

Intravenous Immunoglobulin with Enhanced Polyspecificity Improves Survival in Experimental Sepsis and Aseptic Systemic Inflammatory Response Syndromes.

Iglika Djoumerska-Alexieva, Lubka Roumenina, Anastas Pashov, Jordan Dimitrov, Maya Hadzhieva, Sandro Lindig, Elisaveta Voynova, Petya Dimitrova, Nina Ivanovska, Clemens Bockmeyer, et al.

► **To cite this version:**

Iglika Djoumerska-Alexieva, Lubka Roumenina, Anastas Pashov, Jordan Dimitrov, Maya Hadzhieva, et al.. Intravenous Immunoglobulin with Enhanced Polyspecificity Improves Survival in Experimental Sepsis and Aseptic Systemic Inflammatory Response Syndromes.. *Molecular Medicine*, Feinstein Institute for Medical Research, 2015, 21 (1), pp.1002-1010. <10.2119/molmed.2014.00224>. <pasteur-01450975>

HAL Id: pasteur-01450975

<https://hal-pasteur.archives-ouvertes.fr/pasteur-01450975>

Submitted on 31 Jan 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Intravenous Immunoglobulin with Enhanced Polyspecificity Improves Survival in Experimental Sepsis and Aseptic Systemic Inflammatory Response Syndromes

Iglika Djoumerska-Alexieva,¹ Lubka Roumenina,² Anastas Pashov,¹ Jordan Dimitrov,^{1,2} Maya Hadzhieva,¹ Sandro Lindig,³ Elisaveta Voynova,⁴ Petya Dimitrova,¹ Nina Ivanovska,¹ Clemens Bockmeyer,⁵ Zvetanka Stefanova,¹ Catherine Fitting,⁶ Markus Bläss,³ Ralf Claus,³ Stephan von Gunten,⁷ Sринi Kaveri,² Jean-Marc Cavaillon,⁶ Michael Bauer,³ and Tchavdar Vassilev^{1,3}

¹Department of Immunology, Stefan Angelov Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria; ²INSERM UMRS 1138, Centre de Recherche des Cordeliers, Paris, France; ³Center for Sepsis Control and Care, University Hospital, Friedrich Schiller University, Jena, Germany; ⁴Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America; ⁵Institute of Pathology, Hannover Medical School, Hannover, Germany; ⁶Cytokines and Inflammation Unit, Institut Pasteur, Paris, France; and ⁷Institute of Pharmacology, University of Bern, Bern, Switzerland

Sepsis is a major cause for death worldwide. Numerous interventional trials with agents neutralizing single proinflammatory mediators have failed to improve survival in sepsis and aseptic systemic inflammatory response syndromes. This failure could be explained by the widespread gene expression dysregulation known as “genomic storm” in these patients. A multifunctional polyspecific therapeutic agent might be needed to thwart the effects of this storm. Licensed pooled intravenous immunoglobulin preparations seemed to be a promising candidate, but they have also failed in their present form to prevent sepsis-related death. We report here the protective effect of a single dose of intravenous immunoglobulin preparations with additionally enhanced polyspecificity in three models of sepsis and aseptic systemic inflammation. The modification of the pooled immunoglobulin G molecules by exposure to ferrous ions resulted in their newly acquired ability to bind some proinflammatory molecules, complement components and endogenous “danger” signals. The improved survival in endotoxemia was associated with serum levels of proinflammatory cytokines, diminished complement consumption and normalization of the coagulation time. We suggest that intravenous immunoglobulin preparations with additionally enhanced polyspecificity have a clinical potential in sepsis and related systemic inflammatory syndromes.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2014.00224

INTRODUCTION

Sepsis remains a leading cause of death in intensive care units. It is the result of a severe and uncontrolled activation of inflammatory and coagulation pathways in response to infection, accompanied by a variable degree of immune

paralysis (1–3). Despite adequate antibiotic therapy and the use of sophisticated life-supporting measures, the prognosis of patients with this syndrome has only marginally improved in recent years.

This frustrating lack of progress, especially when novel experimental treatments

aimed to target individual mediators of inflammation were used, has been hard to explain so far (1,2,4,5).

A recent study found significant changes of expression patterns of >80% of human genes (called “genomic storm”) in trauma patients and bacterial lipopolysaccharide (LPS)-injected volunteers with severe inflammatory response syndrome (SIRS) (6). This genomic storm could well explain the discouraging results from the efforts to treat severe generalized inflammatory syndromes by neutralizing a single proinflammatory mediator. Targeting only one or very few components in a system-wide network disturbance may not successfully exert control. A multifunctional therapeutic agent may be

Address correspondence to Tchavdar Vassilev, Department of Immunology, Steffan Angelov Institute of Microbiology, Bulgarian Academy of Sciences, 26, Acad. Georgi Bonchev St., 1113 Sofia, Bulgaria. Phone: +359-2-979-6348; Fax: +359-2-870-0109; E-mail: vassilev@microbio.bas.bg or tchavdarv@gmail.com.

Submitted November 6, 2014; Accepted for publication December 14, 2015; Published Online (www.molmed.org) December 14, 2015.

The Feinstein Institute
for Medical Research 

Empowering Imagination. Pioneering Discovery.®

needed instead. Passive immunotherapy with pooled immunoglobulin preparations (administered as intravenous immunoglobulin [IVIg]) is a logical choice, since they contain a vast array of antibody specificities, some of which could well affect key products of the genomic storm. In addition to antibodies that neutralize pathogens and their virulence factors, IVIg has diverse immunomodulatory and antiinflammatory activities (7). The latter are mediated through versatile interactions with receptors on immune cells, components of the complement system, cytokines, and so on. The outcome is downregulation of T- and B-lymphocyte activity and dendritic cell functions and modulation of the cytokine network (rev. in 7, 8). The results from numerous clinical trials, using IVIg infusions as adjunctive therapy in sepsis patients have been, however, inconclusive (3,9–11).

