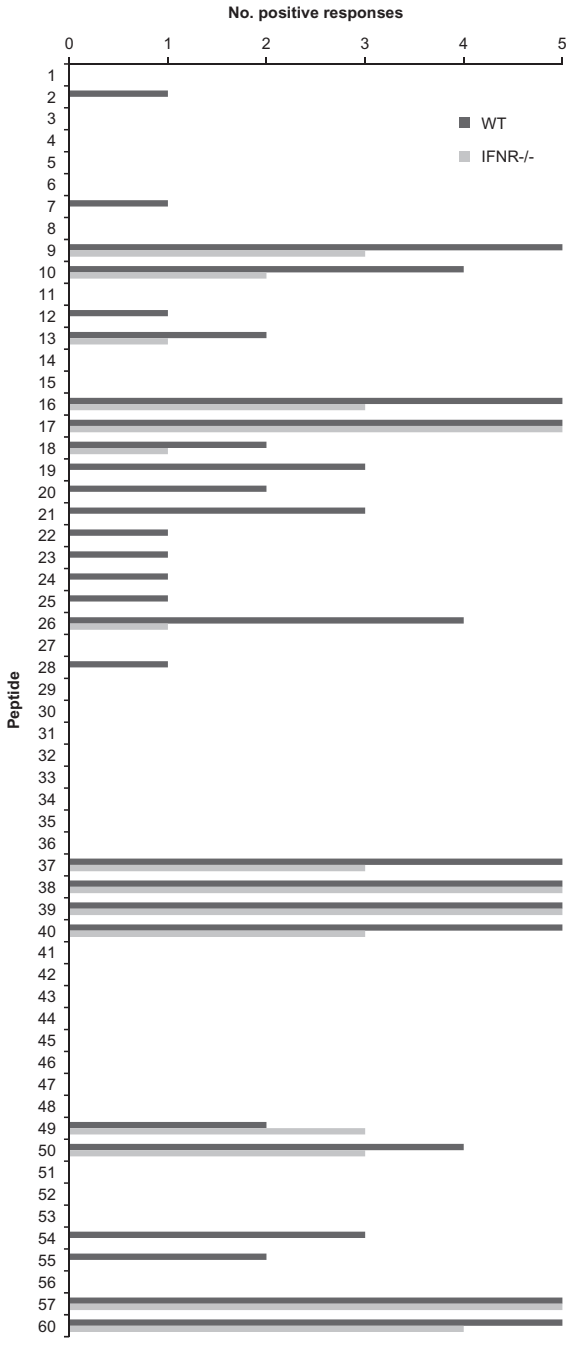


**Fig. 7.** Reactivity of polyclonal sera to EBOV ssGP peptides. Groups of five IFNR$^{-/-}$ and five WT mice were immunised with RAd/ssGP on days 0, 7 and 21. Sera were harvested on day 34 and incubated with peptides derived from ssGP. The figure shows the number of mice reacting to each peptide.

a complete picture of the immunity generated post-vaccination and to allow comparisons with the wild-type mouse model and non-human primate model, analysis of the T-cell responses in IFNR$^{-/-}$ mice is required and will be the subject of future work.

Two areas within the shared 295 N-terminal amino acids of GP, sGP and ssGP appeared to contain linear B-cell epitopes common to the three proteins. The cross-reactivity of immune sera in Western blots can be attributed to antibodies specific for these two regions. However, potential linear B-cell epitopes were also identified that were specific to the immunogen used. This may be due to the fact that GP, sGP and ssGP differ in their glycosylation patterns, and thus their tertiary structures, which likely affects the availability of epitopes (Mehedi et al., 2011 and references within).

**Table 1**
Groups of five IFNR$^{-/-}$ and five WT mice were immunised with RAd/GP on days 0, 7 and 21. Sera were harvested on day 34 and tested for reactivity with peptides derived from EBOV glycoproteins. The *P*-values are Fisher's exact test comparisons of the frequency of epitopes in the shared region compared to those in the GP-specific region.

