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Abbreviations

EBNA1, Epstein-Barr nuclear antigen 1; HMGB1, High-mobility group box 1; HSA, human serum albumin

Abstract

High-mobility group box 1 (HMGB1) concentration in serum or plasma has been proposed as an important biological marker in various inflammation-related pathologies. We previously showed that low titer autoantibodies against HMGB1 could emerge during the course of sepsis. Importantly their presence was positively related with patients' survival. In this study, we focused on plasma samples from 2 patients who survived sepsis and exhibited high titer antibodies to HMGB1. These antibodies were proved to be specific for HMGB1 since they did not bind to HMGB2 or to human serum albumin. Following IgG purification, it has shown that both patients secreted HMGB1-hydrolyzing autoantibodies *in vitro*. These findings suggested that proteolytic antibodies directed against HMGB1 can be produced in patients surviving septic shock.

Keywords: High-mobility group box 1 protein (HMGB1); proteolytic antibodies; sepsis; autoantibodies

1. Introduction

High-mobility group box 1 (HMGB1, also called amphoterin) was initially identified as a conserved, abundant and ubiquitous chromatin-associated protein. It belongs to a family comprising four HMGB proteins (HMGB1-4). HMGB1 is the most abundantly and ubiquitously expressed. In the nucleus, HMGB1 contributes to transcription, DNA replication, recombination and repair [1]. Surprisingly HMGB1 can also be actively secreted by activated immune cells or passively released in the extracellular medium when plasma membranes are damaged, a process that is associated with necrosis [1]. Depending on its redox status, HMGB1 can bind cell specific receptors (TLR2, 4, 9 and RAGE), which modulates cell proliferation, migration, maturation and cytokine release [2]. Some forms of extracellular HMGB1 play a central role during both septic and aseptic forms of inflammation [3]. The direct contribution of HMGB1 to septic inflammation was first demonstrated in a mouse model of endotoxemia [4]. Elevated levels of HMGB1 could be detected in the serum of mice exposed experimentally to endotoxemia or sepsis as well as in sepsis-related conditions in humans [5–7]. In sepsis-related conditions, HMGB1 plasma concentrations are usually higher and persist longer in patients who succumb to the disease than in patients who survive, although some noticeable exceptions have been reported [6–9]. Although highly purified HMGB1 has a weak pro-inflammatory activity by itself [10], it can bind and act in synergy with various Damage-Associated Molecular Patterns (DAMPs) or Pathogen Associated Molecular Patterns (PAMPs) to amplify their pro-inflammatory activities [11]. Accordingly inhibiting HMGB1 activity by various therapeutic agents, including neutralizing antibodies, improves survival in animal models of severe sepsis [12,13]. Nosaka and colleagues also demonstrated that monoclonal antibodies against HMGB1 could provide protection in a mouse model of influenza A virus (H1N1)-induced pneumonia [14].

We previously established that natural antibodies to HMGB1 emerged during the course of sepsis. This emergence was significantly (i) more frequently observed in the plasma of patients surviving septic shock (55%) as compared to patients who did not (20%), (ii) and linked with favorable outcome [15]. In the same cohort of patients, we could identify rare surviving patients that exhibited high-titers autoantibodies against HMGB1, which provided us with the opportunity of characterizing both their specificity and biological activity ex vivo.

2. Patients and methods

2.1 Study population and data collection

The cohort of 42 patients admitted with a septic shock into the medical intensive care unit (ICU) at teaching hospital (Nancy, France) was previously described in details and studied in Gibot et al. [7] and Barnay-Verdier et al. [15]. The diagnosis of septic shock was established on the basis of current definition. Patients were not enrolled if they were over 80 years of age or were immunocompromised. The present study focused on 40 of these 42 critically ill patients corresponding to 178 plasma samples collected longitudinally during the ICU stay. Day 1 was defined as the day of admission into the ICU.

The control group used to establish base-line values for ELISA was composed of 132 plasma samples collected from apparently healthy adult donors who were not matched with critically ill patients [15].

Approval of the institutional review board and informed consent from patients or their relatives were obtained before inclusion.

2.2 Protein expression

Recombinant human HMGB1 (rhHMGB1), human HMGB2 (rhHMGB2) and viral Epstein-Barr Nuclear Antigen (rEBNA) proteins were purified from *Escherichia coli* BL21 (DE3) pLysS strain transformed with pET15b-6His-HMGB1, pET15b-6His-HMGB2 and pET15b-6His-EBNA C terminus [16], respectively. Protein expression and purification were performed as previously described [17]. The removal of contaminating bacterial lipopolysaccharide was performed using Triton X-114 [18]. Finally, recombinant proteins were dialyzed against PBS or 8 M urea (NaH_2PO_4 100mM, Tris HCL 10mM, Urea 8 M, pH 8).