All commercially available IVIg preparations are generally believed to have identical biological and therapeutic properties. This scenario may not be the case, since the licensed therapeutic immunoglobulins, produced using a fractionation step at pH 4.0, have been shown to possess an increased potential to bind to self-antigens (12). Importantly, this increased reactivity to antigens correlates with different functional activity of immunoglobulin preparation (13,14). Thus, the administration of the acid pH-treated IVIg, but not of the same unmodified preparation, significantly decreased mortality in animals with endotoxemia (12,13). Previous studies by our group and others have proven that, in addition to low pH buffers, the exposure to a number of other substances (for example, ferrous ions, heme, reactive oxygen species, and so on) also increases the antigen-binding polyspecificity of some IgG molecules (5,15–17). IVIg modified by Fe(II) exposure could bind to the human proinflammatory cytokine interferon (IFN)- γ and could improve survival in mice injected intravenously with 5×10^8 live *Escherichia coli* or intraperitoneally with bacterial LPS (15,18). Infusions of ferrous ion–modified IVIg were also shown

to have an antiinflammatory activity in an experimental diabetes model (14). On the basis of these preliminary data, we have hypothesized that the passive immunotherapy with pooled immunoglobulin preparations with additionally enhanced polyspecificity could neutralize some of the products of the genomic storm and thus should be beneficial in systemic inflammatory syndromes, regardless of their primary insult. Three models of systemic inflammation in the presence or absence of infection were used to check this hypothesis: induced by LPS, induced by zymosan and induced by cecal ligation and puncture (CLP). Although the infusion of native IVIg had no effect on survival, the administration of the same single dose of the Fe(II)-exposed IVIg significantly improved the survival of mice in all three models. The studies of the mechanisms of beneficial action of the latter preparation revealed its ability to bind to proinflammatory molecules, complement components and extracellular histones.

MATERIALS AND METHODS

Mice

Outbred female ICR mice (8–12 wks old, 18–22 g) were purchased from the Breeding Farm of the Bulgarian Academy of Sciences and kept in a conventional animal facility. C57Bl/6 mice (8–12 wks old, 18–22 g) were from Charles River Laboratories. The latter animals were housed under specific pathogen-free conditions, six per cage, with *ad libitum* access to food and water and maintained in a temperature-controlled environment. The animals were allowed to adapt to laboratory conditions for 3 d. The experimental protocols were approved by the Animal Care Commission of the Institute of Microbiology and the Thuringian State Office for Consumer Protection and Food Safety in accordance with National and European Regulations.

Blood (0.05 mL/mouse) was collected from the retro-orbital sinus by a Pasteur pipette after a local anesthesia with 0.5% tetracaine hydrochloride and let to clot at +4°C. Sera samples were aliquoted and stored at –80°C.

Experimental Sepsis Models

All survival experiments were performed using ICR mice in groups of 12 animals each. Endotoxemia was induced by the intraperitoneal injection of 10 mg/kg *E. coli* LPS (B 055:B5, #L2880, Sigma-Aldrich). CLP was performed as described in the literature (19). Briefly, the mice were anesthetized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg), a 1.5-cm-long laparotomic incision was made and the cecum was ligated at 2/3 of its length and punctured once by a sterile 21G needle. The puncture was confirmed by a delicate pressing, and the abdomen was closed by suture. No antibiotics or fluids were administered. Multiple organ dysfunction was induced by the intraperitoneal injection of 500 mg/kg zymosan (Sigma-Aldrich) (20). The dose of the LPS or zymosan was adjusted to cause 80–100% mortality in the control animals, treated with PBS only.

Modified Pooled Therapeutic Human Immunoglobulins

The intravenous immunoglobulin preparations Endobulin S/D (Baxter) and a special maltose- and albumin-free batch of Immunovenin-intact (BulBio; provided by Julia Nacheva) were used in the experiments. Both preparations are called “native,” since they are produced without using a fractionation step at acid pH (12). The pooled immunoglobulins were exposed to freshly prepared ferrous sulfate solution (1 mmol/L final concentration) for 2 h at 4°C and then dialyzed against phosphate-buffered saline (PBS) (pH 7.4) for at least 48 h as previously described (15). In a separate experiment, the ferrous ion–modified IVIg was dialyzed against 4 mmol/L ethylenediaminetetraacetic acid (EDTA) in PBS and then against PBS.

Treatment Schedules

A single dose of the native, the modified IVIg preparations or PBS alone was injected intravenously (i.v.) 10 min before the administration of LPS or zymosan or at the beginning of CLP procedure. In all experiments, survival was observed daily for 7 d.

In a separate experiment, groups of animals were treated i.v. with 50 mg/kg of the native or Fe(II)-exposed IVIg at different time points: minutes before, 1 h after or 4 h after LPS injection.

Determination of Serum Levels of Cytokines

The levels of tumor necrosis factor (TNF)- α , IFN- γ , IL-6, IL-10 and IL-22 in the sera obtained 4, 24 and 48 h after LPS injection were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (PeproTech EC).

The mouse inflammation antibody array kit (RayBiotech) was used to evaluate semiquantitatively the levels of 40 inflammation-related molecules. Briefly, membranes spotted with 40 capture antibodies were blocked with the manufacturer's bovine serum albumin (BSA) buffer and incubated with the studied serum samples for 2 h at room temperature. After washing, a cocktail of biotinylated anti-cytokine antibodies was incubated with membranes for 2 h at room temperature. Membranes were then washed, incubated with HRP-conjugated streptavidin for 2 h, washed and exposed to the enhanced chemoluminescence (ECL) peroxidase substrate for 2 min. Next, the membranes were drained and exposed for 20 s to X-ray film (Kodak X-Omat AR film). The gray color intensity of the spots was quantified by densitometry and analyzed by using the Image-Tool v2.0 for Windows software package (UTHSCSA).

Measurement of Plasma C3 Levels

The C3 levels in plasma were measured at 4, 24 and 48 h after LPS administration using the Mouse C3 ELISA Kit (GenWay Biotech).

Binding of Modified IVIg to the C1q Complement Component and Biological Activity Assays

Increasing concentrations of native and ferrous ion-modified IVIg were added to the wells of a plate coated with human C1q (Calbiochem, Merck). Bound IgG was detected using anti-human IgG-HRP (Fc-specific, Southern Biotech) and o-phenylenediamine (OPD) substrate.

