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Targeting inside-out phosphatidylserine as a therapeutic strategy for viral diseases

M Melina Soares[1], Steven W King[2] & Philip E Thorpe[1]

There is a pressing need for antiviral agents that are effective against multiple classes of viruses. Broad specificity might be achieved by targeting phospholipids that are widely expressed on infected host cells or viral envelopes. We reasoned that events occurring during virus replication (for example, cell activation or preapoptotic changes) would trigger the exposure of normally intracellular anionic phospholipids on the outer surface of virus-infected cells. A chimeric antibody, bavituximab, was used to identify and target the exposed anionic phospholipids. Infection of cells with Pichinde virus (a model for Lassa fever virus, a potential bioterrorism agent) led to the exposure of anionic phospholipids. Bavituximab treatment cured overt disease in guinea pigs lethally infected with Pichinde virus. Direct clearance of infectious virus from the blood and antibody-dependent cellular cytotoxicity of virus-infected cells seemed to be the major antiviral mechanisms. Combination therapy with bavituximab and ribavirin was more effective than either drug alone. Bavituximab also bound to cells infected with multiple other viruses and rescued mice with lethal mouse cytomegalovirus infections. Targeting exposed anionic phospholipids with bavituximab seems to be safe and effective. Our study demonstrates that anionic phospholipids on infected host cells and virions may provide a new target for the generation of antiviral agents.

Phosphatidylserine, the most abundant anionic phospholipid of the plasma membrane, is segregated to the inner leaflet of the plasma membrane of resting mammalian cells[1,2]. This internal positioning of phosphatidylserine is maintained by ATP-dependent aminophospholipid translocases that catalyze aminophospholipid transport from the external to the internal leaflet of the plasma membrane[3]. Loss of phosphatidylserine asymmetry occurs during apoptosis, cell injury, cell activation and malignant transformation[4] and results from inhibition of the translocases or activation of phosphatidylserine exporters, or lipid-scrambling enzymes, such as scramblases[5,6].

Two lines of reasoning led us to hypothesize that exposure of phosphatidylserine and possibly other anionic phospholipids would commonly occur on the surface of virus-infected cells. First, viruses, including herpesviruses, hepatitis C virus, HIV-1, Pichinde virus, Machupo virus and vaccinia virus, activate host cells to replicate efficiently[7–10]. Virus-induced cell activation may lead to rises in intracellular Ca^{2+} that, in turn, cause phosphatidylserine externalization by activating phosphatidylserine exporters and inhibiting phosphatidylserine import by translocases[4]. This prediction should apply to both enveloped and nonenveloped viruses. Second, phosphatidylserine translocation to the external host cell surface could be an early event associated with virus-induced apoptosis, as occurs with influenza A virus[11], HIV-1 (ref. 12), herpes simplex virus-1 (HSV-1; ref. 13) and vaccinia virus[14].

To detect and target exposed anionic phospholipids, we used a mouse monoclonal IgG_3 antibody, 3G4, which binds with high affinity to complexes of the phosphatidylserine-binding plasma protein β_2-glycoprotein I (β_2GP1) and anionic phospholipids[15]. The antibody binds phosphatidylserine-expressing membranes by crosslinking two molecules of β_2GP1 bound to phosphatidylserine on the membrane[16]. The 3G4-β_2GP1-phosphatidylserine complex is only stably formed on phosphatidylserine surfaces. A chimeric version of 3G4, bavituximab, has been generated, having mouse 3G4 V_H and V_κ domains joined to human IgG1 κ constant domains.

Pichinde virus is an arenavirus that causes lethal hemorrhagic fever in guinea pigs, closely resembling Lassa fever[17] in humans. We first determined whether infection of mouse macrophage P388D1 cells by Pichinde virus induces phosphatidylserine exposure on the cell surface by flow cytometry. Anionic phospholipids began to appear on the cell surface 6 h after infection, at about the same time as viral antigens, and became more abundantly expressed over 72 h (**Fig. 1a**,**b**). Immunofluorescence microscopy showed Pichinde infection induces faint, diffuse plasma membrane staining together with intensely stained discrete regions resembling membrane blebs (**Fig. 1c** and **Supplementary Fig. 1** online). Next, we determined whether Pichinde virions carry external phosphatidylserine on their envelopes. Virus suspensions were incubated with ELISA plates coated with bavituximab or control immunoglobulin. Selective binding to bavituximab-coated plates was observed (**Fig. 1d**). Bead depletion studies established that bavituximab binds infectious virions (**Fig. 1e**). Bavituximab-coated magnetic beads specifically removed infectious Pichinde virus, confirming that infectious virions carry external phosphatidylserine (**Fig. 1e**).