2.3 Antibodies detection by ELISA and immunoblot

Antibodies directed to HMGB1, HMGB2, EBNA1 and commercially available human serum albumin (HSA) (Sigma-Aldrich) were detected by ELISA. Maxisorp polystyrene 96-well plates were coated with 100 ng per well rhHMGB1 or rhHMGB2, 250 ng per well rEBNA recombinant proteins or 85 ng per well HSA in 8M urea at pH 8, and incubated overnight at 4°C. The plates were blocked with phosphate buffer saline, 10% milk, 0.2% Tween 20 for 1h. Plasma samples, diluted 1:10 in blocking buffer, were added in duplicates (100 µl/well) and incubated for 1h at 37°C. After 4 washes, 100 µl of rabbit anti-human immunoglobulin-horseradish peroxidase (HRP) (Rockland 209-4302), diluted at 1:1000, were added to each well and incubated for 1h at 37°C. Detection was achieved by addition of 100 µl of ABTS (Roche Diagnostic) per well and incubated at 37°C for 20 min. Optical densities (OD) were measured at 405 nm and background levels were measured on wells in which plasma was omitted. Cut-off values for each autoantibody were established for each antigen on a group of 100 plasma samples from apparently healthy people. They were defined as the mean background OD values plus three standard deviations.

For indirect immunoblot, a mixture containing rhHMGB1 and rEBNA1 (1ng each per well) was loaded on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond nitrocellulose; GE Healthcare). Following transfer, the membrane was cut into individual strips containing both proteins. Each strip was incubated for 1h in blocking buffer (Tris-buffered saline, 0.2% Tween, 5% milk), washed and incubated overnight at 4°C with a 1:1000 dilution of each patient's plasma. Detection of the primary antibodies was performed using a 1: 5000 dilution of a rabbit anti-human HRP conjugate (GE Healthcare). Immune complexes were detected using ECL detection reagents (GE Healthcare). The quantification was done using ImageJ.

2.4 Measurement of plasma HMGB1

HMGB1 was quantified on plasma samples using a commercial ELISA as previously described by Barnay-Verdier and collaborators with no perchloric acid pretreatment [19].

2.5 Purification of IgG

IgG were purified by affinity-chromatography on protein G Sepharose (GE Healthcare) as previously described [20]. A therapeutic preparation of pooled normal human Ig (IVIg; Sandoglobulin) was used as a source of normal IgG. To exclude potentially contaminating proteases, size-exclusion chromatography of patients IgG and IVIg was performed on a superose-12 column (GE Healthcare), equilibrated with 50 mM Tris, 8 M urea, and 0.02% NaN3 (pH 7.7), at a flow rate of 250 µl/min. IgG-containing fractions were pooled and dialyzed against PBS-0.01% NaN_3 for 48 h at 4°C, followed by dialysis against 50 mM Tris (pH 7.7), 100 mM glycine, 0.02% NaN_3, and 5 mM $CaCl_2$ (catalytic buffer) for 24h at 4°C.

2.6 Hydrolysis assays

rhHMGB1 and HSA were dialyzed against catalytic buffer for 3h at 4°C and stored at –20°C until use, as previously described [20]. Equimolar amounts of rhHMGB1 and HSA were incubated in catalytic buffer with purified IgG and incubated for 24h at 37°C. Intravenous Immununoglobulins (IVIg) was used as a source of IgG and as a negative control. Samples were mixed with Laemmli buffer without mercaptoethanol and a volume corresponding to 1µg of each assayed protein was subjected to SDS-PAGE on a 10% polyacrylamide gel, which was subjected to Coomassie blue staining.

Biotinylation of rhHMGB1 was performed using EZ-Link Sulfo-NHS-LC-biotin reagent (Pierce) following the manufacturer’s instructions. Biotinylated rhHMGB1 was

dialyzed against catalytic buffer for 3 h at 4°C and stored at –20°C until use. Biotinylated rhHMGB1 (90 µg/ml) were incubated in 40 µl of catalytic buffer with purified patient's IgG (20 µg/ml) and incubated for 24h at 37°C. IVIg was used as a source of IgG and as a negative control. Samples were mixed with Laemmli's buffer without mercaptoethanol and 20 µl of each sample was subjected to denaturing SDS-10% PAGE. Protein fragments were then transferred onto nitrocellulose membrane (GE Healthcare). Following overnight blocking in TBS, 0.2% Tween 20 at 4°C, membranes were incubated with streptavidin-coupled HRP diluted 1/2000 in blocking buffer, for 1 h at room temperature. After washing labelled proteins were revealed by using the ECL kit (GE Healthcare).

2.7 Statistical analyses

ELISA were performed at least three times and up to 5 times for each sample. Spearman's correlation coefficient was obtained for correlations.