The ability of modified IVIg to interfere with the binding of human C1q to its ligands was analyzed by ELISA. C-reactive protein (CRP) (Calbiochem) or human IgG₁ were coated to the plates at a concentration of 10 μ g/mL, and the wells were blocked with 2% BSA. IVIg (at 10 mg/mL) was preexposed to ferrous ions (0, 0.5 and 1 mmol/L) and was mixed at 1.5 mg/mL in the wells of a second plate with increasing concentrations of C1q (seven serial dilutions starting from 10 μ g/mL). The mixtures were transferred to the CRP- and human IgG₁-coated plates. After incubation for 1 h at 37°C and extensive washing, anti-C1q biotin-conjugated antibody (purified from goat anti-C1q antiserum from Quidel and biotinylated in-house) was added in a 1:500 dilution. Finally, streptavidin-HRP was added (from DAKO; diluted 1:1,000) for 30 min. After washing, TMB substrate was used, the reaction was stopped with 2 mol/L sulfuric acid and the optical density was read at 450 nm.

Coagulation and Organ Dysfunction Tests

Coagulation time was measured as previously described (21). Animals were bled 4 h after the injection of LPS, and the plasma concentrations of bilirubin and creatinine were measured by commercially available kits (from DIALAB).

Detection of Antibodies to Cytokines and Endogenous Molecules in the Immunoglobulin Preparations

The BioPlex Cytokine Assay (Bio-Rad) was used to detect antibodies to human IL-1Ra, IL-6, IL-8, IL-10, IL-15, TNF, granulocyte colony-stimulating factor (G-CSF), IFN- γ , interferon gamma-induced protein 10 (IP-10), regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-alpha (MIP-1 α) and MIP-1 β in (a) the native IVIg, (b) Fe(II)-exposed IVIg and (c) pooled human IgM (provided by Biotest AG). A human IgG1 monoclonal antibody was used as a control (22). The immunoglobulin preparations were added (at a concentration of 200 μ g/mL) to serial

dilutions of the respective cytokines and incubated for 1 h at 37°C following the manufacturer's protocol. The presence of antibodies to mouse IFN- γ , human monokine induced by gamma interferon (human MIG), high mobility group box 1 (HMGB1) (HMGBiotech), recombinant human heat shock proteins (HSP 60 and HSP 70; Enzo Life Sciences) and human histones H3 and H4 (Roche Diagnostics) were tested with ELISA. Plates (Nunc MaxiSorp) were coated with 2 μ g/mL of the respective antigens in 0.1 mol/L carbonate buffer and incubated overnight at 4°C. After blocking and washing, the plates were incubated for 2 h at room temperature with increasing concentrations of the immunoglobulin preparations under study. The plates were then extensively washed, and goat anti-human IgG antibody coupled to alkaline phosphatase was added and incubated for 1 h at room temperature. Immunoreactivities were revealed with p-nitrophenyl phosphate (Sigma-Aldrich), and the optical density (OD) values were read at OD = 405 nm.

Statistical Analysis

All statistical analyses were performed by using the GraphPad Prism 4 software and the R software (v.3.0.2). Data were expressed as mean \pm standard deviation (SD). Differences in survival curves were compared by using the Mantel-Haenszel test. For other data, the differences in the mean values between groups were analyzed with the two-tailed Student *t* test. Differences were considered significant when *p* < 0.05. Pearson correlation coefficient was used to compare gene expression profiles in different organs.

All supplementary materials are available online at www.molmed.org.

RESULTS

Protective Effect of Ferrous Ion-Modified IVIg in Sepsis and Aseptic SIRS

The intravenous administration of a single dose of 50 mg/kg of the Fe(II) ion-modified IVIg significantly decreased the mortality of animals with