The therapeutic activity of bavituximab was determined in guinea pigs with advanced Pichinde virus infections. Outbred guinea pigs

[1]Department of Pharmacology, 6001 Forest Park Road, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9041, USA. [2]Peregrine Pharmaceuticals Inc., 14272 Franklin Avenue, Tustin, California 92780, USA. Correspondence should be addressed P.E.T. (Philip.Thorpe@UTSouthwestern.edu).

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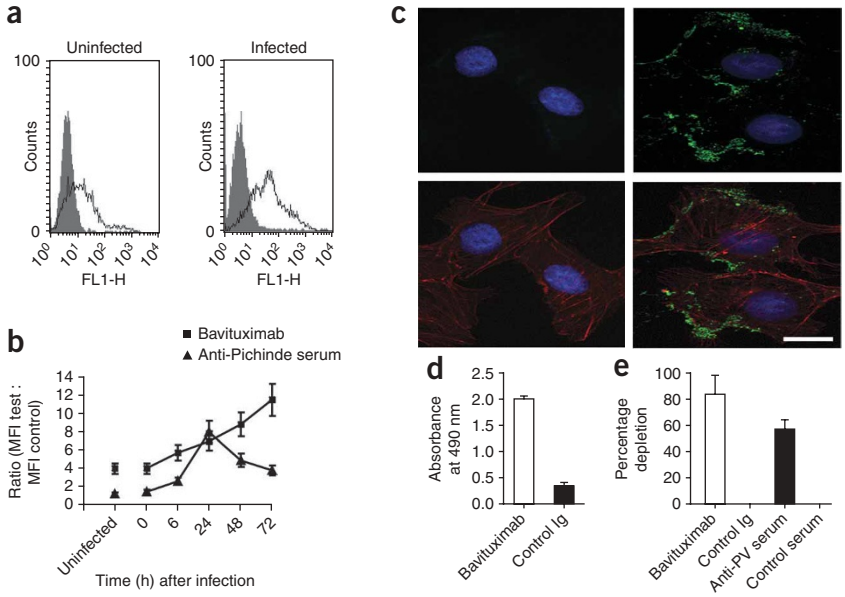

Figure 1 Bavituximab binds Pichinde virus–infected cells and infectious virions. (**a**) Flow cytometric analysis of P388D1 cells after infection at an MOI of 5. Cells were stained at 48 h with bavituximab (open histograms) or control immunoglobulin (filled histograms). Live cells were gated on the basis of their exclusion of 7-AAD. (**b**) Flow cytometric analysis of expression of phosphatidylserine and Pichinde virus antigens over time. P388D1 cells were infected with Pichinde virus at an MOI of 5 and stained at the indicated time points after infection. Phosphatidylserine expression is detected with bavituximab, and Pichinde virus antigen expression is detected with guinea pig Pichinde-specific antiserum. Data are the ratios of mean fluorescence intensity (MFI) ± s.e.m. for cells stained with test antibodies to MFI of cells stained with appropriate negative control antibodies. (**c**) Immunofluorescence staining of uninfected (left) and Pichinde virus–infected monkey kidney fibroblast Vero cells (right) with bavituximab 48 h after infection. The top panels show cells stained with bavituximab (green). The bottom panels show images merged with cytoskeleton (red). Nuclei are in blue (all panels). Control immunoglobulin did not stain and is not shown. Scale bar, 50 μm. (**d**) ELISA detection of virus binding to immobilized bavituximab or control immunoglobulin. Data represent the average absorbance ($n = 3$) ± s.e.m. $P < 0.0001$ (unpaired t-test). (**e**) Quantification of the depletion of infectious Pichinde virus by bavituximab-coated magnetic beads. Beads coated with antibodies to Pichinde virus (anti-PV) were used as a positive control. Data represent percentage depletion ($n = 3$) ± s.e.m. $P = 0.002$ (unpaired t-test).

