



Exosomal vaccines containing the S protein of the SARS coronavirus induce high levels of neutralizing antibodies

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Abstract

Infection with the SARS-associated coronavirus (SARS-CoV) induces an atypical pulmonary disease with a high lethality rate. Although the initial SARS epidemic was contained, sporadic outbreaks of the disease still occur, suggesting a continuous need for a vaccine against this virus. We therefore explored exosome-based vaccines containing the spike S proteins of SARS-CoV. S-containing exosomes were obtained by replacing the transmembrane and cytoplasmic domains of the S protein by those of VSV-G. The immunogenicity and efficacy of the S-containing exosomes were tested in mice and compared to an adenoviral vector vaccine expressing the S protein. Both, S-containing exosomes and the adenoviral vector vaccine induced neutralizing antibody titers. After priming with the SARS-S exosomal vaccine and boosting with the adenoviral vector the neutralizing antibody titers exceeded those observed in the convalescent serum of a SARS patient. Both approaches were effective in a SARS-S-expressing tumor challenge model and thus warrant further investigation.

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Introduction

First reported in 2002, the severe acute respiratory syndrome (SARS) is caused by the newly emerged human coronavirus (SARS-CoV) (Drosten et al., 2003a, 2003b). The disease is characterized by an atypical severe pulmonary disease with high lethality rate (Chan-Yeung, 2004; Chan-Yeung and Yu, 2003; Chan-Yeung et al., 2003; Ho et al., 2003). Within few months after the first outbreak reported in the Guangdong province of China, the SARS-CoV rapidly spread out via international travelers and became a serious problem of public health worldwide. To date several isolated SARS-CoV infections are still occurring mainly in Asia (Peiris et al., 2004) although the major outbreak has been contained since 2003. This indicates that the SARS-CoV infection still represents a threat for the public health. In the absence of an effective therapy against SARS, the development of an effective vaccine is still needed.

The SARS-CoV is an enveloped, positive-stranded RNA virus with a genome of 29,727 nucleotides that encodes four structural proteins including the spike glycoprotein (S), the nucleocapsid protein (N), the membrane protein (M) and the small envelope glycoprotein (E), and several nonstructural proteins most of which, like in other coronaviruses, are of unknown functions (Marra et al., 2003; Rota et al., 2003). The spike S protein binds to members of the DC-SIGN family and to the angiotensin-converting enzyme 2 (ACE2) (Jeffers et al., 2004; Li et al., 2003; Wang et al., 2004), thereby mediating the entry of the virus into the target cells. Therefore, this protein represents a good target for vaccine development against SARS-CoV.

Exosomes released from dendritic cells or cancer cells have been proposed as vaccine candidates for immunotherapy of tumors (Andre et al., 2001, 2002; Chaput et al., 2003, 2005; Delcayre et al., 2005; Taieb et al., 2005; Zitvogel et al., 1998). They are small membrane vesicles with a diameter of 30 to 100 nm, which are released via multivesicular bodies from a variety of different cell types (Schartz et al., 2002). The proteomic profile of exosomes differs substantially from that of cell lysates (Amigorena, 2000; Mears et al., 2004). A number of

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cellular proteins, such as tetraspanins, heat shock proteins or MHC-I molecules are enriched in these vesicles (Denzer et al., 2000; Peche et al., 2003; Admyre et al., 2003; Hemler, 2003; Andre et al., 2004).

Exosomes can be taken-up by dendritic cells, leading to presentation of MHC-I/peptide complexes from the exosomes by the dendritic cells (Andre et al., 2004). Cellular components of the exosomes such as heat shock proteins were reported to enhance the immunogenicity and efficacy of exosome-based cancer vaccines (Chen et al., 2006). We therefore analyzed, whether the exosomal vaccine approach could also be exploited for the development of vaccines against enveloped viruses, such as the SARS coronavirus. To incorporate the S protein of the SARS coronavirus (SARS-S) into exosomes the cytoplasmic and transmembrane domains of SARS-S were replaced by those of the G protein of vesicular stomatitis virus. This chimeric protein (S^{GTM}) was efficiently expressed on the cell surface, allowed entry of pseudotyped retroviral vectors, and was incorporated into exosomes. S^{GTM} -containing exosomes were tested for their immunogenicity in mouse models as a novel protein vaccine against the SARS-CoV. Given the immunostimulatory properties of VSV-G (Marsac et al., 2002; Kuate et al., 2006), we also explored the immunogenicity of exosomes generated from cells coexpressing SARS-S and VSV-G.

Results

Construction and transient expression of wild-type and chimeric S of SARS-CoV

The S protein of the SARS-CoV, a type I transmembrane glycoprotein, is composed of an ectodomain (amino acids 17–1195), a transmembrane domain (TM) (amino acids 1196–1218), and a cytoplasmic domain (CD) (amino acids 1219 to 1255) (Giroglou et al., 2004; Spiga et al., 2003; Zeng et al., 2004). To generate the expression plasmid for the chimeric S protein, the coding region for the transmembrane and cytoplasmic domains of the S protein were replaced by those of VSV-G (S^{GTM}). A schematic representation of the encoded recombinant polypeptides is shown in Fig. 1A.

In order to assess the expression of the recombinant proteins, cell lysates from 293T cells transiently transfected with different SARS-CoV-S expression plasmids were analyzed by Western blot. For the detection of the S protein, a polyclonal antiserum raised against amino acids 441–453 of the S1 subunit was used. As shown in Fig. 1B the wild-type as well as the recombinant proteins were expressed in transfected cells. The S precursor polypeptide with a molecular weight of approximately 180 kDa could be detected in samples transfected with pS^{wt} or pS^{GTM}. The antiserum also reacted with a cellular protein with an approximate molecular weight of 100 kDa (Fig. 1B).

Cell surface expression of the wild-type and chimeric S proteins

The fusion of the ectodomain of the S protein to the N-terminus of the TM of VSV-G should lead to expression of the

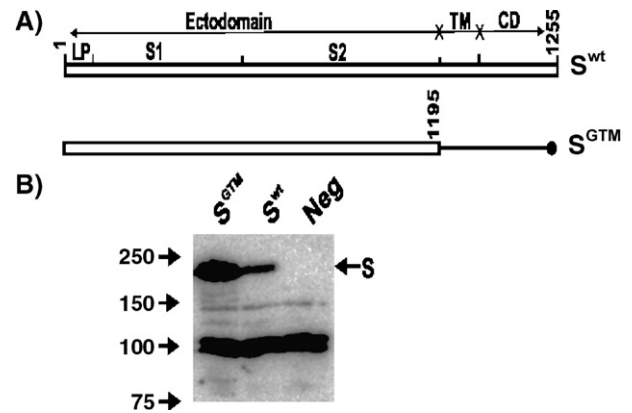


Fig. 1. Construction and expression of the hybrid S protein. (A) Schematic representation of wild-type and chimeric S proteins. The open boxes represent amino acid sequences from the SARS-CoV-S protein, the black line represents amino acid sequences from the VSV-G and the filled circle represents the VSV-G tag sequence. The numbers above the sequences indicate the position of the amino acids in the corresponding parental polypeptide. LP: leader peptide; TM: transmembrane domain; CD: cytoplasmic domain. (B) Expression of wild-type and chimeric S proteins in transiently transfected cells. The expression plasmids for the indicated recombinant proteins were transiently transfected into 293T cells and Western blot analysis of cell lysates were performed using an anti-S1 antiserum. Numbers on the left-hand side of the figure indicate the position of molecular weight size markers. S^{wt}: wild-type S protein; S^{GTM}: hybrid S protein.