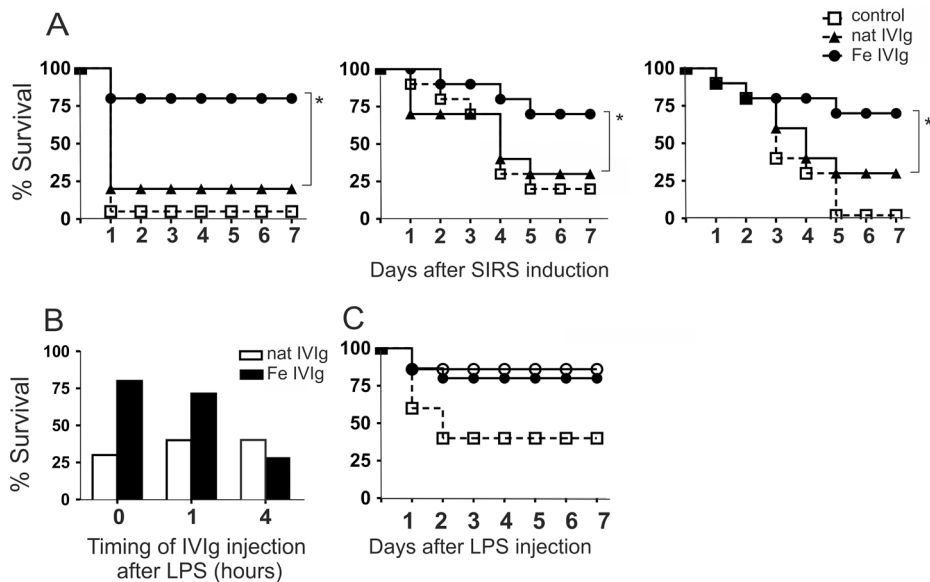


Figure 1. Passive immunotherapy with IVIg modified by Fe(II) exposure improves animal survival in two aseptic SIRS and one polymicrobial sepsis models. Outbred female ICR mice were used in the experiments. (A) Endotoxemia was induced by the intraperitoneal injection of 10 mg/kg *E. coli* LPS (left panel). Zymosan-induced systemic inflammation (middle panel) was caused by intraperitoneal injection of 500 mg/kg zymosan. Polymicrobial CLP-induced sepsis (right panel) was performed as described in Materials and Methods. The survival was compared between the control group, injected i.v. with PBS (open squares, $n = 12$), the group with endotoxemia treated with 50 mg/kg or 250 mg/kg (in zymosan-induced inflammation and CLP sepsis) native IVIg (triangles, $n = 12$), and the group treated with 50 mg/kg (endotoxemia) or 250 mg/kg (zymosan-induced inflammation and CLP sepsis) Fe(II)-exposed IVIg (circles, $n = 12$); * $p < 0.05$, Mantel-Haenszel log-rank test). (B) The beneficial effect of the Fe(II)-exposed preparation on survival was still observed if its administration was delayed. Endotoxemia was induced as described above, and the mice were treated i.v. with 50 mg/kg of the native (open bars) or Fe(II)-exposed IVIg (black bars) at different time points: minutes before, 1 h after the intraperitoneal injection of LPS, or 4 h after the intraperitoneal injection of LPS. (C) Residual iron ions in Fe(II)-exposed IVIg did not contribute to its protective effect in LPS sepsis. ICR mice ($n = 12$) were injected intraperitoneally with 10 mg/kg *E. coli* LPS and treated i.v. with 50 mg/kg Fe(II)-exposed IVIg with high (7.2 mg/g IgG, black circles) or low (0.66 mg/g IgG, white circles) iron concentration or with PBS only (open squares).

endotoxemia (Figure 1A, left panel). The same treatment (at a dose of 250 mg/kg) was protective in zymosan-induced SIRS and in polymicrobial CLP sepsis (Figure 1A, middle and right panels). The survival curves of all animals in the experimental series are shown on Supplementary Figure S1. The native immunoglobulin preparation, regardless of the dose used, had no effect, thus confirming results from numerous clinical trials.

The mechanisms of protective activity of IVIg with enhanced polyspecificity in endotoxemia were studied in more details. The infusion of the modified IVIg

1 h post-LPS injection still had a protective effect, whereas the treatment delayed for 4 h failed to improve the survival (Figure 1B). The iron content of IVIg increased after the Fe(II) treatment from 0.028 to 7.2 mg/g IgG (Supplementary Table S1). Nevertheless, the beneficial effect of the preparation was still present, even upon dialysis against EDTA, which decreased the iron concentration (Figure 1C).

Gene Expression Analysis

Microarray data of organ samples from Fe(II)-exposed IVIg-treated endotoxemic mice were clustered by an

unfiltered unsupervised approach. Unique responses to Fe(II)-exposed IVIg were observed in all organs but were most pronounced in the liver, supported by a weaker correlation—Pearson correlation coefficient r [IVIg versus Fe(II)-exposed IVIg] = 0.83 in kidney and $r = 0.45$ in liver. Although, several differentially expressed genes (DEG) could be identified, they did not constitute an easily interpretable pattern of signaling pathways (Supplementary Figure S2).

Treatment with IVIg with Enhanced Polyspecificity Affects Plasma Levels of Pro- and Antiinflammatory Cytokines

IVIg exposure to Fe(II) ions did not result in its enhanced binding to bacterial LPS (Supplementary Figure S3), indicating that the beneficial effect of modified IVIg was due to its interactions with host defense mechanisms rather than to a direct neutralization of LPS. The observed beneficial effect on survival suggests that the levels of proinflammatory molecules might be influenced by the treatment. Indeed, plasma levels of TNF- α , IL-6 and IFN- γ were significantly reduced in animals injected with modified IVIg (Figure 2A). In contrast, the levels of IL-10 and IL-22 were significantly elevated. A semiquantitative dot-blot protein array technique allowed the analysis of a larger panel of inflammation-related molecules. Significantly lower levels of IL-6, LIX (CXCL5), IL-12p70 and MIP-1 γ (CCL9) were detected after treatment with the IVIg with increased polyspecificity (Figures 2B, C).

Effect of Ferrous Ion-Modified IVIg on the Complement System, Coagulation and Organ Dysfunction of Mice with LPS-Induced SIRS

Complement activation and the release of anaphylatoxins play an important role in the pathogenesis of sepsis (23,24). The levels of C3 complement component in the circulation mirror the severity of ongoing inflammation. The LPS injection resulted in a sharp and prolonged drop in C3 levels, and the administration of