were treated with bavituximab or isotype-matched control antibody 6–7 d after infection, when the guinea pigs showed loss of body weight, ruffled fur and elevated body temperature (data not shown). All guinea pigs in the control groups had to be killed by days 14–18. In contrast, 50% of the bavituximab-treated guinea pigs recovered (**Fig. 2a**). They gained weight and within a week lacked physical signs of disease, and they were healthy on day 135 when the experiments were terminated (**Fig. 2a**). To our knowledge, this is the first report of a therapeutic agent effective against advanced Pichinde virus infections. Fourteen days after infection, virus loads decreased in blood, spleen, lung, liver, kidney and heart from bavituximab-treated guinea pigs compared to control immunoglobulin–treated guinea pigs (**Fig. 2b**). Decreases in virus load were not seen before day 14 (**Supplementary Fig. 2** online). By day 135, the

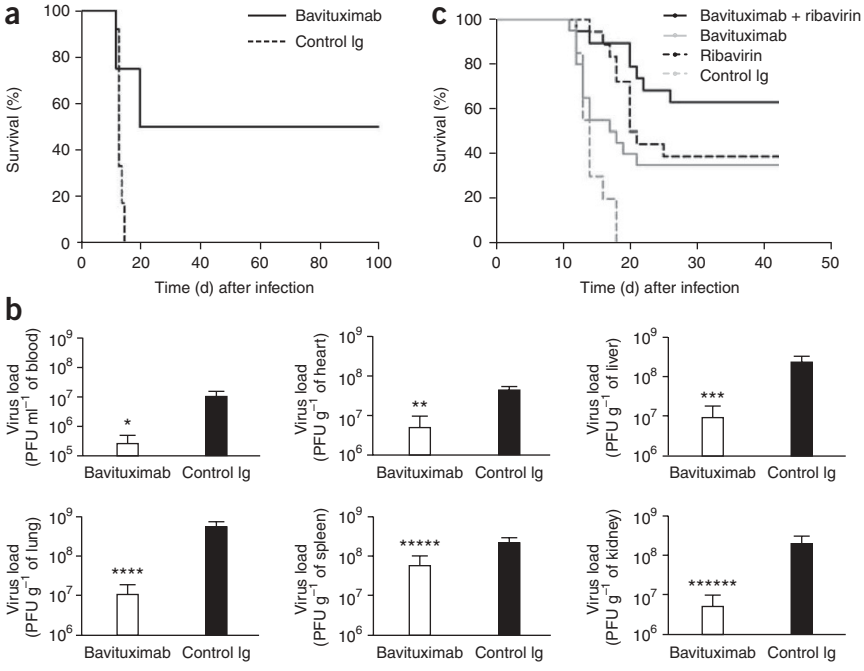

Figure 2 Therapeutic effect against Pichinde virus in lethally infected guinea pigs displaying overt signs of disease. (**a**) Kaplan-Meier survival curves of guinea pigs after lethal infection with Pichinde virus and treatment with bavituximab or control immunoglobulin (Ig). Bavituximab or control immunoglobulin (6 mg kg^{-1}) was administered i.p. to groups of guinea pigs ($n = 8$) beginning after they had developed disease signs (around day 7) and twice a week thereafter. The results are representative of five separate experiments. Survival in the bavituximab group was significantly better than that of the control group ($P = 0.0036$, log-rank Mantel-Cox test). (**b**) Virus load in tissues of treated guinea pigs 14 d after infection. Data represent the mean number of plaque-forming units (PFU) per gram of tissue ($n = 3$) ± s.e.m. The results are representative of two separate experiments. *$P = 0.0164$, **$P = 0.0361$, ***$P = 0.0436$, ****$P = 0.0139$, *****$P = 0.0992$ and ******$P = 0.038$. (**c**) Additive effects of bavituximab and ribavirin treatment on survival. Kaplan-Meier survival curves are shown for Pichinde virus–infected guinea pigs treated with bavituximab and ribavirin ($n = 19$), bavituximab ($n = 20$), ribavirin ($n = 18$) or control immunoglobulin ($n = 20$). Bavituximab or control immunoglobulin (6 mg kg^{-1}) was administered i.p. three times per week and ribavirin (8 mg kg^{-1}) was administered i.p. daily beginning after the guinea pigs developed disease signs. The combination was significantly more effective than bavituximab alone ($P = 0.011$). All treatments were significantly different from control immunoglobulin ($P < 0.0001$). The results are representative of two separate experiments.