hybrid protein in a membrane-bound form. The results of immunofluorescence staining (Fig. 2A) of transfected 293T cells with a SARS-convalescent serum indeed suggest such a localization for S^{wt} and S^{GTM}. To further confirm cell surface expression, 293T cells transfected with S expression plasmids were stained with a SARS-convalescent serum without disrupting the cellular membrane. As shown in Fig. 2B the wild type as well as the hybrid S proteins were expressed on the cell surface. The mean fluorescence intensity of cells expressing S^{GTM} was higher compared to that of cells expressing S^{wt} (Fig. 2B). To further compare expression levels of S^{GTM} and S^{wt} on the surface of the cell, surface proteins of transfected cells were biotinylated, lysed and precipitated by avidin-coupled beads. Western blot analysis of the precipitates revealed efficient expression of S^{GTM} but not S^{wt} on the surface of the cell (Fig. 2C, right panel). Both proteins could be detected in total cell lysates (Fig. 2C, left panel). These results indicate that the transmembrane and cytoplasmic domains of VSV-G mediate more efficient transport of the S protein ectodomain to the cell membrane than the homologous domains.

Functional assay for the chimeric S protein

The production of a fully functional hybrid S protein retaining much of the conformation of the parental S protein is important for vaccine design, since neutralizing antibodies generated after immunization with the hybrid S protein should also be able to neutralize the wild-type SARS-CoV. In addition, it could be beneficial if receptor induced conformational changes of the hybrid S protein also mimic those of the parental S protein. To test whether the S^{GTM} protein is functionally

active, SIV, HIV, and MLV-based vectors were pseudotyped with either the wild-type or the hybrid S protein. Vector titers were determined on ACE2-transfected 293T cells, which were more susceptible for vector transduction than VeroE6 cells (data not shown). Infectious SIV, HIV and MLV vectors were obtained using the hybrid S protein (S^{GTM}). Titers in the absence of a viral surface protein were below 5 GFU/ml. Incorporation of S^{GTM} into vector particles (Fig. 3A) seemed to correlate with the titers obtained for SIV, HIV-1, or MLV vectors (Fig. 3B), while similar titers were obtained for all three

vectors after pseudotyping with VSV-G. Vector titers of S^{GTM} pseudotypes were only 1.5- to 2.7-fold higher than those obtained by pseudotyping with S^{wt} , although the latter could hardly be detected in the particle preparations.

Incorporation of S proteins into exosomes

The high efficiency of expression of S^{GTM} on the surface of cells may also lead to increased incorporation into exosomes released from transfected cells. In order to assess this, Western blot analyses were performed on pelleted vesicles from pS^{wt} - and pS^{GTM} -transfected cells using anti-S1 antibodies. As shown in Fig. 4A, the S protein could not be detected in the sample from pS^{wt} -transfected cells. In contrast, the S^{GTM} protein could be detected in the pelleted supernatant of pS^{GTM} -transfected cells. We also checked for the presence of proteins characteristic for exosomes including a heat shock protein (HSP90) and the tetraspanin protein CD82. Western blot analysis of vesicles derived from cells transfected with pS^{GTM} revealed the presence of both proteins with a molecular weight of 90 kDa and 45 kDa respectively (Fig. 4A). In addition to these expected proteins, the Western blot analysis with the anti-CD82 antibody revealed incorporation of an unknown protein with a molecular weight of about 85 kDa. In contrast, HSP90, CD82, and the CD82 related protein were not detectable in blots of samples from pS^{wt} - or mock-transfected cells (Fig. 4A). The presence of HSP90 and CD82 proteins correlated with incorporation of the S^{GTM} into pelletable vesicles, indicating that S^{GTM} , but not the wild-type S protein (S^{wt}) may enhance release of exosomes into the conditioned media.

In order to check if S^{GTM} was membrane bound, S^{GTM} was pelleted from the supernatant of transfected cells through a 20% sucrose cushion, resuspended, and incubated with *n*-octyl- β -D-glucopyranoside (OG), a mild detergent solubilizing lipid membranes. The OG-treated sample was layered on a 20% sucrose cushion followed by ultracentrifugation and Western blot analysis of the pellet and the concentrated supernatant (Fig. 4B). After OG treatment, S^{GTM} was almost exclusively found in the supernatant. The absence of S^{GTM} in the pellet was not due to loss of S^{GTM} during the whole procedure, since S^{GTM} could