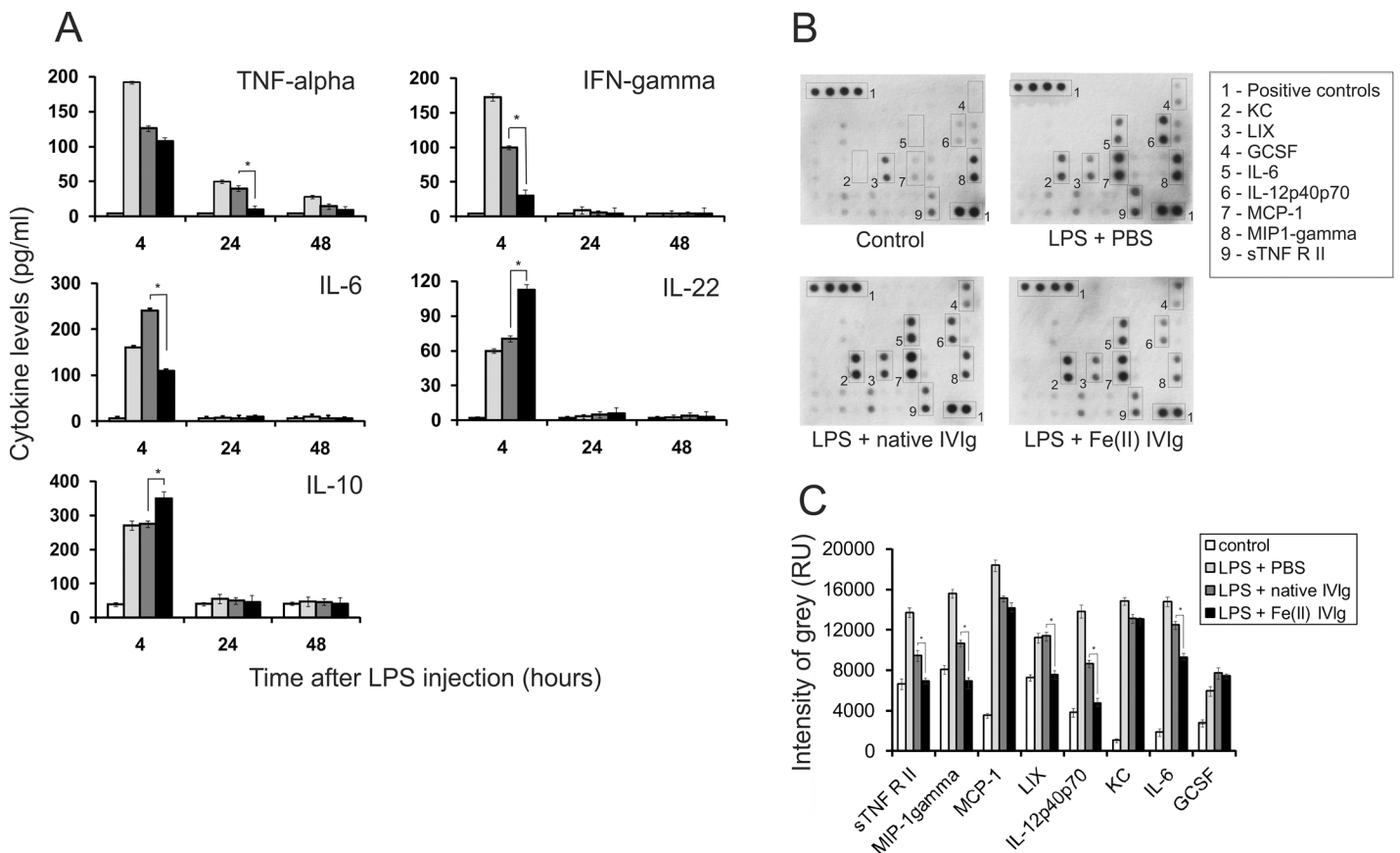


Figure 2. Serum levels of inflammation-related molecules in treated endotoxemic mice. (A) Endotoxemia was induced by the intraperitoneal injection of 10 mg/kg *E. coli* LPS. A control group was injected i.v. with PBS (n = 10, light gray bars), a second group was injected i.v. with 50 mg/kg native IVIg (n = 10, dark gray bars), and a third group with 50 mg/kg of Fe(II)-exposed IVIg (n = 10, black bars). Cytokine levels in untreated mice are represented with white bars. Sera were obtained 4, 24 and 48 h after LPS injection, and cytokine levels were measured by ELISA. Data represent mean pg/mL values ± SD of quadruplicate wells; *p < 0.05 (paired Student *t* test). (B) Scan of dot blots evaluating semiquantitatively serum levels of a larger panel of inflammatory molecules 2 h after LPS injection. Each blot is representative of three individual experiments. (C) The individual dots (B) were subjected to densitometry, and the data were analyzed by using the Image-Tool v2.0 for Windows software package (UTHSCSA) and were represented in relative units of gray intensity (n = 3, mean ± SD, *p < 0.05, paired Student *t* test).

the native IVIg did not significantly affect this level. Interestingly, the infusion of the same single dose of the IVIg with enhanced polyspecificity led to a significantly diminished consumption of C3 (Figure 3A). We analyzed in more detail the interaction of the immunoglobulin preparations with human complement components. The binding of the pooled human IgG to the C1q component was enhanced after its exposure to ferrous ions (Figure 3B). Whereas the preincubation of C1q with Fe(II)-exposed IVIg did not affect the interaction of the latter with immobilized IgG1 (not shown), it

significantly inhibited C1q binding to CRP (Figure 3C). Interestingly, the modified immunoglobulin preparation bound to C3a, but not to C5a anaphylatoxins (Figure 3D).

Systemic inflammation in sepsis is accompanied by an overactivation of the coagulation cascade and results in disseminated clots in small blood vessels, contributing to organ failure. A single infusion of ferrous ion-modified IVIg to endotoxemic mice normalized the blood clotting time (Figure 3E). This result could well explain the observation that liver injury was significantly ameliorated

in the group treated with the modified IVIg, as assessed by the capacity of ferrous ion-modified IVIg to decrease the LPS-induced elevation of serum bilirubin levels (Figure 3F). Creatinine levels at the same early time point were not affected.

Fe(II)-Modified IVIg Acquires Additional Polyspecificity That Includes the Ability to Bind Proinflammatory Mediators and Endogenous “Danger” Molecules

The exposure of therapeutic IVIg to increasing concentrations of ferrous ions results in its ability to bind to a