3. Results

3.1 High-titer autoantibodies against HMGB1 in two patients surviving septic shock

HMGB1 is regarded both as a marker and as an important player in septic shock. We previously identified natural anti-HMGB1 antibodies in a cohort of patients surviving septic shock, , up to ten days after admission in ICU. The detection of anti-HMGB1 antibodies, during the course of the disease was significantly associated with patient's survival, suggesting that they may possibly counteract HMGB1 deleterious effects [15]. In order to characterize the specificity of this humoral response, we investigated the presence of autoantibodies directed against two control proteins i.e. human serum albumin (HSA), a non-related protein, and HMGB2, which shares 79% sequence similarity with HMGB1, in the same plasma samples. As shown on Fig. 1, most plasma samples containing anti-HMGB1 also contained anti-HMGB2 antibodies (Fig. 1A) whereas no reactivity was observed against HSA (Fig. 1B). More surprisingly, 5 plasma samples exhibited high-titer antibodies to HMGB1 but not to HMGB2 (Fig. 1A). These 5 samples belonging to plasma samples sequentially collected from two patients surviving septic shock (thereafter designated as patients 1 and 2). Among them, 4 were detected in the patient 1 (at day 2, 3, 4 and 5 post-admission) and one in the patient 2 (at day 7 post-admission) (Fig. 2A). The kinetics of anti-HMGB1 antibodies was similar in both patients and characterized by a very low level of anti-HGMB1 IgG at the day of admission followed by a rapid and important increase during the ICU stay (Fig. 2A). In both patients, plasma HMGB1 was lower than 20ng/ml (Fig. 2A). The sudden appearance of high-titer antibodies to HMGB1 may result from a non-specific polyclonal B-cell activation [21]. To test this hypothesis, we designed an ELISA assay to monitor antibodies against Epstein-Barr virus (EBV) protein EBNA1. Indeed, EBV latently infects almost 90% of the adult

population, where it induces a persistent IgG response against several viral antigens, including EBNA1 [22]. Therefore, we assumed that a non-specific polyclonal B-cell activation would likely be associated with an increase in anti-EBNA1 antibodies as well. As expected, IgGs against EBNA1 were detected at early time points when anti-HMGB1 antibodies were still undetectable both by ELISA and indirect immunoblotting (Fig. 2). More strikingly, the emergence of anti-HMGB1 was not associated with a corresponding increase in the level of anti-EBNA1 antibodies. These results suggested that the emergence of anti-HMGB1 did not result from a global and non-specific B-cell activation. Rather we observed a drastic reduction in the level of anti-EBNA1 antibodies that almost perfectly mirrored the appearance of anti-HMGB1 antibodies in both patients (Fig. 2).

3.2 Detection of anti-HMGB1 antibodies endowed with a specific proteolytic activity against HMGB1

Previous work demonstrated that natural antibodies endowed with a proteolytic activity against coagulation factors could arise during sepsis [20]. This led us to speculate that high-titer antibodies to HMGB1 detected in patients 1 and 2 could interact with plasma HMGB1 and degrade it. To test this hypothesis, plasma IgG from 3 samples from each patient were purified and evaluated for their ability to degrade recombinant HMGB1 (rhHMGB1) *in vitro*. As a control, these purified IgGs were also evaluated for their ability to degrade purified HSA in the same assay. As shown in Fig. 3A and 3B (and Supp Fig.1), a marked reduction of rhHMGB1 was observed in one plasma sample for each patient, corresponding to samples with high-titer antibodies to HMGB1 (day 5 post-admission in patient 1 and day 7 post-admission in patient 2). This proteolytic activity was specific since HSA was not degraded in the same conditions (Fig. 3A-3B). To confirm these results, IgGs purified at day 5 (patient

1) and day 7 (patient 2) were incubated with a biotinylated form of rhHMGB1 (Fig. 3C and Supp Fig.2). This led us to detect rhHMGB1 breakdown products thus confirming the presence of antibodies that could degrade rhHMGB1 in this samples.

As a conclusion, we demonstrated here for the first time that high-titer antibodies endowed with a specific proteolytic activity against HMGB1 could emerge in patients surviving a septic shock.

4. Discussion

We previously demonstrated that plasma autoantibodies against HMGB1 could arise during septic shock and were associated with a favorable outcome. Such antibodies were detected in 37.5% septic patients and were significantly less frequent in apparently healthy blood donors (15%) [15].

In most patients the presence of antibodies to HMGB1 was associated with antibodies to HMGB2. Except for 5 samples the relative titer of anti-HMGB2 IgG was well correlated with the titer of autoantibodies to HMGB1. Considering that HMGB2 protein shares a high degree of similarity with HMGB1 this suggested that antibodies directed against HMGB1 could cross-react with HMGB2 [23], although work with polyclonal preparations of IgG does not allow to reach a definitive conclusion. In most instances, anti-HMGB1 and -HMGB2 autoantibodies appeared between 2 and 10 days post-admission. This is reminiscent of previous studies where antibodies to self-antigens where detected in about 46% septic patients 1 to 7 days after admission into ICU [24]. Similarly Bakhiet et al. [25] reported the rapid emergence of anti-cytokine autoantibodies in a rodent model of *Haemophilus influenzae* type b self-limiting meningitis.