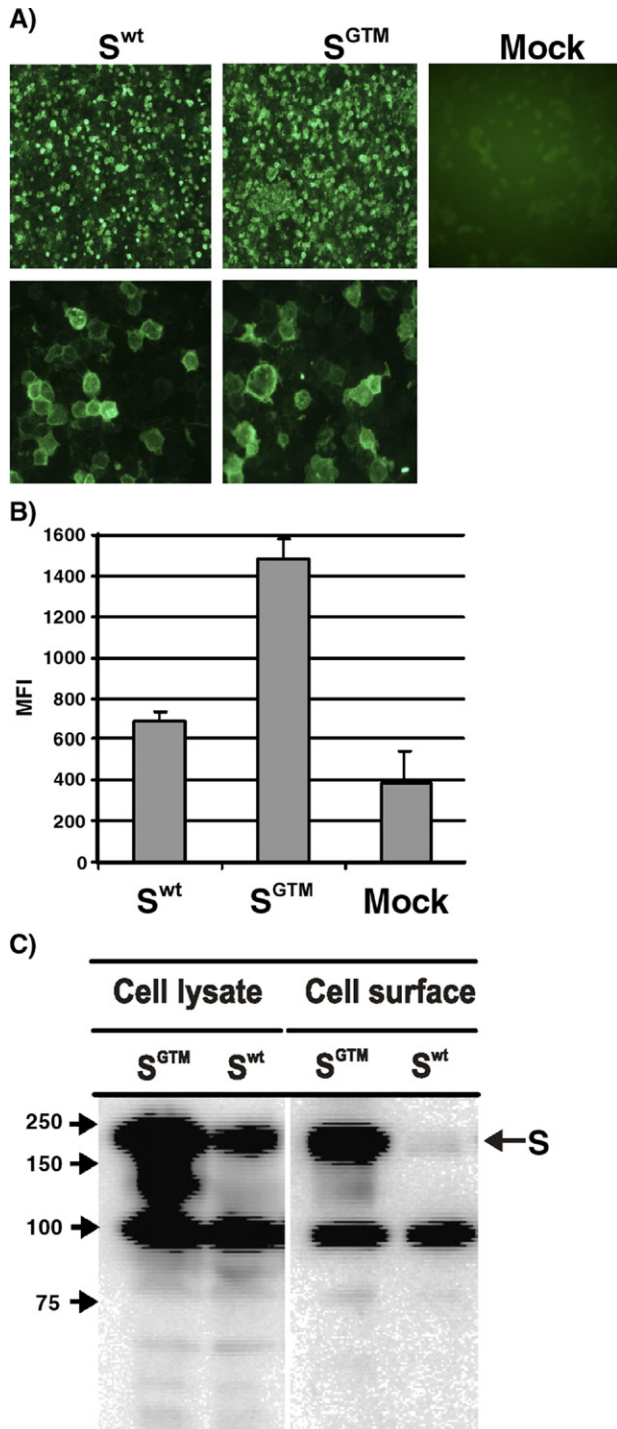


Fig. 2. Cell surface expression of S^{wt} and S^{GTM} . (A) Immunofluorescence assay. Cells transfected transiently with the indicated expression plasmids were incubated with a convalescent serum of a SARS patient and bound antibodies were detected using an FITC-conjugated anti-human secondary antibody. The upper panel represents the 10-fold magnification of the image and the lower panel a 40-fold magnification. (B) FACS analysis. 293T cells were transiently transfected with plasmid encoding the wild-type S protein (S^{wt}) or the hybrid protein (S^{GTM}) and FACS analysis was performed on intact cells using SARS patient serum and FITC-conjugated anti human IgG. The level of protein expression was expressed as mean fluorescence intensity (MFI). As negative control, mock-transfected cells were used. (C) Biotinylation assay. 293T cells were transfected with the expression plasmid for the indicated S proteins. Half of the transfected cells were lysed directly, while the extracellular domains of the membrane proteins of the other half were first biotinylated and then precipitated by avidin beads. Both, lysates from transfected cells (cell lysate) and the biotinylated and precipitated surface proteins (cell surface) were analyzed by Western blot with the S1 antiserum. Numbers on the left-hand side of the figures indicate the position of molecular weight size markers.

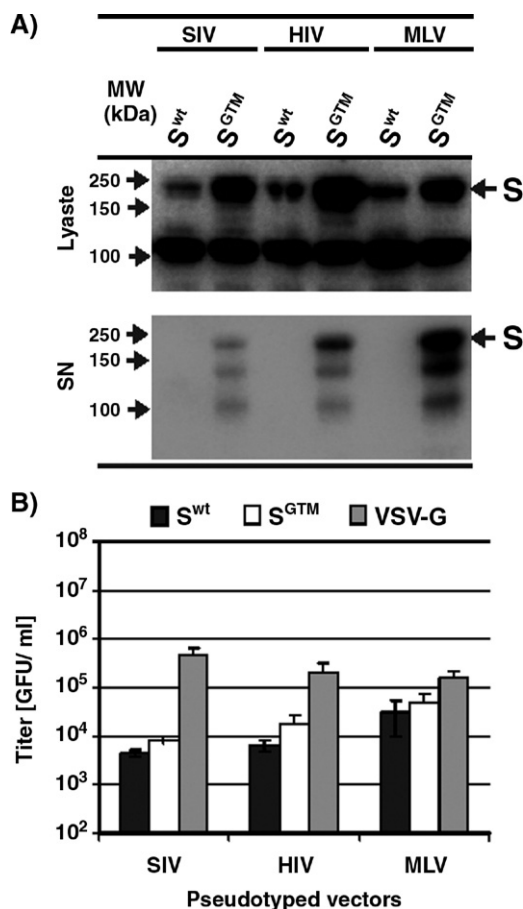


Fig. 3. Infectivity of retroviral vector particles pseudotyped with the SARS-S or the S^{GTM} protein. (A) SIV, HIV and MLV-based vectors pseudotyped with either the S protein (S^{wt}) or the hybrid S^{GTM} were obtained by transient transfection of 293T cells. Cell lysates from transient transfections (upper panel) as well as purified vector particles (SN) were subjected to Western blot analysis with the anti-S1 antiserum. Numbers on the left-hand side of the figures indicate the position of molecular weight size markers. (B) For the determination of the vector titers the conditioned media from transfected cells were used to infect 293T cells transiently expressing the ACE2 receptor. Two days later, the GFP-positive cells were counted and the titers were calculated as green fluorescence forming unit (GFP)/ml.

be repelled in the absence of OG (Fig. 4B). Thus, S^{GTM} seems to be incorporated into membrane vesicles released from transfected cells and resembling exosomal structures.

Immunogenicity of SARS-S-containing exosomes

To test the immunogenicity of the SARS-S-containing exosomes, 293T cells were transiently transfected with the S^{GTM} expression plasmid. The serum-free conditioned media were harvested and the exosomes were partially purified by ultracentrifugation through a 20% sucrose cushion. Since we previously observed that incorporation of VSV-G into immunodeficiency virus-like particles enhanced immune responses to HIV-1 Gag (Kuate et al., 2006), we also coexpressed VSV-G and S^{GTM} in 293T cells and purified the released exosomes by ultracentrifugation. Both exosome preparations contained similar amounts of S^{GTM} (Fig. 4C, left panel). The preparation of

exosomes from cells coexpressing VSV-G and SARS-S (G+S^{GTM}) contained both proteins (Fig. 4C, right panel). In this Western blot analysis, both proteins were detected using an antibody recognizing the cytoplasmic domain of VSV-G. The ratio of the band intensities of the S^{GTM} and VSV-G proteins therefore indicates that in the vaccine preparation VSV-G is present at molar excess (Fig. 4C, right panel). These exosome-based vaccines were compared to an adenoviral vector expressing S^{GTM}, since adenoviral vector vaccines were shown to induce strong immune responses to a broad spectrum of antigens encoded by the adenoviral vector. The adenoviral S^{GTM} vector was constructed using a modified pAd-Easy system (He et al., 1998; Kuate et al., 2004). The adenoviral vector was purified by CsCl gradient centrifugation and expression of S^{GTM} was confirmed by Western blot analysis (data not shown). We also explored a prime–boost regimen of priming with the exosomal vaccine and boosting with the adenoviral vector vaccine.

A single injection of the adenoviral vector expressing S^{GTM} induced antibodies binding to SARS-S expressed in murine B16 cells (Fig. 5A). For the exosomal vaccines two injections were required to induce similar antibody levels to SARS-S (Fig. 5B). Coexpression of VSV-G and SARS-S (G+S^{GTM}) only led to a slight increase in antibody levels to SARS-S, while the highest antibody levels were obtained after priming with S^{GTM} exosomes and boosting with the adenoviral vector vaccine (Fig. 5, right panel). Although the binding antibody levels were significantly higher in all vaccinated groups compared to the non-vaccinated control group ($p < 0.001$, one-way ANOVA on log-transformed MFI), the differences between the vaccinated groups did not reach statistical significance.