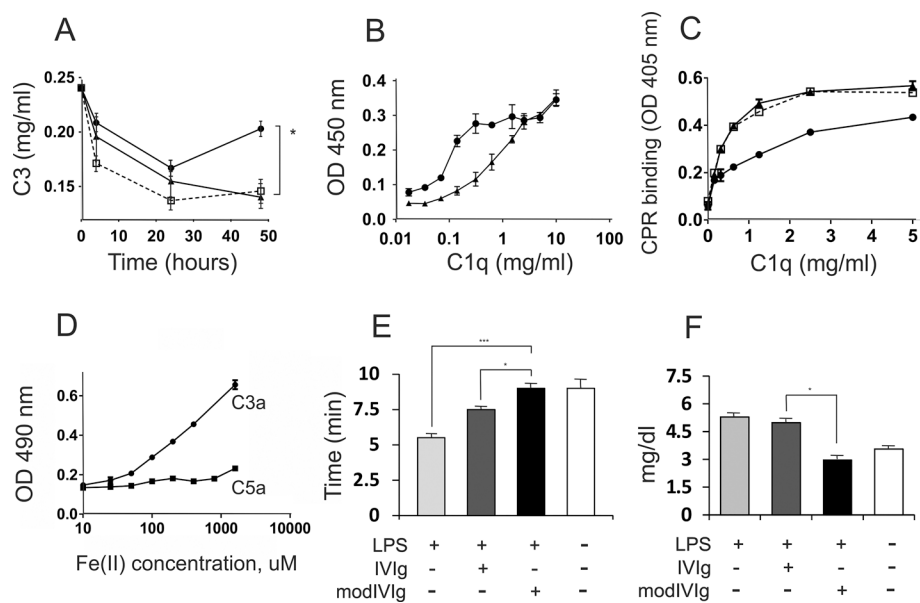


Figure 3. A single administration of Fe(II) exposure-modified IVIg overcomes the coagulation and complement abnormalities in LPS-induced sepsis. (A) Endotoxemia was induced to ICR mice by the intraperitoneal injection of 10 mg/kg *E. coli* LPS. A control group ($n = 10$, open squares) was injected i.v. with PBS, a second group was treated with 50 mg/kg native IVIg ($n = 10$, black triangles), and a third was treated with 50 mg/kg Fe(II)-exposed IVIg ($n = 10$, black circles). Serum samples were collected at 4, 24 and 48 h after LPS administration, and the C3 levels in plasma were measured using a commercial Mouse C3 ELISA kit: $*p < 0.05$, paired Student *t* test. (B) The exposure of IVIg to Fe(II) ions results in its increased binding to C1q molecules (black circles) compared with that of native IVIg (black triangles) (mean \pm SD). (C) C1q pretreatment with Fe(II) exposure-modified IVIg inhibits the binding of the former to CRP (black circles), in comparison to C1q pretreated with native IVIg (black triangles). The binding of untreated C1q is presented with open squares. (D) The exposure of IVIg to increasing concentrations of ferrous ions results in an enhanced binding to the C3a, but not the C5a component. Anti-C3a and anti-C5a reactivity was compared by ELISA in IVIg, exposed to increasing concentrations of ferrous ions. (E) Endotoxemia was induced to ICR mice by the intraperitoneal injection of 10 mg/kg *E. coli* LPS. A control group was treated i.v. with PBS ($n = 10$, light gray bars), a group was treated with 50 mg/kg native IVIg ($n = 10$, dark gray bars) and another was treated with 50 mg/kg Fe(II)-exposed IVIg ($n = 10$, black bars). Animals were bled 4 h after the injection of LPS, and coagulation time was measured as previously described (21): $*p < 0.05$; $***p < 0.001$, paired Student *t* test. (F) Bilirubin serum levels measured 4 h after LPS administration in groups of mice treated with native ($n = 10$, dark gray bars), Fe(II)-exposed IVIg ($n = 10$, black bars) or PBS alone ($n = 10$, light gray bars). $*p < 0.05$, *t* test.

broader number of antigens in a human umbilical vein endothelial cell (HUVEC) lysate (Supplementary Figure S4). In addition, anti-human IP-10, IFN- γ , anti-MIG and anti-HMGB1 reactivities were observed in Fe(II)-exposed IVIg by using a BioPlex assay or ELISA, whereas native IVIg and pooled IgM preparations had no detectable antibodies to the studied cytokines. Antibodies to endogenous "danger" molecules (free histone 3 and

histone 4) reported to possess a toxic effect on endothelial cells in sepsis (25), as well as to heat shock proteins, were also present in the modified preparation (Table 1 and Supplementary Figure S5).

DISCUSSION

Passive immunotherapy with commercially available therapeutic IVIg exposed *in vitro* to a low concentration of prooxidative ferrous ions significantly

improved survival in animals with experimental sepsis and aseptic SIRS. The same immunoglobulin preparations were not protective in their native form. The improved survival was accompanied by lower serum levels of proinflammatory cytokines, normalization of the coagulation time, diminished consumption of C3 complement component and attenuated organ injury or dysfunction. These multimodal effects strongly suggest that the ferrous-exposed IVIg has acquired novel biological properties. Pooled IVIGs are highly polyspecific therapeutic preparations. Previous studies indicate that the exposure of IVIg to Fe(II)-ions or ROS, released in inflammation sites, further amplifies this polyspecificity (15).

IVIg is known to suppress the harmful effects of activated complement cascade components by preventing their binding to the respective receptors (26). In the fluid phase, IVIg with enhanced polyspecificity did not perturb the capacity of C1q to recognize IgG immune complexes. The latter is in agreement with previous studies showing that, even at high doses, IVIg does not alter C1q binding and complements activation on sensitized sheep erythrocytes used as a model of cell-bound immune complexes (27). Therefore, the administration of Fe(II) ion-modified IVIg will not affect C1q functions during the early phase of defense against pathogens. Moreover, C1q binds better to the surface immobilized Fe(II) ion-modified IVIg (as a model of immune complex) than to the native IVIg, suggesting that the modified preparation could be more effective in opsonizing invading bacteria. When allowed to interact in a fluid phase with C1q, Fe(II) ion-exposed IVIg inhibited the binding of C1q to surface-bound CRP, suggesting that attenuation of the complement activation on damaged host cells or on bacteria could result. Because CRP levels are increased up to 50-fold in sepsis (28), the inhibition of its effector functions may be beneficial by reducing the ongoing inflammation. In our experiments, the Fe(II) ion-modified IVIg also showed enhanced binding to C3a

Table 1. Exposure of IVIg to ferrous ions results in the appearance of reactivity to some of the tested human cytokines, complement components and danger molecules.

Numbers	Inflammation-related and danger molecules	Binding appears after IVIg exposure to ferrous ions	Method used	Reference
Cytokines				
1	MIG	Yes	ELISA	Supplementary Figure S5
2	IP-10	Yes	BioPlex Array	Supplementary Figure S5
3	HMGB1	Yes	ELISA	Supplementary Figure S5
4	IFN- γ	Yes	BioPlex Array	Supplementary Figure S5 and Ref. 15
5	Mouse IFN- γ	No	ELISA	Supplementary Figure S5
6	IL-8	No	BioPlex Array	NS
7	IL-10	No	BioPlex Array	NS
8	IL-15	No	BioPlex Array	NS
9	G-CSF	No	BioPlex Array	NS
10	MCP-1	No	BioPlex Array	NS
11	MIP- α	No	BioPlex Array	NS
12	MIP- β	No	BioPlex Array	NS
13	TNF- α	No	BioPlex Array	Supplementary Figure S5
14	IL-6	No	ELISA	NS
15	IL1-RA	No	ELISA	NS
16	RANTES	No	BioPlex Array	NS
Complement components				
17	C1q	Yes	ELISA	Figure 3B
18	C3a	Yes	ELISA	Figure 3D
19	C5a	No	ELISA	Figure 3D
Danger molecules				
20	Histone 3	Yes	ELISA	Supplementary Figure S5
21	Histone 4	Yes	ELISA	NS
22	HSP 60	Yes	ELISA	Supplementary Figure S5
23	HSP 70	Yes	ELISA	Supplementary Figure S5
24	HSP 90	Yes	ELISA	NS

Antibody reactivity to mouse IFN- γ were measured in a control ELISA experiment. NS, not shown.

anaphylatoxin, expected to result *in vivo* in its rapid neutralization and elimination.