| Serum sample | Frequency of positive responses | | | |
|---|---|---|---|---|
| | Shared region | GP-specific region | Peptide 59 of sGP library | Peptide 60 of ssGP library |
| WT | 5/57 | 19/75$^{(P<0.05)}$ | 1/1 | 1/1 |
| WT | 6/57 | 20/75$^{(P<0.05)}$ | 1/1 | 1/1 |
| WT | 4/57 | 24/75$^{(P<0.001)}$ | 1/1 | 1/1 |
| WT | 7/57 | 18/75$^{(P>0.05)}$ | 1/1 | 1/1 |
| WT | 6/57 | 21/75$^{(P<0.05)}$ | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 4/57 | 12/75$^{(P>0.05)}$ | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 4/57 | 17/75$^{(P<0.05)}$ | 0/1 | 1/1 |
| IFNR$^{-/-}$ | 5/57 | 16/75$^{(P>0.05)}$ | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 3/57 | 8/75$^{(P>0.05)}$ | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 2/57 | 13/75$^{(P<0.05)}$ | 1/1 | 1/1 |

**Table 2**
Groups of five IFNR$^{-/-}$ and five WT mice were immunised with RAd/sGP on days 0, 7 and 21. Sera were harvested on day 34 and tested for reactivity with peptides derived from EBOV glycoproteins. The *P*-values are Fisher's exact test comparisons of the frequency of epitopes in the shared region compared to those in the sGP-specific region.

| Serum sample | Frequency of positive responses | | | |
|---|---|---|---|---|
| | Shared region | sGP-specific region | Peptide 58 of GP library | Peptide 60 of ssGP library |
| WT | 3/57 | 1/14$^{(P>0.05)}$ | 1/1 | 1/1 |
| WT | 5/57 | 3/14$^{(P>0.05)}$ | 1/1 | 1/1 |
| WT | 4/57 | 2/14$^{(P>0.05)}$ | 1/1 | 1/1 |
| WT | 4/57 | 1/14$^{(P>0.05)}$ | 1/1 | 1/1 |
| WT | 11/57 | 3/14$^{(P>0.05)}$ | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 3/57 | 2/14$^{(P>0.05)}$ | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 4/57 | 1/14$^{(P>0.05)}$ | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 2/57 | 1/14$^{(P>0.05)}$ | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 4/57 | 2/14$^{(P>0.05)}$ | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 6/57 | 1/14$^{(P>0.05)}$ | 1/1 | 1/1 |

**Table 3**
Groups of five IFNR$^{-/-}$ and five WT mice were immunised with RAd/ssGP on days 0, 7 and 21. Sera were harvested on day 34 and tested for reactivity with peptides derived from EBOV glycoproteins.

| Serum sample | Frequency of positive responses | | | |
|---|---|---|---|---|
| | Shared region | ssGP-specific region | Peptide 58 of GP library | Peptide 59 of sGP library |
| WT | 10/57 | 1/1 | 1/1 | 1/1 |
| WT | 23/57 | 1/1 | 1/1 | 1/1 |
| WT | 12/57 | 1/1 | 0/1 | 0/1 |
| WT | 20/57 | 1/1 | 1/1 | 1/1 |
| WT | 14/57 | 1/1 | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 11/57 | 1/1 | 1/1 | 1/1 |
| IFNR$^{-/-}$ | 5/57 | 0/1 | 0/1 | 0/1 |
| IFNR$^{-/-}$ | 5/57 | 1/1 | 0/1 | 0/1 |
| IFNR$^{-/-}$ | 10/57 | 1/1 | 0/1 | 0/1 |
| IFNR$^{-/-}$ | 12/57 | 1/1 | 1/1 | 1/1 |

The use of the IFNR$^{-/-}$ mouse as a small animal model for evaluating medical countermeasures to other viruses is increasing. For example, it currently offers the only animal model for studying disease caused by Crimean–Congo hemorrhagic fever virus (Zivcec et al., 2013) and it provides an alternative to ruminant animal

models for the study of Schmallenberg virus (Wernike et al., 2012) and Bluetongue virus (Calvo-Pinilla et al., 2009). Although other knock-out mouse strains have been tested for their suitability as small animal models for filovirus infection (Bray, 2001; Raymond et al., 2011), the majority of research has been performed with the IFNR$^{-/-}$ mouse. It is now known that this strain is susceptible to the airborne route of infection (Lever et al., 2012) and that the mice can be protected from challenge with naturally occurring ebolaviruses by vaccination with recombinant adenoviruses. The protective mechanisms against infection with naturally occurring ebolaviruses can now be elucidated in the IFNR$^{-/-}$ mouse model and compared to the mechanisms of protection in non-human primates (Sullivan et al., 2011). This will provide further evidence of the relevance of the IFNR$^{-/-}$ mouse as a small animal model for ebolavirus infection. In addition, other filovirus therapeutics, that have already been shown to be effective in non-human primates (Bradfute and Bavari, 2011 and references within; de Wit et al., 2011 and references within), should be tested in the IFNR$^{-/-}$ mouse. The results of these studies will allow a decision to be made on the future use of the IFNR$^{-/-}$ mouse as a small animal model for infection with naturally occurring filoviruses.