We identified five serial samples from two patients surviving sepsis, which presented with high-titer IgG to HMGB1. IgG from these samples were purified by affinity chromatography. Although these purified IgG could not neutralize HMGB1 chemotactic activity in MRC5 fibroblasts migration assay ([26] and data not shown), some of these purified antibodies were endowed with a proteolytic activity against rhHMGB1 *in vitro*. The proteolytic activity demonstrated some degree of specificity for HMGB1 since HSA used as a control antigen was not hydrolyzed in the same experimental conditions. Interestingly, plasma samples from these two patients

contained only minor amount of HMGB1 (below 20ng</ml). Nevertheless, we could not correlate the presence of autoantibodies against HMGB1 and low plasma concentration of HMGB1 (Supp Fig. 3).

To our knowledge, this report is the first characterization of natural antibodies directed to HMGB1 that are endowed with a specific proteolytic activity. As these antibodies were identified in two patients who survived septic shock, we could hypothesize that the transient emergence of high-titer proteolytic antibodies may attenuate HMGB1 deleterious effect during sepsis. This is reminiscent of previous work by Lacroix-Desmazes et al. that correlated the survival of septic patients with the presence of plasma IgG endowed with a high hydrolytic activity against the peptide PFR-MCA, a generic synthetic–substrate for kallikrein-like serine proteases [20]. Due to their therapeutic potential, many studies have been conducted to characterize catalytic antibodies targeting antigens from influenza virus [27,28], HIV [29–31], Cryptococcus neoformans [32] and Helicobacter pylori urease [33]. The production of catalytic antibodies directed against cytokines may be of great interest to neutralize these cytokines when they exhibit a deleterious activity. Antibodies to vasoactive intestinal peptide [34] and to TNF-alpha have already been produced and characterized [35]. The isolation of catalytic antibodies against HMGB1 would likely be possible from isolated B-cells of patients, although the prevalence of such antibodies is likely low as suggested by the present study. The effectiveness of monoclonal antibodies to protect mouse from deleterious H1N1 infection [14] would suggest to look for catalytic antibodies against HMGB1 in other pathologies, including viral infections with Influenza virus A.

Several non-exclusive mechanisms could be proposed to explain the appearance of natural antibodies to HMGB1. The generation of auto-reactive antibodies and

concomitant disappearance of anti-EBNA1 IgG might result from the exposure of circulating antibodies to reactive oxygen species [36], a set of molecules that are produced early during sepsis [37]. Reactive oxygen species (ROS) and/or prooxidative compounds are per se protein-destabilizing factors. They may exert similar effects on the variable regions of the antibodies as the agents that are able to induce *in vitro* appearance of new antigen specificities. This model could explain both the appearance of IgG with new reactivity against HMGB1 and the concomitant disappearance of antibodies directed against the viral protein EBNA1. Alternatively, the secretion of autoantibodies may rise from the polyclonal activation of B cells producing antibodies to HMGB1 [21]. Indeed, upon ROS generation circulating HMGB1 may be oxidized and then become a ligand for both BCR, TLR-4 and TLR-9 on autoreactive B cells [38], [39] which may induce a rapid differentiation into plasmablasts/plasmocytes.

5. Conclusion

We demonstrate here for the first time that plasma antibodies endowed with a specific proteolytic activity against HMGB1 may emerge in patients surviving a septic shock. Further investigations should be carried out in larger number of plasma samples from different cohorts of patients with septic shock in order to systematically compare the presence of antibodies endowed with HMGB1 proteolytic activity in surviving and non-surviving patients.

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Conflict of interest

The authors declare that they have no conflict of interest.

Figure legends

Fig 1

Correlation between anti-HMGB1 and anti-HMGB2 (A), and anti-HSA (B) IgG titers. Autoantibodies directed to HMGB1, HMGB2 and HSA were detected by indirect ELISA. Results are expressed as optical density (OD) values at 405 nm. The dotted lines correspond to the cut-off values defined as the mean OD plus three standard deviations obtained on a group of 100 plasma samples from apparently healthy blood donors.

Dots represent 178 measurements performed in 40 patients with septic shock at various time intervals ranging from 1 to 18 days. Spearman coefficient (r) is depicted within the graph.

Fig 2

(A) Time course detection of HMGB1 and IgG against anti-HMGB1 (black triangle), anti-HMGB2 (black square) and anti-EBNA1 (black circle) on sequential plasma from patients 1 and 2. OD ratios were defined as the ratio of the OD measured for a given antigen over the OD value obtained anti-HSA. HMGB1 concentration (open triangle) is indicated. (B) The same samples were subjected to an indirect immunoblot by using independent filter strips loaded with both rhHMGB1 and rEBNA1.