For the determination of the neutralizing antibody titers, S-pseudotyped retroviral vector-based (Figs. 6A, B) and wild-type SARS-CoV-based (Fig. 6C) neutralization assays were performed. When using S-pseudotypes for the neutralization assay, the highest serum dilution reducing vector infectivity by more than 90% was determined, in order to avoid minor unspecific inhibitory effects of sera at high concentrations. The highest neutralizing antibody titers were obtained in animals that were primed with S^{GTM} and boosted with Ad-S^{GTM} vectors (Fig. 6A). Comparison of neutralizing antibody titers of this group to the group immunized once with Ad-S^{GTM} indicates that S^{GTM} immunization had a priming effect (Fig. 6A). Two injections of the exosomal S^{GTM} vaccine also induced substantial neutralizing antibody titers that were slightly higher if VSV-G was included in the exosomal vaccine (Fig. 6A). The neutralizing antibodies specifically inhibited SARS-S-mediated entry, since the same sera did not reduce the titer of retroviral vectors containing the amphotropic MLV-Env instead of SARS-S (Fig. 6A).

To compare the neutralizing activity of the antibodies obtained from immunized mice to that obtained during natural SARS coronavirus infection in humans, a convalescent serum of a SARS patient obtained 10 weeks after onset of symptoms was analyzed side by side with selected mouse sera using the pseudotyped retroviral vector. Sera from S^{GTM}+Ad-S^{GTM}-immunized mice inhibited vector infectivity more efficiently than the convalescent serum of the SARS patient (Fig. 6B).

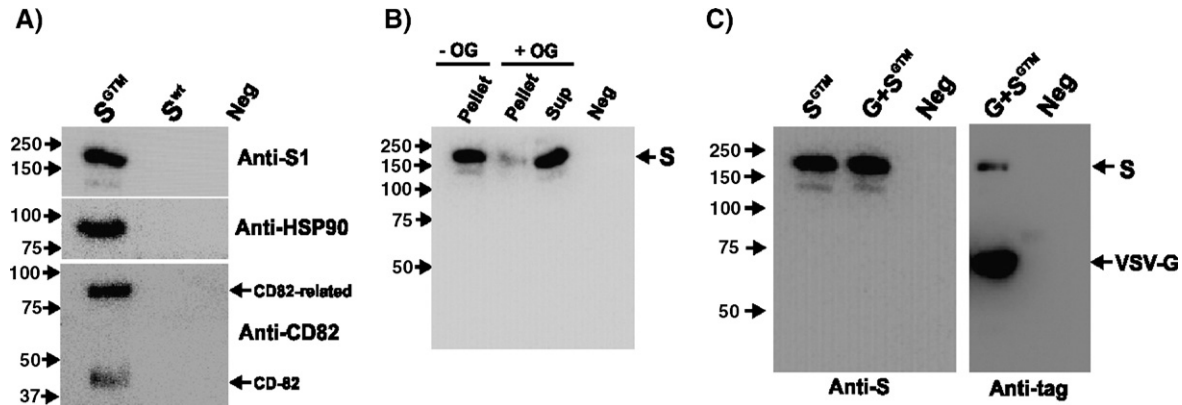


Fig. 4. Characterization of the S exosomal vaccines. (A) Incorporation of wild-type and chimeric S protein in exosomes. 293T cells were transfected with plasmids expressing the indicated proteins and vesicles pelleted from the conditioned media were analyzed by Western blot for the presence of the S (upper band), HSP90 (middle band) or CD82 (lower band) proteins using the indicated antibodies. (B) Membrane association of S^{GTM}. Pelleted S^{GTM} was resuspended, treated (+OG) with *n*-octyl- β -D-glucopyranoside, and repelleted through a sucrose cushion by ultracentrifugation. After centrifugation, the pellet was harvested and supernatant (sup) was concentrated by ultrafiltration. The samples obtained (pellet and sup) were analyzed for the presence of the S protein by Western blot using the S1 antiserum. As controls, untreated (-OG) pelleted S^{GTM}, and pellets from the conditioned media of mock-transfected cells (Neg) were processed the same way. (C) Western blot analysis of the exosomal vaccines. Conditioned media from 293T cells transfected with plasmid expressing the hybrid protein together (G+S^{GTM}) or not (S^{GTM}) with that expressing the VSV-G were concentrated by ultracentrifugation and the exosomes were analyzed by Western blot using the S1 antiserum (left panel) or an anti-VSV-G antibody (right panel).

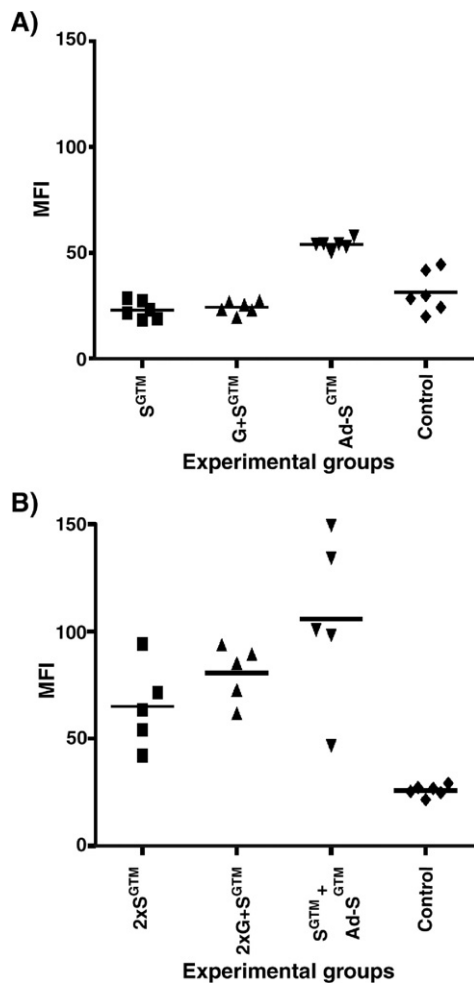


Fig. 5. Binding antibody levels. B16 cells stably expressing the S protein were incubated with sera obtained after a single immunization (A) or the indicated prime–boost regimen (B). After washing to remove unbound antibodies, cells were incubated with FITC-labeled anti-mouse antibodies and FACS analysis was performed to determine the mean fluorescence intensity of the samples.

To confirm, that the antibodies inhibiting entry of SARS-S-pseudotyped retroviral vectors, also neutralize wild-type SARS-CoV, pooled antisera from the group of mice receiving two S^{GTM} injections or an S^{GTM} prime and adenoviral vector boost immunization were tested in a virus neutralization assay. Consistently, the highest neutralizing activity was obtained for the S^{GTM} prime adenoviral vector boost regimen, which induced higher titers of neutralizing antibodies than those detected in the convalescent serum of the SARS patient (Fig. 6C).