## Materials and methods

### *Reagents, cells and viruses*

All reagents were supplied by Sigma (UK) unless indicated otherwise. The HEK 293 (human embryonic kidney) and Vero C1008 (simian kidney) cell lines (European Collection of Animal Cell Cultures, UK) were propagated by standard methods using the recommended culture media. The EBOV GP, sGP and ssGP sequences (NCBI accession numbers AAB81004.1, NP_066247.1 and NP_066248, respectively) were gene optimised for mammalian expression and synthesised by GeneArt (Life Technologies, UK). The three DNA sequences were cloned into the pShuttle-CMV vector (supplied in the AdEasy™ Adenoviral Vector system; Agilent Technologies, UK). RAds expressing GP, sGP and ssGP were then constructed using the AdEasy™ Adenoviral Vector system and were designated RAd/GP, RAd/sGP and RAd/ssGP, respectively. The empty adenovirus vector (RAd) has been described previously (Phillpotts et al., 2005) and was used in experiments as a negative control. Stocks of recombinant adenoviruses were produced by The Native Antigen Company (UK). Human-derived EBOV strain E718 (Ellis et al., 1979) was kindly supplied by Dr G Lloyd (Health Protection Agency, Porton Down, UK). EBOV was propagated in Vero C1008 cells for 7d prior to harvesting of infected tissue-culture supernate and clarification by centrifugation at 900 × *g* for 15 min. Clarified supernate was titrated by end-point dilution assay (Lever et al., 2012) in 96-well cell culture microplates (Corning Incorporated, USA). The 50% tissue-culture infectious dose ($TCID_{50}$) was calculated from the proportion of infected wells at each dilution using the formula of Reed and Muench (1938). All work with live EBOV was performed under UK Advisory Committee on Dangerous Pathogens (ACDP) and UK Scientific Advisory Committee on Genetic Modification (SACGM) Level 4 containment (Smither and Lever, 2012).

### *Immunofluorescence*

RAds were tested for expression of GP, sGP or ssGP by immunofluorescence. HEK 293 cells were infected with RAd, RAd/GP, RAd/sGP or RAd/ssGP for 24 h at a multiplicity of infection (moi) of 1000. Cells were then harvested, washed twice in PBS by centrifugation and resuspended in PBS. The suspension (5 μl) was spotted onto glass slides (CA Hendley Ltd., UK) which were then air dried and fixed in acetone at −20 °C for 15 min. The fixed cells were reacted for 1 h at 37 °C with 10 μg/ml polyclonal anti-EBOV (generated by immunising a rabbit with gamma-irradiated EBOV) in PBS/1% (v/v) foetal calf serum (FCS). After three washes in PBS, cells were stained for 1 h at 37 °C with FITC-labelled goat anti-rabbit IgG (whole molecule) diluted 1/1000 in PBS/1% (v/v) FCS. The slides were washed a further four times in PBS before being mounted in 50% (v/v) glycerol and examined under UV illumination.

### *Animals, immunisation and challenge with EBOV*

IFNR$^{-/-}$ (A129) mice and mice from the parental strain (129S7/SvEv; designated WT) were obtained from B&K Universal Ltd. (UK). Adult mice, aged 6–16 weeks, were caged in separate groups of males or females. IFNR$^{-/-}$ mice were housed in flexible-wall isolators within ACDP/SACGM Level 2 containment and WT mice were housed in open cages within ACDP/SACGM Level 2 containment. Mice were immunised intranasally under halothane anaesthesia on days 0, 7 and 21 with $10^7$ plaque forming units (pfu) RAd, RAd/GP, RAd/sGP or RAd/ssGP in 50 μl PBS. Numbers of male and female mice were distributed equally within treatment groups. Prior to challenge, vaccinated mice were transferred to rigid-wall isolators within ACDP/SACGM Level 4 containment and, 11d after the final immunisation (day 40), 10 $TCID_{50}$ EBOV (equating to approximately 1 plaque forming unit [pfu] and 10 50% lethal doses [$LD_{50}$]; Lever et al., 2012) were administered by the intraperitoneal route in a volume of 100 μl. Infected mice were weighed daily and observed twice daily for 21d for clinical signs (piloerection, hunched posture, immobility, eye disorders) or mortality. Mice exhibiting severe clinical signs were humanely culled. All animal studies were performed in accordance with the UK Scientific Procedures Act (Animals) 1986 and UK Codes of Practice for the Housing and Care of Animals Used in Scientific Procedures 1989.