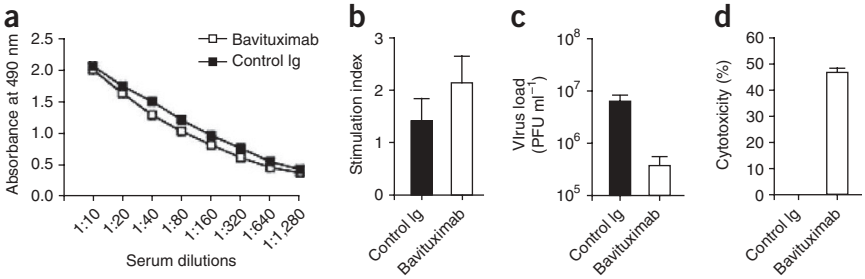

Figure 3 Mechanism of anti-viral effects of bavituximab. (**a**) Quantification of antibodies to Pichinde virus (IgG and IgM) by ELISA. Plasma from Pichinde virus–infected guinea pigs ($n = 3$) was collected 7 d after onset of treatment (14 d after infection). The titer of serum from bavituximab-treated guinea pigs was not significantly different from that of control immunoglobulin–treated guinea pigs. Data represent the average absorbance ($n = 3$) ± s.e.m. (**b**) Quantification of Pichinde virus antigen–specific proliferative response in splenocytes from bavituximab-treated guinea pigs. Spleens from bavituximab- or control-treated Pichinde virus–infected guinea pigs ($n = 3$) were removed 7 d after onset of treatment (14 d after infection). Splenocytes were stimulated with Pichinde virus antigen or mock antigen and their ability to incorporate [^{3}H]-thymidine was determined. Bavituximab treatment did not significantly increase the stimulation index. Data represent the average stimulation index ($n = 3$) ± s.e.m. (**c**) Quantification of Pichinde virus in the blood of guinea pigs treated with bavituximab. Blood samples from groups of four guinea pigs were harvested 1 d after treatment with bavituximab or control immunoglobulin. Bavituximab treatment significantly reduced virus burden. $P = 0.0145$ (unpaired t-test). Data represent the mean PFU ml^{-1} ($n = 3$) ± s.e.m. (**d**) Quantification of antibody-dependent cellular cytotoxicity of Pichinde virus-infected guinea pig kidney fibroblasts 48 h after infection. Specific lysis was determined by quantifying ^{51}Cr release. Bavituximab induced specific lysis of virus infected cells as compared to control immunoglobulin ($P < 0.001$, unpaired t-test). Data represent average percentages ($n = 3$) ± s.e.m.

bavituximab-treated survivors had completely cleared virus from their tissues (data not shown). Combining bavituximab and ribavirin (the current drug of choice for treating Lassa fever) had additive activity (**Fig. 2c**), as expected for drugs with nonoverlapping mechanisms of action. With the combined treatment, 63% of guinea pigs survived, compared to 39% or 35% of guinea pigs treated with ribavirin or bavituximab alone, respectively (**Fig. 2c**).