Efficacy of SARS-S-containing exosomes in a tumor challenge model

A tumor challenge model was established to explore the relative efficacy of different SARS-S-based vaccines. To this end B16 melanoma cells were established stably expressing the wild-type SARS-S protein. Non-immunized mice inoculated with these cells develop tumors within 1 or 2 weeks after injection (Fig. 7A, 7B). Immunized mice were challenged with the tumor cells 2 weeks after a single immunization or 1 week after the booster immunization. For single immunizations, the rate of tumor growth was slightly delayed in vaccinated animals compared to the controls (Fig. 7A). This delay was more pronounced in Ad-S^{GTM}-immunized animals. To compare the rate of tumor growth in the different vaccine groups, one-way ANOVA test was performed on log-transformed tumor volume at day 15 after challenge. At this time point the tumor volume in some of the control animals already reached 3500 mm³ (Fig. 7A). Larger tumor volumes were observed in control animals compared with that obtained in the vaccine groups. However, a statistically significant difference in tumor growth was found only between control group and Ad-S^{GTM}-immunized animals ($p < 0.05$).

For prime–boost immunization regimen, the same pattern of tumor growth in the control mice as in single immunization regimen was observed. In all vaccinees, the rate of tumor

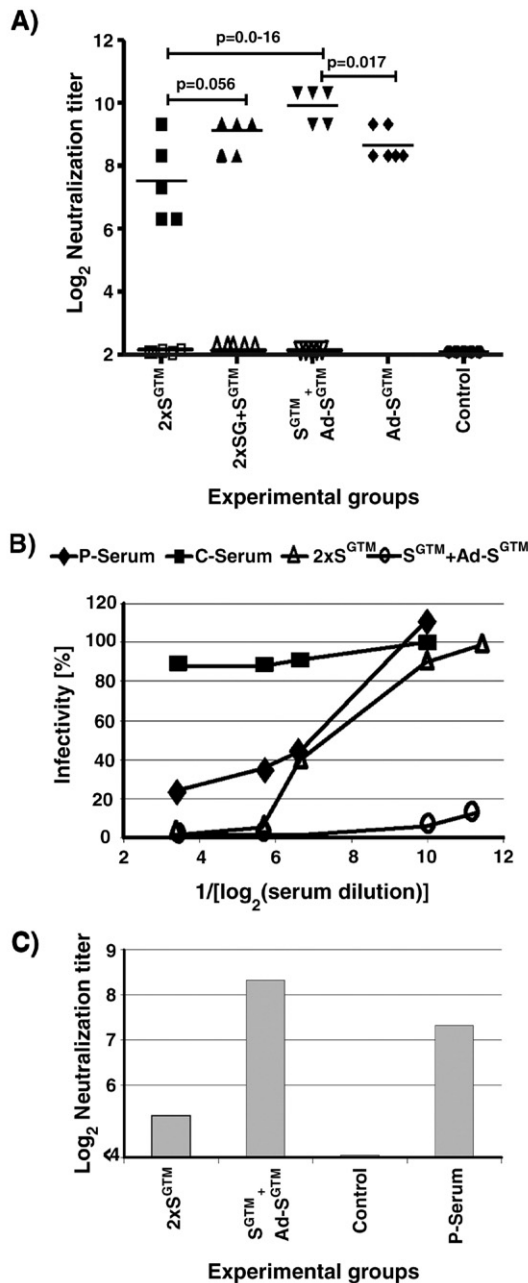


Fig. 6. Neutralization assay. (A) Sera from mice immunized with the indicated vaccines were diluted serially into serum-free DMEM medium and incubated with S-pseudotyped MLV vectors. The cocktail was added in triplicates to 293T cells transiently expressing the ACE2 receptor. Two days later, GFP-positive cells were counted under fluorescence microscope and the highest dilution that led to >90% inhibition of the vector infectivity compared to control infection was taken as the neutralizing antibody titer (closed symbols). The log₂ of the titers is given. Control infections for the specificity of the neutralizing antibodies were performed using MLV-based vectors containing the amphotropic MLV Env (open symbols). (B) Comparison of the neutralization activity of antisera from immunized mice and that of a convalescent SARS patient. The inhibition of S-pseudotyped MLV vectors incubated with different dilutions of a control serum (C-Serum), a serum from a SARS-recovered patient (P-Serum), or sera of mice immunized as indicated was determined. The log₂ of the serum dilution is given on the x-axis. (C) Virus neutralization assay. Vero cells were infected in triplicates with the wild-type SARS-CoV, incubated with different dilutions of antisera from the indicated experimental animal groups, and monitored 3 to 5 days later for formation of cytopathic effects. The neutralizing antibody titer is presented as the highest dilution of the antisera that prevent cytopathic effects in 66% of the wells.

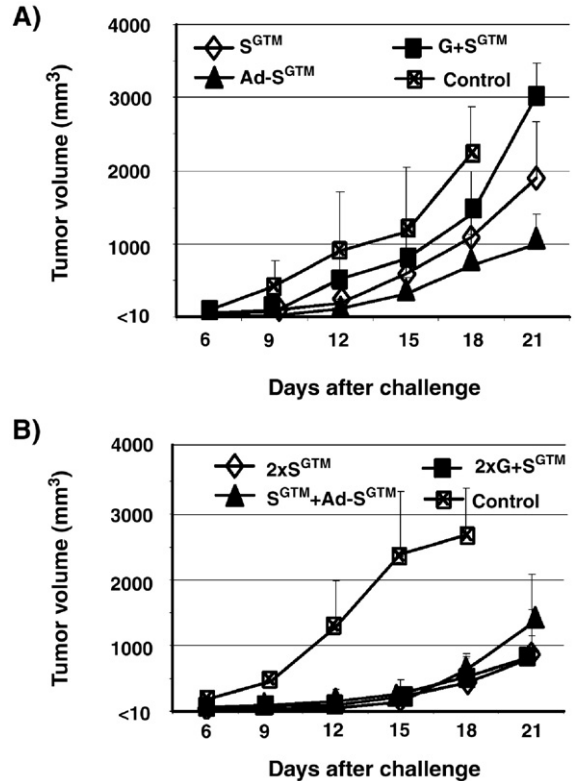


Fig. 7. Determination of the tumor growth after challenge. (A) Single immunization. Mice were challenged 2 weeks after a single immunization with the indicated vaccines with B16 cells stably expressing the S protein. The tumor growth was monitored daily and the volume was determined at a 3-day interval. (B) Prime-boost immunization. Mice immunized as indicated were challenged with the tumor cells 1 week after the second immunization and the tumor growth was monitored.

growth was strongly retarded compared to the control animals (Fig. 7B). As for single immunization, animals that received Ad-S^{GTM} as booster immunization (group S^{GTM}+Ad-S^{GTM}) controlled efficiently the tumor growth. Control of tumor growth was SARS-S specific as mice immunized twice with S^{GTM} did not control growth of B16 cells not expressing the S protein (data not shown). A comparison of the tumor volume at day 15 post challenge showed no statistically significant difference between vaccine groups (Fig. 7B). However, statistically significant differences were found when compared the volume of the tumor in control animals to those immunized with S^{GTM} ($p < 0.01$), G+S^{GTM} ($p < 0.001$) and S^{GTM}+Ad-S^{GTM} ($p < 0.01$).

