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TITLE: "Potential Application of Viral Empty Capsids for the Treatment of Acute Lung Injury/Acute Respiratory Distress Syndrome"

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14. ABSTRACT The goal of this project has been to test the hypothesis that recombinant empty capsids of SV40, Virus Like Particles (VLPs), may attenuate ARDS, increasing survival and recovery from this severe clinical condition. We used the rat 2CLP (cecal ligation and two punctures) model for sepsis-induced ARDS. The VLPs, or vehicle (saline) control, were administered in 3 equal portions (total dose 0.3 mg/kg VLPs, in saline) for 3 days prior to the 2CLP operation. 2CLP was performed 24 hours later. While 100% the untreated rats died in 4 days, 75% of the VLP-treated ones survived. During the first 48 hours the treated rats presented similar symptoms and weight loss to the untreated ones. However afterwards their deterioration decelerated and they proceeded to full recovery. Lung pathology was normal in both treated and untreated rats sacrificed 24 hours after the 2CLP operation. Typical sepsis-induced lung pathology, which was mild, was seen in VLP-treated survivors 4 days post operation, suggesting that those rats were after the ARDS climax. Blood tests, including liver functions, showed a similar trend: partial recovery on day 4 progressing to full recovery on day 12. Studies using RNAseq predicted that 2CLP injury increases the level of Pattern recognition receptors for bacteria and viruses, Toll-like receptor signaling, leading to cytokine production, innate immune response, and neutrophil and monocyte infiltration of affected organs. The major effect VLP-treatment seen 24 hours after the insult is stabilization of blood vessels via Rac1 signaling. In conclusion, the study provides proof of concept for the potential value of SV40 VLPs for the treatment of severe sepsis, justifying further studies in large animals under Intensive Care conditions. SV40 VLPs may thus provide a desperately needed answer to a wide range of critical clinical conditions with high mortality, including sepsis, trauma and MODS (multiple organ dysfunction syndrome), for which only supportive treatment is presently available.					
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1. INTRODUCTION:

The proposal is addressed at Acute Respiratory Health Problems, ARDS. It is aimed in particular to develop preventive therapeutics to reduce the incidence of acute respiratory distress syndrome after acute lung injury in trauma patients, by treatment with SV40 Virus Like Particles (VLPs). The rationale is based on the beneficial effect of SV40 VLPs on an Acute Kidney Injury (AKI) model in mice, previously demonstrated by our group (Butin-Israeli et al., 2008). Both AKI and ARDS are severe clinical conditions that require treatment in intensive care units (ICU). Both are characterized by high morbidity and mortality, in spite of high cost optimal supportive care. All therapeutic treatments attempted so far failed. Previous therapeutic approaches have been single-targeted, aimed at a single target mostly within the immune response. Our novel development is multi-targeted: The VLPs induce complex network when entering cells, including anti-apoptotic signaling which, as was demonstrated, protect mice kidneys from apoptosis, necrosis and consequent damage induced by a toxic (mercury) insult, increasing survival significantly. The results of the present project shown below demonstrate dramatic effect on survival of rats with sepsis-induced ARDS.

2. KEYWORDS:

Acute Respiratory Distress Syndrome (ARDS); Acute Lung Injury (ALI); Peritoneal sepsis; SV40 virus-like particles (VLPs); Cecal ligation and puncture (2CLP); Immune response.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Efficacy of VLPs in treatment of the LPS-ARDS rat model.

Our experiments showed that the endotoxin model was too mild for our purpose. Therefore after we stopped the studies planned on that model and moved forward to Specific Aim 2. This change was approved by our officer Dr. Usamah Kayyali (letter dated May 11 2016). The change is reflected in the updated timeline shown in the **modified SOW**.

Specific Aim 2: Efficacy of VLPs in treatment of the 2CLP-ARDS rat model.

Major tasks and milestones were completed on time, month 16.

Specific Aim 3: Development of a simplified reagent (“lead compound”).

Milestones were completed 2 months after the expected date, on month 16 instead of month 14. The results showed that dose of the derivatives required for therapeutic effect a significantly higher than the dose of VLPs. It appears that our rationale in designing the lead compound was incorrect.

Specific Aim 4: The mechanism of ARDS attenuation by VLP in the 2CLP model.

Because of technical problems the RNAseq facilities did not operate for a number of weeks. Nevertheless the data collection and conclusions were drawn. However specific pathways were not validated.

What was accomplished under these goals?