The protective effect of bavituximab seems not to be due to direct neutralization. Bavituximab only inhibited Pichinde virus replication in P388D1 macrophages by 60% in virus-yield assays *in vitro*, even in physiological concentrations of β_2GP1 (**Supplementary Fig. 3a** online). Phosphatidylserine on Pichinde virus may not be required for virus entry, in contrast to vaccinia virus, where viral phosphatidylserine is required for infectivity[14]. However, the possibility remains that bavituximab inhibits viral replication in macrophages or another cell reservoir for Pichinde virus *in vivo*. Also, protection seems not to be due to induction of neutralizing antibodies to Pichinde virus (**Fig. 3a** and **Supplementary Fig. 4a** online) or Pichinde virus–specific T cells (**Fig. 3b** and **Supplementary Fig. 4b**), as neither was detectable 6–7 d after onset of treatment (14 d after infection) when the treated guinea pigs began to recover. Two mechanisms seem to be responsible for the protective effect. First, bavituximab causes opsonization and clearance of infectious virus from the bloodstream, leaving less virus to infect other tissues, as bavituximab treatment of viremic guinea pigs reduced the amount of infectious virus in the bloodstream by 95% within 24 h (**Fig. 3c**). Second, bavituximab induces antibody-dependent cellular cytotoxicity of virus-infected cells, as bavituximab mediated lysis of virus-infected primary guinea pig fibroblasts by primary guinea pig macrophages *in vitro* (**Fig. 3d**). Because phosphatidylserine exposure is an early event during virus infection, antibody-dependent cellular cytotoxicity may limit virus spread.

To explore whether phosphatidylserine exposure is a common feature of virus-infected cells, we tested bavituximab binding to cells infected with influenza A, vaccinia, vesicular stomatitis virus (VSV) and mouse cytomegalovirus (mCMV). All four viruses induced phosphatidylserine exposure, as detected by flow cytometry (**Fig. 4a**) and fluorescence microscopy (**Fig. 4b** and **Supplementary Fig. 5** online). This is in accordance with previous studies using annexin A5, showing phosphatidylserine externalization on the surface of cells infected with influenza A virus[18], HIV-1 (refs. 12,19), HSV-1 (ref. 13) and vaccinia virus[14]. Fluorescence microscopy showed that infection induced diffuse staining of the plasma membrane and the formation of membrane blebs similar to those on Pichinde virus–infected cells (**Fig. 4b** and **Supplementary Fig. 5**).

Phosphatidylserine also seems to be commonly exposed on enveloped virions. As with Pichinde virus, we found that bavituximab-coated beads specifically bound to and removed infectious VSV virions from suspensions (**Supplementary Fig. 6** online). Other groups have reported that annexin A5 binds to the external surface of HIV-1 (ref. 19) and vaccinia virus[14]. Many nonicosohedral viruses that bud out from the plasma membrane are assembled in, and bud out of, membrane microdomains, commonly called 'rafts'[20]. HIV-1 (ref. 21), influenza virus[22], VSV[23], Moloney murine leukemia virus[23], Ebola virus[24], Marburg virus[24] and respiratory syncytial virus[25] all appear to egress from rafts. Phosphatidylserine is highly enriched in rafts[26] and may stabilize raft formation[27]. Co-capping and colocalization studies have demonstrated that phosphatidylserine is present on the outer surface of rafts in activated B cells[28] and activated neutrophils[29]. It is possible that, in cells activated to expose phosphatidylserine by virus infection, phosphatidylserine likewise becomes incorporated into raft outer surfaces, from where it is assimilated into the outer viral envelope during budding and egress. Additionally, the herpesviruses, CMV, HSV-1 and HSV-2, which acquire their envelopes from intracellular organelles, have externalized phosphatidylserine[30,31]. Herpesviruses obtain their final envelope when they bud into vesicles derived from the trans-Golgi network[32]. Perhaps Golgi-associated structures into which the virions bud have not yet developed full lipid asymmetry, or they lose phosphatidylserine asymmetry along with the plasma membrane during virus infection.

Our observation that phosphatidylserine is externalized on CMV-infected cells prompted us to test 3G4 against lethal mCMV infections. Bavituximab does not directly neutralize mCMV *in vitro* (**Supplementary Fig. 3b**). BALB/c mice were infected with an 80% lethal infectious dose (LD_{80}) of mCMV and treated with 3G4 18 h later. Most control mice had to be killed by day 5, and only 25% were alive at day 96 (**Fig. 4c**). In contrast, 3G4-treated mice initially lost weight but then recovered, and all were alive at day 96 when the experiment was terminated (**Fig. 4c**). These results suggest that the protective effect of bavituximab is not unique to Pichinde virus or guinea pigs.