The net beneficial effect of the blunted host response upon administration of IVIg with enhanced polyspecificity was reflected in less organ damage, primarily in kidneys (Supplementary Figure S6). The transcriptome analysis showed a high variability between the individual animals, suggesting that detection of the differential expression of particular genes might be a transitory phenomenon in highly disordered (chaotic) gene network dynamics, characteristic of the septic state (29). For instance, some of

the untreated septic animals clustered with the IVIg treated; and some, with the modified IVIg. Nevertheless, the experiment showed detectable changes in the gene expression patterns, specific for the modified IVIg. A different setup with larger experimental groups will be necessary to trace these changes of the cascade, ultimately dampening the genomic storm (Supplementary Figure S2).

The idea that polyspecific natural antibodies may act as a buffering system that prevents brisk changes in the levels of circulating cytokines, hormones, and so on, was proposed 20 years ago (30).

The immune-suppressive properties of plasma during sepsis have been discussed extensively before. Diverse factors are currently considered in this context: IL-10, extracellular ubiquitin, circulating CD14, epinephrine and other inducible signals (31). Such a convergence of different activities toward an antiinflammatory plasma compartment indicates the importance of this principle. It is thus not surprising that the quasi-complete repertoire of circulating natural antibodies can be conditionally recruited to that function, too.

It is counterintuitive that naturally polyspecific IgG antibodies should generally limit or block cytokine activities in the tissues. On the other hand, *in vivo*-induced polyspecificity would be limited in space (to the site of inflammation) and time (IgG polyspecificity would result in a short half-life [32] and molecules gaining systemic access would be quickly diluted). Thus, its effect would be tightly controlled. It is possible that the *in vitro* treatment of IVIg with protein-destabilizing agents serendipitously activated this function on a large scale. The thus-modified IVIg would have at a systemic level a buffering effect that has evolved as a local phenomenon. During the event of uncontrolled systemic inflammation (sepsis), this step may be helpful, since it would enhance the inherent antiinflammatory activity of plasma. Our data provide evidence to support this hypothesis, since only the modified, but not the native, IVIg preparation binds to (a) a panel of antigens in a HUVEC lysate, (b) mediators of inflammation (IFN- γ , IP-10/CXCL10, MIG/CXCL9 and HGMB1), (c) the complement components C1q and C3a and (d) the hydrophobic “danger signals” heat shock proteins and the free extracellular histones H3 and H4 (15,25) (Figure 3; Supplementary Figures S4, S5). Importantly, our studies revealed that Fe(II) ion exposure is associated with a considerable increase in the hydrophobicity of the antigen-binding sites of IgG (15). Because endogenous danger signals frequently have a

hydrophobic nature, antibody-mediated nonspecific quenching of hydrophobicity may also explain the systemic antiinflammatory effect of modified IVIg (33,34).

Furthermore, the elimination of the formed immune complexes by Fc receptor-expressing phagocytes may contribute to the downregulation of the ongoing severe generalized inflammatory reaction.

Although the dependence of the observed therapeutic effect of modified IVIg on lymphocytes and macrophages is compatible with the “immunosomatics” mechanism (30), an effect, mediated by a particular mononuclear cell population, cannot be ruled out. Innate response activator (IRA) CD19^{low}CD5^{low}CD93⁺ B cells have been implicated recently in the control of experimental sepsis in mice (35). Indeed, our recent studies show that, unlike native IVIg, ferrous ion–modified IVIg induced in the LPS-injected mice had higher levels of IRA B cells, and the beneficial effect of the treatment on survival correlated with this increase (36).

CONCLUSION

This work contains evidence that pleiotropic therapeutic approaches offer a viable strategy for treatment of sepsis and aseptic SIRS. Apart from the mechanistic explanation, the pleiotropy principle also justifies the use of mouse models, despite the controversy on differences in transcriptome profiles in humans and mice with severe inflammatory syndromes (37,38).

ACKNOWLEDGMENTS

This work was supported by grants from the Bulgarian-Swiss Research Programme (BSRP IZEBZO_142967), the Bulgarian National Science Fund (DFNI B02/29), the Pasteur Institute (ACIP A07-2012), the NATO Science for Peace Program (SfP 982158), and the German Federal Ministry of Education and Research (BMBF; grant 01EO1002) and grant 03Z2J521 (Meta-ZIK), both to the Centre for Sepsis Control and Care. We thank D Himsel for technical assistance.

The microarray data generated in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through the accession number GSE55964.

DISCLOSURE

T Vassilev is an applicant and T Vassilev and J Dimitrov are inventors of a patent (European patent EP1838731).