Discussion

Exchanging the transmembrane domain and cytoplasmic domain of SARS-CoV-S by the corresponding domains of VSV-G altered the cellular fate of the S protein substantially. The cytoplasmic domain of VSV-G has previously been shown to determine the transport pathway (Hart et al., 1994; Andersson et al., 1997) and the basolateral localization of VSV-G in polarized cells (McQueen et al., 1986; Brown et al., 1989; Compton et al., 1989). Fusing heterologous proteins to

membrane spanning and cytoplasmic domains of VSV-G targeted the heterologous protein to the cell surface and allowed efficient incorporation into viral vector particles (Owens and Rose, 1993; Jin and Wright, 2003). This was now also observed for the ectodomain of SARS-CoV-S. The S^{GTM} fusion protein was expressed at higher levels at the cell surface compared to the wild-type protein. In comparison to S^{GTM}, incorporation of wild-type S into viral particles was severely reduced. S^{GTM} was not only incorporated into viral particles but was also functionally active as it allowed transduction of ACE2-expressing cells with pseudotyped retroviral and lentiviral vectors. This indicates that SARS-S-mediated fusion of the viral membrane with the cellular membrane is not disturbed by the heterologous transmembrane and cytoplasmic domains and suggests that neutralizing epitopes of SARS-S are maintained in S^{GTM}.

Transfection of 293T cells with the expression plasmid for S^{GTM} also led to secretion of S^{GTM}. Consistent with an exosomal localization, secreted S^{GTM} seemed to be membrane bound and could be pelleted through a 20% sucrose cushion. Western blot analysis of S^{GTM}, but not S^{wt} vesicles or vesicles from mock-transfected cells, revealed the presence of HSP90 and CD82, two of several proteins that are characteristic for exosomes (Wubbolts et al., 2003; Clayton et al., 2005). Although exosomes are constitutively secreted from several cell types as a consequence of fusion of multivesicular bodies with the plasma membrane (Schartz et al., 2002), the lack of detection of these proteins in S^{wt} or mock-derived vesicles suggests that S^{GTM} either induces exosome formation or enhances incorporation of HSP90 and CD82 into the exosomes. It remains to be determined whether this function can be mapped to the transmembrane or more likely to the cytoplasmic domain of VSV-G.

In contrast to soluble protein vaccines, exosomes containing membrane-anchored ectodomains of viral surface proteins should have multiple copies of the same viral protein exposed on the surface of the same exosome thereby facilitating cross-linking of the B cell receptor. In this respect, they mirror virosomal vaccines that are made by reconstitution of purified viral membrane proteins into liposomes. They differ from virosomal vaccines by the fact that exosomes do not only contain the viral protein, but also a wide array of cellular proteins, some of which have been shown to support induction of immune responses. Although exosomal vaccines have been primarily raised interest due to the fact that they allow cross presentation leading to the induction of CD8⁺ T cell responses, the multimeric presentation of viral surface proteins in their natural confirmation might also efficiently trigger viral surface protein-specific B cell receptors, particularly if cellular components of the exosome provide additional stimulatory signals. Since this could also favor the induction of neutralizing antibodies, we explored the potential of S^{GTM}-containing exosomes as a novel vaccine approach against SARS coronavirus infections. Two injections of S^{GTM}-containing xenogenic exosomes without adjuvants were sufficient to induce neutralizing antibody titers. After priming with the SARS-S exosomal vaccine and boosting with an

adenoviral vector the neutralizing antibody titers exceeded those of a SARS-convalescent patient serum (Fig. 5C). In previous immunization studies using soluble S proteins, several immunizations in the presence of adjuvant were necessary to generate reasonable neutralizing antibody titers (Keng et al., 2005).

We previously observed that incorporation of fusion-competent, but not fusion-deficient VSV-G into HIV-1-based VLPs led to an approximate 100-fold increase of antibody titers against the HIV-1 capsid protein (Kuate et al., 2006). This indicates that the fusion activity of VSV-G is required for its immunostimulatory properties and suggests that VSV-G-mediated shuttling of VLP-associated antigens into particular cellular compartments can dramatically enhance humoral immune responses. However, incorporation of VSV-G into S^{GTM}-containing exosomes did not lead to a significant increase in binding or neutralizing antibodies against SARS-S. Since S^{GTM} itself is fusion-competent, the enhancing effect of fusion-competent VSV-G might be masked. Alternatively, the immunostimulatory properties of VSV-G might differ depending on the type of co-administered antigen. After fusion of the exosomal membrane with the endosomal membrane, the large ectodomain of the membrane anchored S^{GTM} should be in the lumen of the endosome, while HIV-1 capsid proteins localized within the VLP should reach the cytoplasm. Although VSV-G and S^{GTM} contain the same cytoplasmic domain directing intracellular trafficking we cannot formally exclude the possibility that VSV-G and S^{GTM} are localized on different exosomes, which might also explain the poor enhancing effect of VSV-G on SARS-S antibody titers.

Since the S^{GTM}-containing exosomes were prepared from human cells, they are expected to contain a number of human proteins, which might be targeted by the murine immune response. We therefore also determined the immunogenicity of an adenoviral vector expressing S^{GTM}. Injection of this adenoviral vector into mice should lead to expression of S^{GTM} in the vaccinees and subsequent release of exosomes containing S^{GTM}, but no other heterologous proteins. A single injection of the S^{GTM}-expressing adenoviral vector induced neutralizing antibodies and reduced the growth of SARS-S-expressing tumor cells confirming the immunogenicity of adenoviral vector vaccines. Whether the release of S^{GTM}-containing exosomes *in vivo* contributes to the strong immune responses observed remains to be determined. Immune responses to the adenoviral vector particle limit repeated application of adenoviral vector vaccines (Bangari and Mittal, 2006; Barouch et al., 2004; Yang et al., 2003). We therefore used a prime–boost regimen consisting of S^{GTM}-containing exosomes followed by the adenoviral vector boost. This prime–boost regimen induced the highest neutralizing antibody titers, which exceeded the titers obtained after a single adenoviral vector immunization or two doses of S^{GTM}-containing exosomes.

The neutralizing antibody titers induced by immunization paralleled the efficiency of control of growth of SARS-S-expressing tumor cells. All mice receiving the heterologous or

homologous prime–boost regimens were able to inhibit tumor growth. A single injection of the S^{GTM}-expressing adenoviral vector inhibited tumor growth to a similar extent as the prime–boost regimens. However, only transient suppression of the tumor growth was observed. This growth pattern was previously described for ovalbumin-immunized mice challenged with B16 cells stably expressing ovalbumin (MO4) (Bonifaz et al., 2004). Selection of tumor cells not expressing SARS-S and their subsequent outgrowth might explain this observation.