Fig 3

(A) Hydrolysis assay on Human recombinant HMGB1 (rhHMGB1) or HSA proteins. Proteins were incubated in the presence of purified IgG from patients 1 and 2 collected at different day following their admission into the ICU, for 24 h at 37°C. As a negative control both proteins were incubated in the presence of the buffer alone

(CTL) or in the presence of IVIg. Following incubations, the proteins were separated by SDS-PAGE and revealed by Coomassie blue staining. (B) Quantification of hydrolysis assay. Residual amounts of rhHMGB1 or HSA protein were quantified by ImageJ.

(C) Hydrolysis assay on biotinylated recombinant form of rh-HMGB1. Biotinylated rh-HMGB1 was incubated alone (*lane 1*) or in the presence of IgG from two sepsis patients (*lanes 2–3*) for 24 h at 37°C. Pooled IgG from healthy donors (IVIg) *(lane 4)* was used as control IgG. Biotinylated HMGB1 and degradation products were subjected to 10% SDS-PAGE, transferred onto a nitrocellulose membrane, incubated in the presence of streptavidine-HRP and detected by ECL revelation.

Supplementary figure 1

Enlarged view of the Coomassie stained gel presented in figure 3A.

Supplementary figure 2

Enlarged view of the nitrocellulose membrane presented in figure 3C.

Supplementary figure 3

ELISAs were performed to measure HMGB1 plasma concentration as well as IgG directed HMGB1 on a series of 55 plasma samples from 11 patients samples – including plasma from patients 1 and 2 – containing low or high level of autoantibodies to HMGB1. Spearman correlation coefficient was 0.17.

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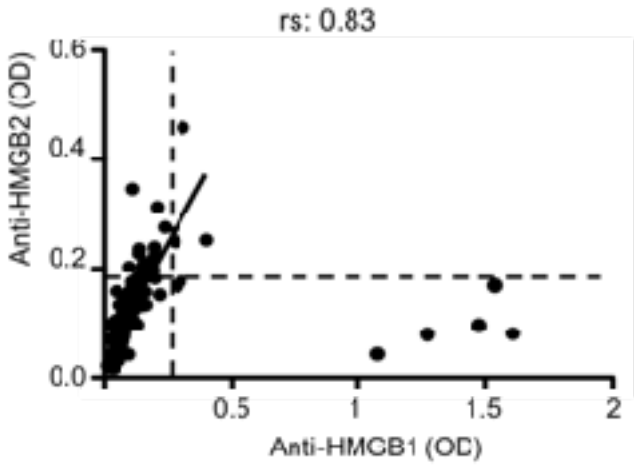
A.


rs: 0.83
Anti-HMGB2 (OD)
0.6
0.4
0.2
0.0
0.5
1
1.5
2
Anti-HMGB1 (OD)

B.

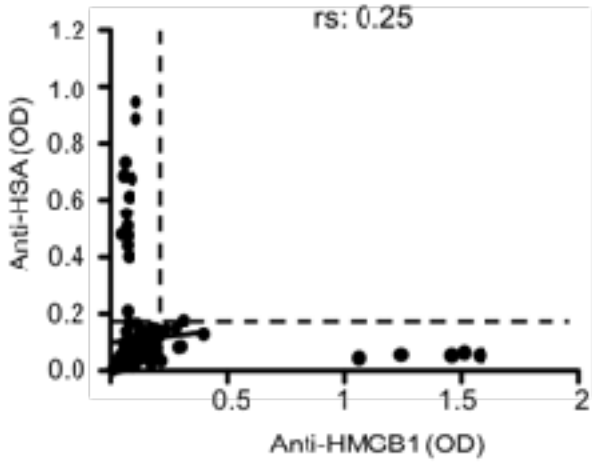
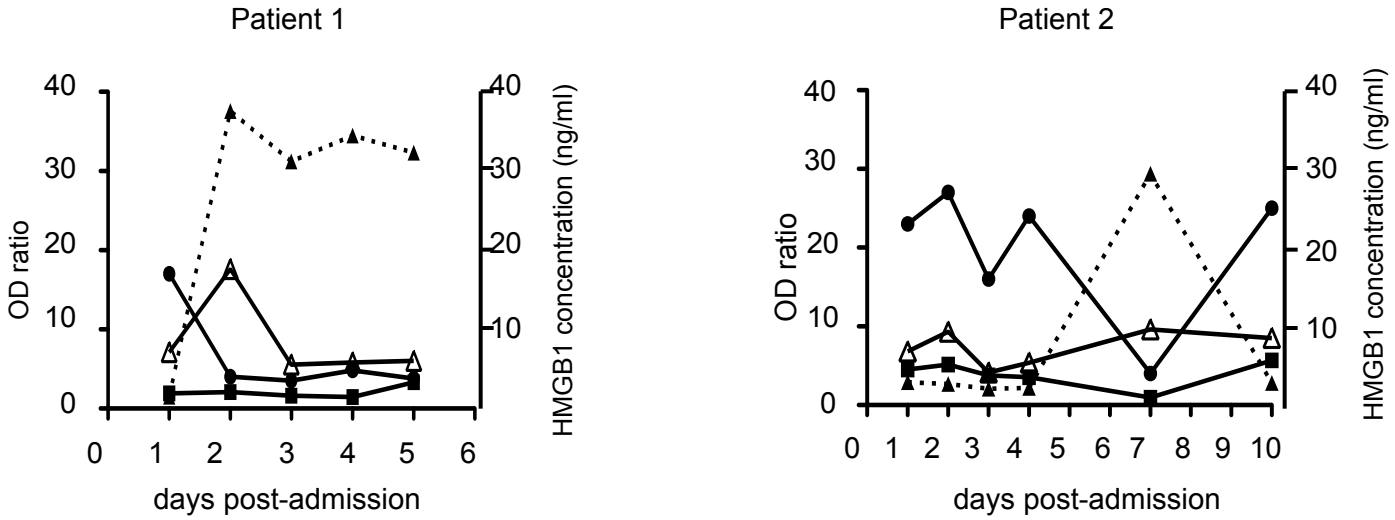