Bavituximab therapy seems to be well tolerated. Treated animals retained normal body weight, appetite, appearance and physical activity (data not shown). No evidence of toxicity was visible histologically (data not shown). Coagulation parameters remained within the normal range (**Supplementary Fig. 7** online). 3G4 recognizes domain II of

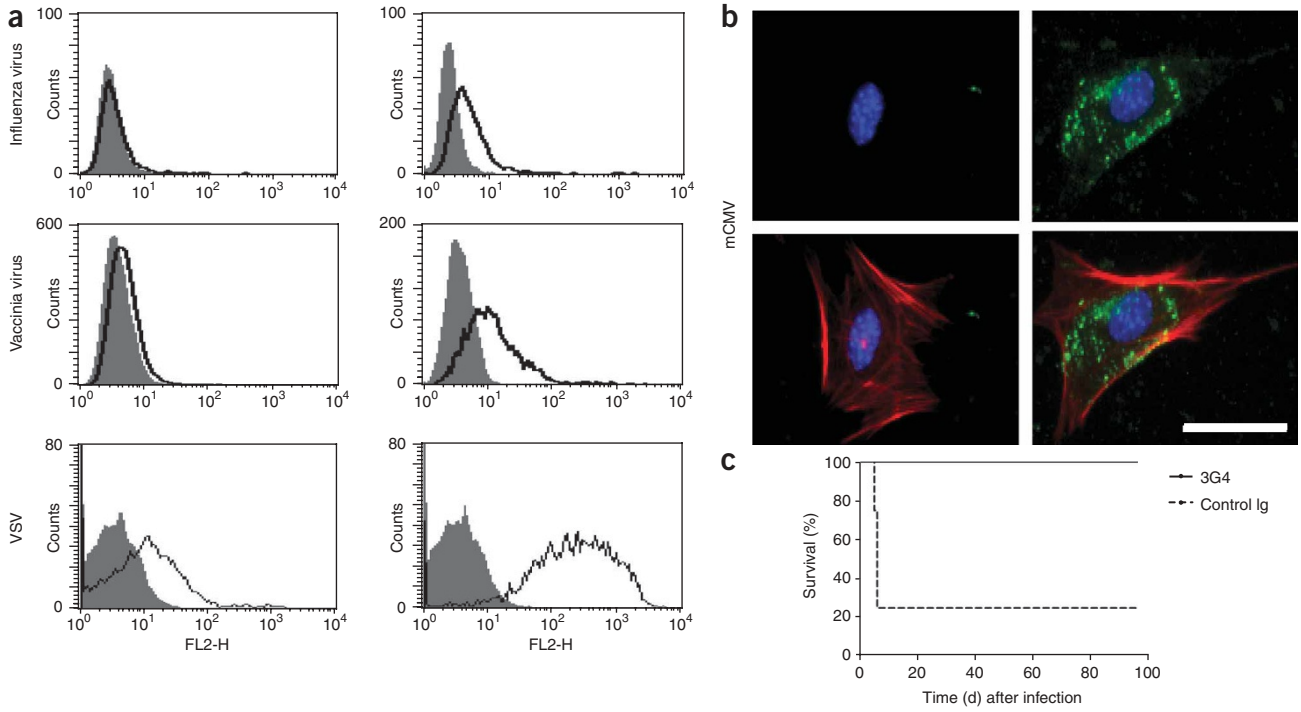

Figure 4 Broad-spectrum recognition of virus-infected cells and protection against cytomegalovirus infection in mice. (**a**) Flow cytometric analysis of virus-infected cells (right) and control uninfected cells (left). Cells were infected at an MOI of 5. Cells were harvested at 24 h for influenza A virus and VSV and at 48 h for vaccinia virus and stained with bavituximab (open histograms) or control immunoglobulin (filled histograms). Live intact cells were gated on the basis of their exclusion of 7-AAD. (**b**) Immunofluorescence staining of mCMV-infected cells (right) and uninfected cells (left) with bavituximab. M2-10B4 cells on chamber slides were infected with mCMV at an MOI of 5 and stained with bavituximab 48 h later. The top panels show bavituximab staining of externalized phosphatidylserine on infected cells (green). The bottom panels show images merged with cytoskeleton staining (red). Nuclei are in blue (all panels). Control immunoglobulin did not stain and is not shown. Scale bar, 50 μm. (**c**) Kaplan-Meier survival curves of BALB/c mice after infection with an LD_{80} dose of mCMV and treatment with mouse 3G4 ($n = 10$) or control immunoglobulin ($n = 8$). Treatment with 3G4 (4 mg kg^{-1}) or control immunoglobulin was initiated 18 h after infection and administered three times a week thereafter. Survival in the 3G4 group was significantly superior to the control group ($P < 0.0001$). The results are representative of two separate experiments.

β_2GP1, which is not known to be associated with antiphospholipid syndromes[33]. In phase 1 clinical studies, bavituximab treatment of subjects with chronic hepatitis C infections seemed safe and well tolerated. Reductions in serum HCV RNA levels were observed[34,35].

Phosphatidylserine on virions and virally infected cells may enable viruses to evade immune recognition and dampen inflammatory responses to infection. Viruses may have subverted physiological mechanisms by which apoptotic cells avoid inducing inflammation and autoimmunity. Phosphatidylserine suppresses activation and maturation of dendritic cells[36] and inhibits inflammatory responses of macrophages[37,38] and adaptive immune responses *in vivo*[39]. Bavituximab treatment may mask phosphatidylserine on virus-infected cells, on viruses or both, leading to the development of effective antiviral immune responses.