We have chosen this tumor challenge model to avoid work with infectious SARS coronavirus. Although we do not know at present, whether this model predicts vaccine efficacy against SARS coronavirus infections in animal models or in humans, it represents an additional parameter allowing preclinical ranking of different vaccine approaches to be further evaluated. Since S^{GTM}-containing exosomes and S^{GTM}-expressing adenoviral vectors induce neutralizing antibody titers that exceed those observed in the convalescent serum of a SARS patient if combined in prime–boost regimen and since humoral immunity alone could control coronavirus replication in a murine model (Yang et al., 2004), both approaches warrant further investigation.

Materials and methods

Cell culture

The human embryonic kidney cell derivative 293T (DuBridg et al., 1987) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS), L-glutamine, penicillin and streptomycin. Vero cells were maintained in minimal essential medium (MEM) (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS.

Plasmids

The plasmids pLEGFP-N1 and pEGFP-C1 were purchased from Clontech, Heidelberg, Germany. The construction of the plasmids VCGΔBH (Kuate et al., 2004) SgpΔ2 (Schnell et al., 2000) Hgpsyn (Wagner et al., 2000), pHIT-G (Fouchier et al., 1997), HIV-CL-CG (Miyoshi et al., 1998), pcTatRev (Southgate et al., 1990), pCAGG-S (Hofmann et al., 2004) and pCDNA-ACE/Z (Hofmann and Pohlmann, 2004) has been described. For the construction of the expression plasmids for the chimeric S proteins, the transmembrane and cytoplasmic domains (Chapple and Jones, 2002) of a codon-optimized version of the G protein of vesicular stomatitis virus were amplified from the plasmid pCD-Gsyn (unpublished data) using the primers 5'atttaaatgaatcactcattgaccttcaagaattgggaaataggaatataatggccttcggcgacaccggcctgagcaagaaccccatc and 5'acaagctgtagccagccatagagccaccgcatccccagcat, flanked with the restriction enzymes *SwaI* and *NheI* respectively. The PCR product was digested with *SwaI* and *NheI*, and inserted in frame into the *SwaI-NheI* digested pS^{wt} (pCAGG-S (Hofmann et al., 2004)) generating pS^{GTM}.

Western blot analysis for S protein expression

293T cells were plated into 25 cm² flasks and transfected 1 day later with 10 μg of pS^{wt} or pS^{GTM} together with 1 μg of GFP-expressing plasmid (pEGFP-C1) in order to monitor transfection efficiency. Two days later, transfected cells were resuspended into 200 μl of protein loading buffer and samples were denatured at 95 °C for 10 min. Fifteen microliters of each sample was thereafter separated using 10% SDS-PAGE and the proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell Bioscience, Dassel, Germany) using standard techniques. Blots were subsequently incubated with a 1:1000 dilution of a rabbit antiserum raised against S1 (Biomol GmbH, Hamburg, Germany), then with 1:2000 diluted goat anti-rabbit antiserum conjugated with horseradish peroxidase (Sigma, Taufkirchen, Germany). After washings, protein bands were detected using a commercially available chemiluminescence's Western blot detection kit (Biozym Scientific GmbH, Oldendorf, Germany) according to the manufacturer's instructions.

Immunofluorescence assay

293T cells were plated into 25 cm² flasks and transfected 1 day later with 5 μg of pS^{wt}, pS^{GTM} or carrier DNA. Two days later, cells were detached from the flasks using 20 mM of EDTA in PBS, washed two times with PBS and transferred onto slides by centrifugation using a cytospin centrifuge (Shandon, Biomedical polymer Inc., Gardner, USA). Cells were thereafter fixed for 10 min in cold acetone, and then incubated for 1 h with 30 μl of 1:50 diluted serum obtained from a SARS patient 10 weeks after his hospitalization. After washing to eliminate unbound antibodies, cells were incubated for 30 min at 37 °C with 30 μl of FITC-conjugated anti-human IgG (DakoCytomation GmbH, Hamburg, Germany), diluted 1:20 into 0.1% of Evan's blue solution. After another washing step the presence of green cells was monitored under a fluorescence microscope and pictures were taken.

Characterization of cell surface expression

Cells were plated and transfected as described above with 5 μg of the pS^{wt} or pS^{GTM}. Two days after transfection, cells were detached from the flask as described for the immunofluorescence assay and washed three times with the FACS buffer (2% FCS in PBS). 10⁶ cells/well were transferred into 96-well, v-bottom microtiter plates and incubated for 30 min at 4 °C with 100 μl of patient serum diluted 1:50 into FACS buffer. After three washes with FACS buffer, cells were incubated for 20 min in the dark at 4 °C with 100 μl of FITC-conjugated anti-human IgG diluted 1:100 in FACS buffer containing propidium iodide. After washing, cells were analyzed using FACS Calibur (Becton Dickinson, Heidelberg, Germany). For the biotinylation assay, 293T cells were transfected with 10 μg of the plasmids expressing the wild-type S proteins or its mutant. Two days later half of the cells were lysed in loading buffer and used for SDS-PAGE, while the other half was used for biotinylation

of surface proteins essentially as previously described (Sandrin et al., 2004).

Functional analysis of the hybrid S protein

To produce S-pseudotyped vectors, 293T cells were plated into 175 cm² flasks at a density of 8×10^6 cells/flask and transfected 1 day later with 20 µg each of the vector plasmid (VCGΔBH, HIV-CL-CG or pLEGFP-N1) and the packaging plasmid (SgpΔ2, Hgpsyn or pHIT-60 respectively), and 10 µg of the envelope expressing plasmid (pS^{wt}, pS^{GTM} or pHIT-G) as described above. For the production of HIV-based vectors, 5 µg of the plasmid pcTatRev was also cotransfected. Eight hours after transfection, the conditioned media were discarded and cells were fed with 20 ml of fresh serum-free medium (AIM-V Invitrogen, Karlsruhe, Germany). One day later, the conditioned media were harvested, cleared from cell debris by low speed centrifugation and passed through a 0.45 µm filter. To determine the vector titer, 293T cells transiently transfected with a plasmid expressing ACE2 were plated into 24-well plates at the density of 5×10^4 cells/well and infected 1 day later with serial dilutions of the conditioned media. Two days later, GFP-positive cells were counted under a fluorescence microscope and the vector titers were calculated from the number of GFP-positive cells/well.

Preparation and characterization of S exosomal vaccines

293T cells were plated in 175 cm² tissue culture flask at a density of 8×10^6 cells/flask and transfected 1 day later with either S^{wt}- or S^{GTM}-expressing plasmid. Two days after transfection, the conditioned media were harvested and the vesicles containing in these media were pelleted by ultracentrifugation through 20% sucrose cushion. Western blot analysis using an anti-S1 antibody was performed to directly compare the incorporation of the wild-type S (S^{wt}) and the hybrid (S^{GTM}) proteins into the vesicles. The pelleted vesicles were also subjected to Western blot analysis as described above, using 1:300 diluted mouse monoclonal antibodies directed against HSP90 or CD82 (Santa Cruz Biotechnology, Heidelberg, Germany) respectively.