rs: 0.25
Anti-HSA (OD)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.5
1
1.5
2
Anti-HMGB1 (OD)

Figure 1

A.


Patient 1
Patient 2
OD ratio
HMGB1 concentration (ng/ml)
days post-admission
0
10
20
30
40
0 1 2 3 4 5 6
0 1 2 3 4 5 6 7 8 9 10

B.

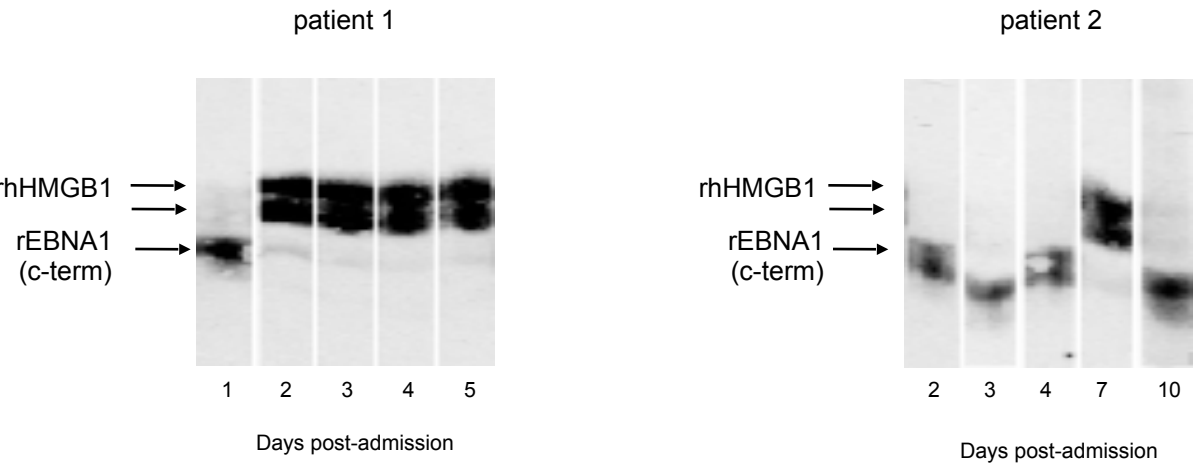
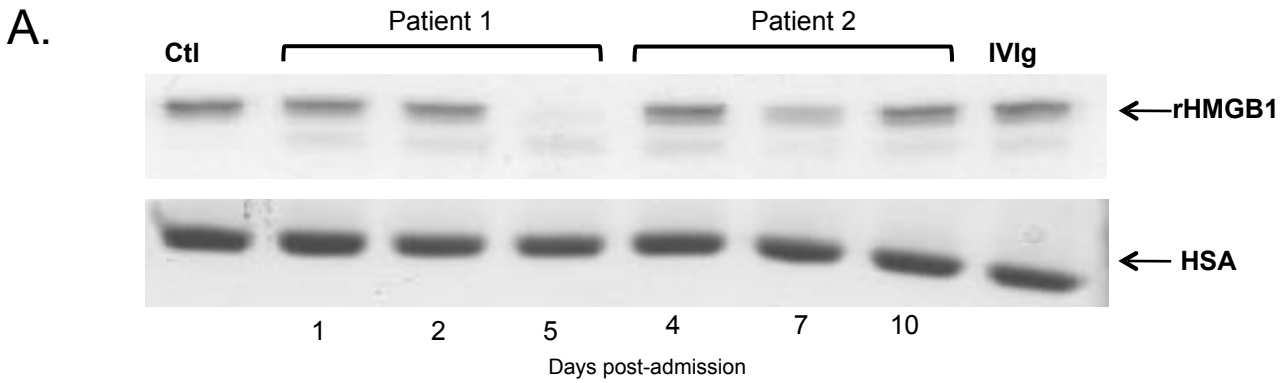
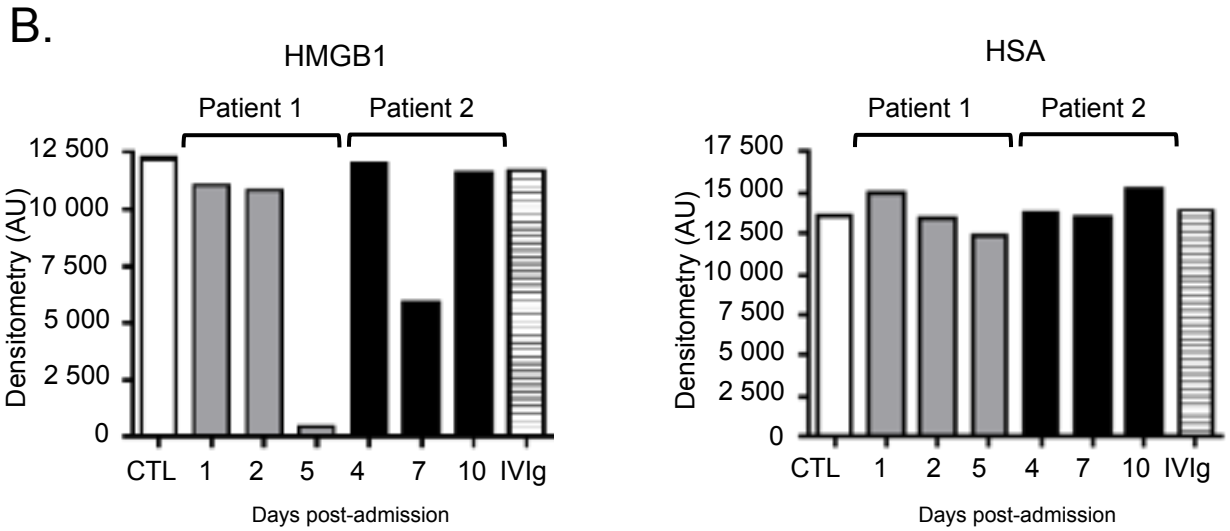