In conclusion, targeting exposed phosphatidylserine on virus-infected cells and on the virions themselves shows promise as an antiviral strategy. Another potential target is phosphatidylethanolamine, which is often exposed along with phosphatidylserine[4,40]. Because these lipids are host derived and are independent of the viral genome, the acquisition of drug resistance should be less problematic than it is with agents that target virus-encoded components.

METHODS

Flow cytometry. We infected nonadherent mouse macrophage P388D1 cells (American Type Culture Collection, ATCC) at a multiplicity of infection (MOI) of 5 (adherent cells could not be used because cell detachment disturbs phosphatidylserine distribution in the membrane). We washed the cells with FACS buffer (5% FBS and 0.01% sodium azide in PBS) and blocked Fc receptors with mouse serum. We stained the cells with bavituximab (Peregrine Pharmaceuticals) and human β_2GP1 (Haematologic Technologies) at 4°C. We used rabbit Pichinde-specific antiserum (a gift from J. Aronson) to detect Pichinde antigen. We washed the cells and incubated them with FITC- or phycoerythrin-conjugated antibodies (Jackson ImmunoResearch). We added 7-amino-actinomycin D (7-AAD; BD Biosciences) before analyzing cells on a FACScan (Becton Dickinson). We analyzed results with CellQuest (Becton Dickinson).

Immunofluorescence staining. We infected monkey kidney fibroblast Vero cells (ATCC) or mouse bone marrow fibroblast M2-10B4 cells (ATCC) growing on chamber slides (BD Biosciences) with Pichinde virus or mCMV (MOI of 5), respectively, and stained them with bavituximab or control immunoglobulin at 37 °C in the presence of β_2GP1. We fixed the cells with 4% paraformaldehyde and incubated them with FITC-labeled antibodies to human IgG (Jackson ImmunoResearch). We permeabilized the cells with 0.1% Triton X-100 and stained the cytoskeleton with Texas Red–labeled phalloidin (Invitrogen) and nuclei with Hoechst 33342 (Invitrogen). We captured images with a CoolSNAP digital camera and analyzed them withMetaVue software (MDS Analytical Technologies).

Virus binding studies. For the virus ELISA, we coated Immulon plates with Pichinde virus (**Supplementary Methods** online), washed them and blocked them. We added bavituximab or control antibody in the presence of β_2GP1. We detected binding with horseradish peroxidase–labeled antibodies to human IgG and substrate *O*-phenylenediamine dihydrochloride (Sigma-Aldrich). We measured the absorbance at 490 nm.