### *Western blot*

HEK 293 cells were infected with RAd/sGP or RAd/ssGP (moi 100) and, after 24 h, supernates were harvested and centrifuged to remove cell debris. Western blots were performed with either recombinant GP or infected cell supernates. An equal volume of recombinant GP (approximately 30 μg) or supernate and Laemmli sample buffer were heated at 95 °C for 5 min. Proteins were separated on a 4–20% polyacrylamide gel (Thermo Scientific, UK) and transferred to a PVDF membrane (Invitrogen, UK). Non-specific antibody-binding sites were blocked with 1% (w/v) skim milk powder in PBS/0.1% (v/v) Tween-20 for 1 h. The membrane was then incubated for 1 h with pooled serum samples from IFNR$^{-/-}$ mice that had been vaccinated with RAd, RAd/GP, RAd/sGP or RAd/ssGP (1/100 dilution in blocking buffer; sera obtained from the marginal tail vein on day 24). After three washes in PBS/0.1% (v/v) Tween-20, the membrane was incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG (whole molecule) diluted to 1/100 in blocking buffer. Protein bands were visualised using 3,3′-diaminobenzidine, after three washes in PBS/0.1% (v/v) Tween-20.

### *Enzyme immunoassay*

Enzyme immunoassay (ELISA) was performed by standard methods. Sera, obtained by cardiac puncture of vaccinated mice on day 34, were assayed for specific antibodies using gamma-irradiated EBOV antigen diluted to approximately $2 \times 10^7$ pfu/ml in carbonate–bicarbonate buffer. The secondary antibodies were peroxidase-conjugated goat anti-mouse IgG isotypes (AbD Serotec, UK) diluted to 1/4000. Immunoglobulin concentrations were estimated by comparison of

the absorbance values generated by diluted serum samples with a standard curve prepared from dilutions of murine IgG isotypes.

*Binding of immune sera to peptide libraries*

The reactivity of immune sera (obtained by cardiac puncture of vaccinated mice on day 34) to peptides derived from the GP, sGP and ssGP proteins was assessed by ProImmune (UK) using synthetic peptide libraries (15-mer peptides, overlapping by 10 amino acids) immobilised onto ProArray Ultra™ slides. The GP peptide library consisted of 134 peptides, the sGP library of 71 peptides and the ssGP library of 58 peptides (the numbers assigned to each peptide within a library for identification purposes are not sequential). Two peptides (numbers 148 [VIIAVIALFCICKFV] and 149 [IIAVIALFCICKFVF]) located at the extreme C-terminus of GP failed to synthesise correctly and these peptides were discarded from further analysis. Peptide microarrays were incubated with dilutions of sera (1/200–1/60,000) harvested from IFNR$^{-/-}$ and WT mice immunised with RAd/GP, RAd/sGP and RAd/ssGP, as described above, followed by incubation with a fluorescently-labelled anti-mouse IgG antibody (DyLight 649 AffiniPure goat anti-mouse IgG [H+L], Jackson ImmunoResearch Laboratories, USA). After several washing steps, the arrays were dried and scanned using a high-resolution fluorescence microarray scanning system. Image analysis software was then used to quantify the fluorescence intensity associated with each fluorescent spot on the microarray slide.

*Statistical methods*

Statistical analysis was performed by using SPSS software (version18.0). Survival data were analysed using the Logrank test. Data concerning the concentration of isotypes in sera were found to be suitable for parametric analysis when transformed to the logarithm of 10 by comparison to the normal distribution in normal plots and Levene's tests for unequal variance. Antibody concentration was then analysed by multivariate linear model analysis. The frequency of epitopes was found to be suitable for parametric analysis after normalisation to the maximum and an arcsine transformation. Where multiple tests have been performed, the Bonferroni's correction was used and reported. These data were then analysed by univariate linear model analysis. Direct comparison of the response of IFNR$^{-/-}$ and WT mice to each epitope was compared by Fisher's exact test. In order to compare the proportions of epitopes recognised in shared and specific regions, two methods were used. First, data were prepared by calculating the proportion of the recognised to unrecognised peptides for each serum sample and transformed using arcsine; this rendered the data suitable for parametric analysis (confirmed by comparison to the normal distribution in normal plots and Levene's tests for unequal variance). The data were then analysed by a repeated measures univariate linear model. Also, individual serum samples were compared by Fisher's exact test. At no point was there statistical evidence to suggest that gender affected results.

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