patient 1
patient 2
rhHMGB1
rEBNA1
(c-term)
1 2 3 4 5
2 3 4 7 10
Days post-admission

Figure 2


A.
Patient 1
Patient 2
Ctl
IVIg
rHMGB1
HSA
1
2
5
4
7
10
Days post-admission


B.
HMGB1
Patient 1
Patient 2
12 500
10 000
7 500
5 000
2 500
0
Densitometry (AU)
CTL
1
2
5
4
7
10
IVIg
Days post-admission
HSA
Patient 1
Patient 2
17 500
15 000
12 500
10 000
7 500
5 000
2 500
0
Densitometry (AU)
CTL
1
2
5
4
7
10
IVIg
Days post-admission

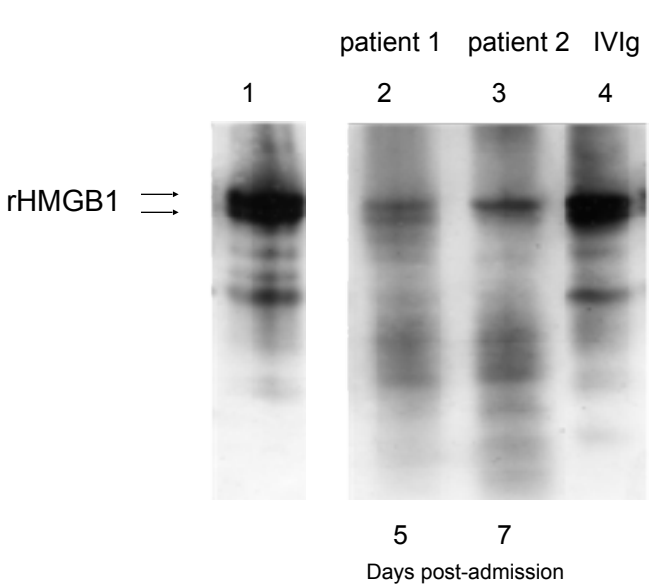
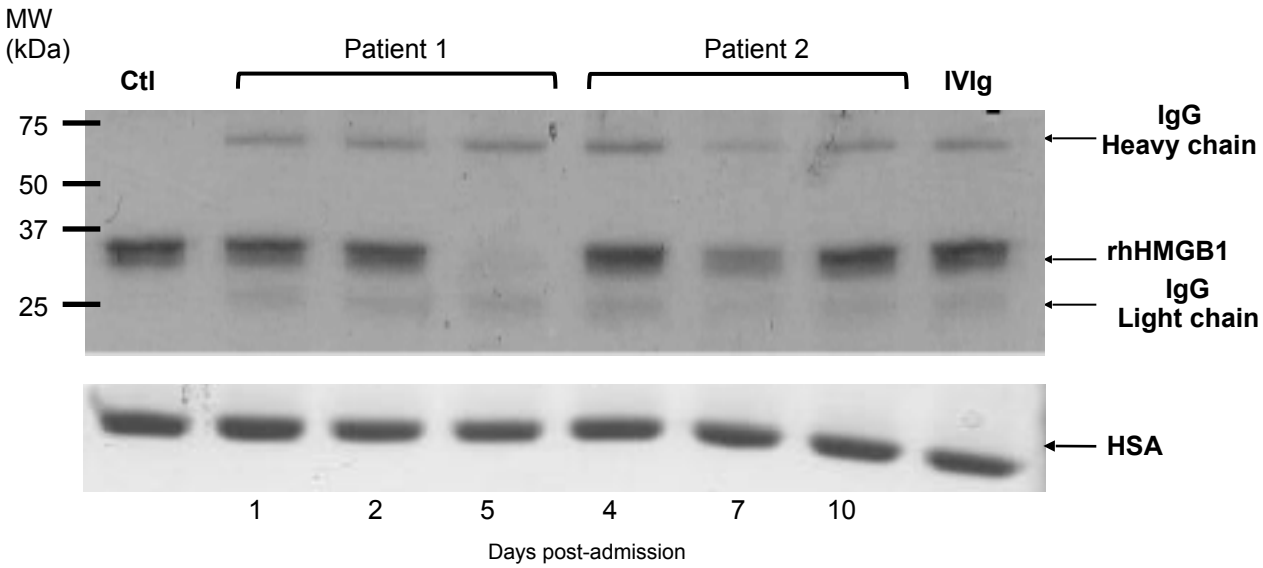
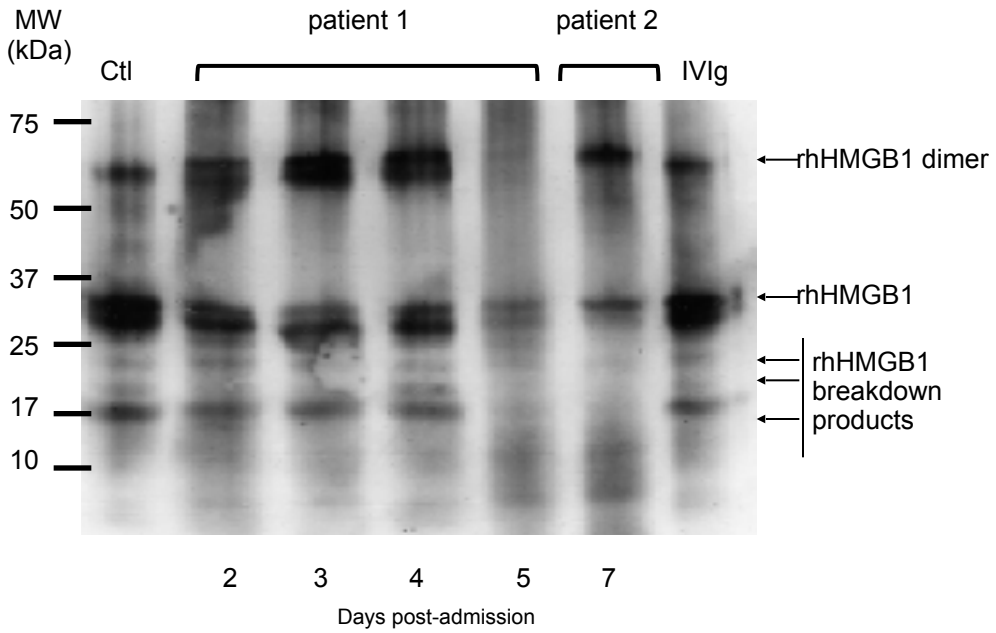

C.
patient 1
patient 2
IVIg
1
2
3
4
rHMGB1
5
7
Days post-admission

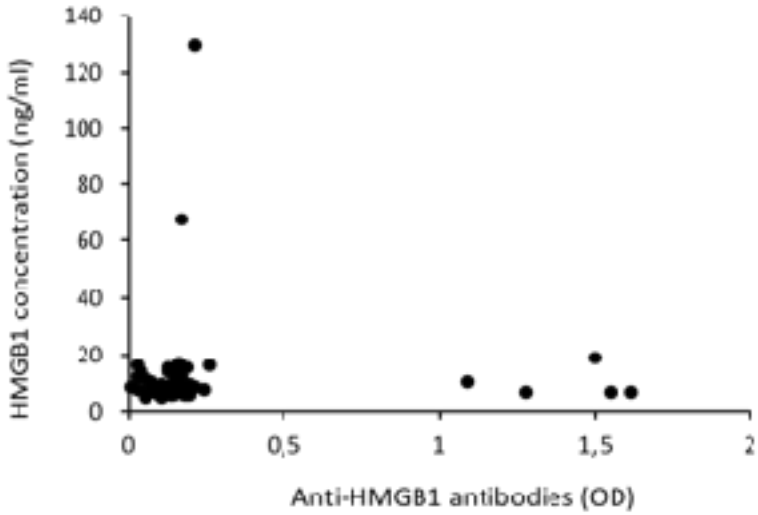
Figure 3



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3