REFERENCES

- Angus DC, van der Poll T. (2013) Severe sepsis and septic shock. *N. Engl. J. Med.* 369:840–51.
- Cavaillon J-M, Adrie C (eds.). (c2009) *Sepsis and Non-infectious Systemic Inflammation: From Biology to Critical Care*. Weinheim: Wiley-VCH Verlag.
- The INIS Collaborative Group. (2011) Treatment of neonatal sepsis with intravenous immune globulin. *N. Engl. J. Med.* 365:1201–11.
- Rittirsch D, Flierl MA, Ward PA. (2008) Harmful molecular mechanisms in sepsis. *Nat. Rev. Immunol.* 8:776–87.
- Bouvet J, et al. (2001) Induction of natural auto-antibody activity following treatment of human immunoglobulin with dissociating agents. *J. Autoimmun.* 16:163–72.
- Xiao W, et al. (2011) A genomic storm in critically injured humans. *J. Exp. Med.* 208:2581–90.
- Kazatchkine MD, Kaveri SV. (2001) Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *N. Engl. J. Med.* 345:747–55.
- Lutz HU (ed.). (c2012) *Naturally Occurring Antibodies (NAbs)*. New York: Springer Science+ Business Media; (Austin) TX: Landes Bioscience. pp. 267.
- Laupland KB, Kirkpatrick AW, Delaney A. (2007) Polyclonal intravenous immunoglobulin for the treatment of severe sepsis and septic shock in critically ill adults: a systematic review and meta-analysis. *Crit. Care Med.* 35:2686–92.
- Soares MO, et al. (2012) An evaluation of the feasibility, cost and value of information of a multicentre randomised controlled trial of intravenous immunoglobulin for sepsis (severe sepsis and septic shock): incorporating a systematic review, meta-analysis and value of information analysis. *Health Technology Assessment.* 16:1–186.
- Werdan K, et al. (2007) Score-based immunoglobulin G therapy of patients with sepsis: the SBITS study. *Crit. Care Med.* 35:2693–701.
- Djoumerska I, Tchobanov A, Pashov A, Vassilev T. (2005) The autoreactivity of therapeutic intravenous immunoglobulin (IVIg) preparations depends on the fractionation methods used. *Scand. J. Immunol.* 61:357–63.
- Djoumerska-Alexieva IK, et al. (2010) Exposure of IgG to an acidic environment results in molecular modifications and in enhanced protective activity in sepsis. *FEBS J.* 277:3039–50.
- Pavlovic S, et al. (2011) Intravenous immunoglobulins exposed to heme (heme IVIG) are more efficient than IVIG in attenuating autoimmune diabetes. *Clin. Immunol.* 138:162–71.
- Dimitrov JD, et al. (2006) Ferrous ions and reactive oxygen species increase antigen-binding and anti-inflammatory activities of immunoglobulin G. *J. Biol. Chem.* 281:439–46.
- Dimitrov J, et al. (2007) Antibodies use heme as a cofactor to extend their pathogen-eliminating activity and to acquire new effector functions. *J. Biol. Chem.* 282:26696–706.
- McIntyre J, Wagenknecht D, Faulk W. (2006) Redox-reactive autoantibodies: detection and physiological relevance. *Autoimmun. Rev.* 5:76.
- Djoumerska-Alexieva IK, Dimitrov JD, Nacheva J, Kaveri SV, Vassilev TL. (2009) Protein destabilizing agents induce polyreactivity and enhanced immunomodulatory activity in IVIg preparations. *Autoimmunity.* 42:365–7.
- Hubbard WJ, et al. (2005) Cecal ligation and puncture. *Shock.* 24 Suppl 1:52–7.
- Volman TJ, Hendriks T, Goris RJ. (2005) Zymosan-induced generalized inflammation: experimental studies into mechanisms leading to multiple organ dysfunction syndrome. *Shock.* 23:291–7.
- Ivanovska ND, et al. (2008) Properdin deficiency in murine models of nonseptic shock. *J. Immunol.* 180:6962–9.
- Haeflner-Cavaillon N, Klein M, Dorrington KJ. (1979) Studies on the Fc gamma receptor of the murine macrophage-like cell line P388D1. I. The binding of homologous and heterologous immunoglobulin G1. *J. Immunol.* 123:1905–13.
- Ward PA, Gao H. (2009) Sepsis, complement and the dysregulated inflammatory response. *J. Cell Mol. Med.* 13:4154–60.
- Markiewski MM, DeAngelis RA, Lambris JD. (2008) Complexity of complement activation in sepsis. *J. Cell Mol. Med.* 12:2245–54.
- Xu J, et al. (2009) Extracellular histones are major mediators of death in sepsis. *Nat. Med.* 15:1318–21.
- Basta M. (2008) Ambivalent effect of immunoglobulins on the complement system: activation versus inhibition. *Mol. Immunol.* 45:4073–9.
- Basta M, Fries LF, Frank MM. (1991) High doses of intravenous Ig inhibit in vitro uptake of C4 fragments onto sensitized erythrocytes. *Blood.* 77:376–80.
- Devran O, et al. (2012) C-reactive protein as a predictor of mortality in patients affected with severe sepsis in intensive care unit. *Multidiscip. Respir. Med.* 7:47–52.
- Tang BM, Huang SJ, McLean AS. (2010) Genome-wide transcription profiling of human sepsis: a systematic review. *Crit. Care.* 14:R237.
- Coutinho A, Avrameas S. (1992) Speculation on immunosomatics: potential diagnostic and therapeutic value of immune homeostasis concepts. *Scand. J. Immunol.* 36:527–32.
- Cavaillon JM, Adib-Conquy M. (2007) Determining the degree of immunodysregulation in sepsis. *Contrib. Nephrol.* 156:101–11.

32. Sigounas G, Harindranath N, Donadel G, Notkins AL. (1994) Half-life of polyreactive antibodies. *J. Clin. Immunol.* 14:134–40.
33. Matzinger P. (2002) The danger model: a renewed sense of self. *Science.* 296:301–5.
34. Seong SY, Matzinger P. (2004) Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat. Rev. Immunol.* 4:469–78.
35. Rauch PJ, *et al.* (2012) Innate response activator B cells protect against microbial sepsis. *Science.* 335:597–601.
36. Djoumerska-Alexieva I, Pashova S, Vassilev T, Pashov A. (2013) The protective effect of modified intravenous immunoglobulin in LPS sepsis model is associated with an increased IRA B cells response. *Autoimmun. Rev.* 12:653–6.
37. Seok J, *et al.* (2013) Genomic responses in mouse models poorly mimic human inflammatory diseases. *PNAS.* 110:3507–12.
38. Takao K, Miyakawa T. (2015) Genomic responses in mouse models greatly mimic human inflammatory diseases. *PNAS.* 112:1167–72.

Cite this article as: Djoumerska-Alexieva I, *et al.* (2015) Intravenous immunoglobulin with enhanced polyspecificity improves survival in experimental sepsis and aseptic systemic inflammatory response syndromes. *Mol. Med.* 21:1002–10.