To detect binding of bavituximab to infectious Pichinde virus, we coated MagPrep beads conjugated to human IgG–specific antibodies (Novagen) with bavituximab or control immunoglobulin. We coated MagPrep-streptavidin beads (Novagen) with biotinylated antibodies to guinea pig IgG and guinea pig antibodies to Pichinde virus (**Supplementary Methods**). We added the beads to virus in the presence of β_2GP1 and incubated at 37 °C on a rotator. We removed the beads, together with bound virus, with a magnet. We quantified the virus remaining in the supernatant by plaque assay and calculated the percentage removal of virus.

Animal studies. We infected male Hartley guinea pigs (Charles River Laboratories) intraperitoneally (i.p.) with a lethal dose of Pichinde virus. We initiated treatment after the onset of fever (usually 7 d after infection). For single-agent therapy, we treated the guinea pigs i.p. with 6 mg kg^{-1} of bavituximab or control antibody twice a week. For combination therapy, we treated the guinea pigs i.p. with 6 mg kg^{-1} of bavituximab or control antibody twice a week and with 8 mg kg^{-1} ribavirin daily. We killed the guinea pigs when their body weights decreased by greater than 20% or when their disease was scored as 'severe' on the basis of appearance, clinical signs or unprovoked behavior. All guinea pig studies were performed in accordance with University of Texas Southwestern's Institutional Animal Care and Use Committee guidelines.

We infected female BALB/c mice (National Cancer Institute) i.p. with an LD_{80} dose of mCMV (**Supplementary Methods**). We treated mice i.p. with 4 mg kg^{-1} of 3G4[15] or control immunoglobulin beginning 18 h after infection and three times a week thereafter. We killed the mice when their disease was severe. All mouse studies were performed in accordance with UT Southwestern guidelines.

Tissue virus loads. We killed guinea pigs 14 d after infection and froze their blood and major organs. Pichinde virus infects all organs other than the brain and salivary gland[17]. We quantified virus by plaque assay. Previous experiments had shown that bavituximab at the concentrations present in the blood has no effect on plaque formation (data not shown).

Antibody-mediated cellular cytotoxicity. We used primary guinea pig kidney fibroblasts as target cells and peritoneal exudates from thioglycolate-treated guinea pigs as effector cells. For the assay, we infected fibroblasts with Pichinde virus (MOI of 5). We added bavituximab or control antibody to the cultures 24 h after infection in the presence of β_2GP1. We added effector cells 48 h after infection. We determined cytotoxicity after 18 h with the CytoTox 96 non-radioactive assay (Promega).

Proliferative responses to Pichinde virus antigens. We prepared Pichinde virus antigen or mock antigen by multiple freezing and thawing cycles of extracellular Pichinde virus or mock-infected medium. We distributed splenocytes into 96-well round-bottomed plates at 5×10^4 cells per well in RPMI 1640 medium containing 10% FBS. We added Pichinde virus antigen (10 μg ml^{-1}) or mock antigen to the cells. On day 3, we pulsed the cells with 1 μCi per well of [^{3}H]-thymidine (Amersham Biosciences). The stimulation index was calculated as the ratio of the c.p.m. obtained for Pichinde virus to the c.p.m. obtained for mock antigen.

Pichinde virus–specific humoral responses. We tested guinea pig sera for virus-specific antibodies by ELISA. We coated Immulon 4 plates (Dynatech) with Pichinde virus antigen or mock antigen, washed them and incubated them with guinea pig serum. We detected binding with peroxidase-conjugated goat antibodies to guinea pig IgG and IgM (Jackson Laboratories) followed by substrate *O*-phenylenediamine dihydrochloride. We measured the absorbance at 490 nm.

Statistical analyses. We calculated the significance between two means with Student's unpaired *t*-test. We analyzed survival data with the Mantel-Cox log-rank test. $P \leq 0.05$ was considered significant.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

M.M.S. designed and supervised experiments, interpreted results and wrote the manuscript. S.W.K. provided reagents and contributed to experimental design and interpretation of results. P.E.T. conceived the idea of phosphatidylserine-directed antiviral therapy, oversaw experiments and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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