For the preparation of the exosomal vaccines, 293T cells were plated as described above and transfected 1 day later with 80 µg of plasmid expressing the S^{GTM} together or not with 40 µg of plasmid expressing the G protein of VSV-G. Eight hours after transfection, cells were fed with 20 ml fresh serum-free medium (AIM-V, Invitrogen, Karlsruhe, Germany). One day later the conditioned media were harvested and the exosomes were concentrated by ultracentrifugation as described above. The pellets were resuspended into PBS and used for Western blot analysis or kept at -80 °C for further experiments. In order to assess the membrane-bound nature of the S^{GTM}, resuspended pellets obtained from the supernatant of transfected cells by ultracentrifugation through a 20% sucrose cushion, were incubated for 4 h at 4 °C with 30 mM of *n*-octyl-β-D-glucopyranoside (OG) (Applichem, Darmstadt, Germany) under gentle shaking in order to solubilize the cell membrane.

The treated and untreated (used as control) samples were repelleted through 20% sucrose (containing 60 mM of OG) as described above. To assess the solubilization of the S protein, the supernatant of the OG-treated sample was collected after centrifugation and concentrated approximately 50-fold using Vivaspin ultrafiltration spin column with a cut-off molecular weight of 10, 000 (Vivaspin AG, Goettingen, Germany) as described by the manufacturer. The presence of the S protein in different sample preparations was assessed by Western blot analysis. For all experiments, the conditioned media from mock-transfected cells were concentrated by ultracentrifugation and used as negative control.

For animal experiments, 30 µg total protein of each exosomal vaccine was used per inoculation, as similar amount of the S protein was observed in Western blot analysis of different vaccine preparations. For the production of the recombinant adenoviral vector expressing the hybrid S^{GTM} protein, the S^{GTM} gene was subcloned from the pS^{GTM} plasmid into the pShuttle-CMVteto2 plasmid (Kuate et al., 2004) to generate pS-TetO2-S^{GTM}. For the homologous recombination, BJ-5183 bacteria cells were co-transformed by electroporation with the linearized pS-TetO2-S^{GTM} recombinant plasmid and the pAd-easy1 (He et al., 1998). The resulting recombinant plasmid was digested with *PacI* and transfected into 293 T-Rex cells for viral production. Adenoviral particles were amplified in the same cells and purified by standard cesium–chloride gradient centrifugation as previously described (Kuate et al., 2004). The particle concentration of the vector particles was determined by optical density measurement as previously described (Mittereder et al., 1996).

Immunization and challenge of animals

For the immunization five groups of 6- to 7-week-old BL57/6 mice (Charles River Wiga GmbH, Sulzfeld, Germany) were injected at the footpad, each with 30 µg (total protein) of the exosomal vaccines. For single immunization, animals ($n=6$ /group) received either S^{GTM} (group S^{GTM}), S^{GTM} with VSV-G (group G+S^{GTM}) or 5×10^9 particles of recombinant adenoviral vector expressing S^{GTM} (group Ad-S^{GTM}). Two weeks later blood samples were collected retro-orbitally and animals were challenged by injection at the right flank of 2×10^6 B16 melanoma cells stably expressing the wild-type S protein (B16-S). As negative control, six naïve mice were also challenged with B16-S cells. For the prime–boost experiment, animals ($n=5$ /group) either received two injections of S^{GTM} (group 2×S^{GTM}) or two injection of S^{GTM} with VSV-G (group 2×G+S^{GTM}) on weeks 0 and 5. One group (S^{GTM}+Ad-S^{GTM}) was primed with S^{GTM} on week 0 and boosted 5 weeks later with Ad-S^{GTM}. One week after the last immunization, blood samples were collected and animals were challenged with B16-S cells as described above. The tumor growth was monitored daily and the volume of the tumor was determined every 3 days. The animals were sacrificed when the tumor volume exceeded 3500 mm³. To assess the specificity of tumor suppression, mice ($n=3$) were immunized on weeks 0 and 5 with S^{GTM} vaccines and

challenged on week 6 with 2×10^6 B16 cells not expressing the S protein.

Analysis of the immune responses

For the analysis of the binding antibodies, B16-S cells were plated at a density of 5×10^5 cells/well into U-bottom 96-well plates and incubated for 30 min at 4 °C with 1:50 dilution of sera collected from immunized and naïve control animals. Cells were thereafter washed twice with FACS buffer and incubated for 20 min at room temperature with 100 µl of 1:100 diluted FITC-labeled monoclonal antibodies against mouse immunoglobulin (DakoCytomation GmbH, Hamburg, Germany). After another series of 2 washes, cells were analyzed by flow cytometry as described above and the amounts of antibodies were expressed as mean fluorescence intensity.

For the determination of the neutralizing antibody titers, S-pseudotyped MLV vectors were produced as described above. For the infection, 293T cells transiently expressing the ACE2 proteins were plated into 96-well plates at a density of 2×10^4 cells/well. One day later, different dilutions of the sera were incubated for 30 min at 37 °C with vector particles and the mixtures were added in triplicate to the cells. Two days after infection GFP-expressing cells were counted under fluorescence microscope and the 90% neutralization titer was determined as the highest serum dilution inhibiting infection by more than 90%. In order to check the specificity of the neutralization assay, the same assay was performed using MLV vectors containing amphotropic MLV-Env. We further compared the neutralizing properties of the antisera from immunized mice to that from a SARS-recovered patient. Two antisera were chosen for the experiment: one with the lowest neutralizing antibody titer (from the S^{GTM} group) and another one with the highest titer (from the S^{GTM}+Ad-S^{GTM} group). These antisera together with that from a SARS-recovered patient were serially diluted and incubated with S-pseudotyped MLV vectors. Subsequently, 293T cells expressing the ACE2 receptor were infected with these samples. Two days later the number of GFP-positive cells was used to calculate the percentage of the vector infectivity. A control human antiserum was used to assess the specificity of the inhibition.

An additional neutralization assay was performed using wild-type SARS-CoV (Frankfurt 1 strain). Antisera from each group of immunized animals from the prime/boost regimen were pooled and serial dilutions of these antisera were incubated for 1 h at 37 °C with 100 TCID₅₀/well of SARS-CoV, and the mixtures were added in triplicates to fresh Vero cells. As a negative control fresh media instead of virus/antisera mixtures were added to the cells. After 3 to 5 days of incubation, cells were monitored under the microscope for CPE formation. The neutralizing antibody titer was given as the inverse of the serum dilution that prevents cytopathic effect in 66% of wells. An antiserum from a convalescent SARS patient, differing from the patient the Frankfurt 1 SARS strain was isolated from, was also included in the assay for a direct comparison of its neutralizing properties with those of the antisera from immunized mice.

Statistical analysis

For the statistical analysis, the software GraphPad Prism 4 (GraphPad software Inc., San Diego, CA, USA) was used. One-way ANOVA analysis was performed on log-transformed data and the post test was performed if the overall *P* was less than 0.05. For the post test, the Bonferroni's multiple comparison test was performed. To compare the neutralizing antibody titers between groups, the non-parametric Mann–Whitney test was performed.

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