Specific Aim 1: Efficacy of VLPs in treatment of the LPS-ARDS rat model.

The experiments demonstrated that the endotoxin model was too mild for our purpose. The insult caused death in only a small proportion of the animals, not allowing us to see whether VLPs had any effect without performing a number of tests, such as cytokines, on a large number of animals to achieve statistical power. Furthermore, there was no clear correlation between the LPS dose and death rate (Figure 1).

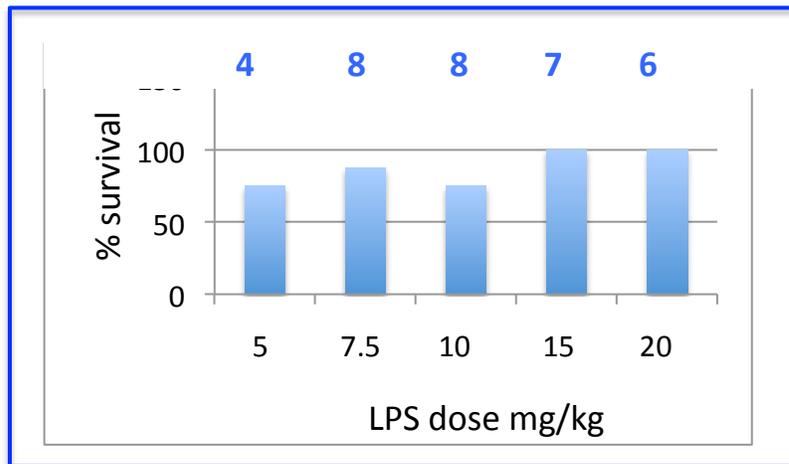


Figure 1. The effect of LPS dose on rat survival. The number of animals that received each dose is designated above. Note that at the higher LPS doses, 15 and 20 mg/kg, survival was 100%.

We decided to move forward to the other ARDS model that was included in the proposal, the 2LCP model, Specific Aim 2. This change was approved by our officer Dr. Usamah Kayyali (letter dated May 11).

Specific Aim 2: To test the efficacy of VLPs in the treatment of ARDS in the 2CLP model, similarly to Aim 1.

Major Task 6: Setting up the 2CLP-ARDS rat model

Subtask 1: Obtaining Local IACUC Approval

ACURO approval was obtained on time.

Both milestones, obtaining and establishment of the animal model were achieved earlier than planned, on Month 9th instead of month 10th.

Subtask 2: Establishing the animal model

The 2CLP model used in the present study was essentially as described by Rittirsch et al (Rittirsch et al., 2009). The cecum was ligated below the ileocecal valve. In order to cause sepsis the cecum was perforated twice. After a number of trials we used 14G IV catheter (BD Venflon, orange) for the two punctures. This model led to rapid death of all the vehicle-treated 2CLP rats (see Fig. 2 below).

*** Milestones**

Milestone 1. Local IACUC Approval achieved on time.

Milestone 2. Animal model for 2CLP-induced ARDS was achieved 1 month earlier than planned.

Major Task 7: VLP dose response and delivery time.

Subtask 1: Protocol for protective VLP administration for 2CLP induced ARDS

Essentially we found that the protocol that was optimal in our study on the mouse AKI model were also optimal with the 2CLP rat model. VLPs were injected on 3 subsequent days, 1/3 dose on each day, prior to the insult.

The protocol that was established is detailed here:

Days -3,-2,-1: Weigh rats.

Tail vein injection of either vehicle (saline) or VLPs (in saline). VLP concentration was adjusted so that each rat received 100 µl per 100 gram of its weight. Saline was injected at the same times and at the same proportional volume per rat. The daily injection VLP dose is 0.1 mg/kg, a total of 0.3 mg/kg in 3 days.

Day 0: Weigh rats. Perform the 2CLP procedure. Start observing the rats in the late afternoon.

From Day 1: Observe the rats several times during the day. Record death.

Subtask 2: Data both on effective dose range and on VLP toxicity

Dose response was tested within the range of 0.1 to 1.0 mg/kg. The optimal dose was found to be 0.3 mg/kg. Because of the high expense in producing large quantities of VLPs we did not go beyond 1.0 mg/kg. Therefore, at this stage we do not know at which dose VLPs become toxic to rats.

Subtask 3: Proof of principle for therapeutic effect of VLP for 2CLP induced ARDS

The dramatic increase in survival by VLP treatment is shown in Fig. 2. Rats were injected with a total dose of 0.3 mg/kg VLPs, or vehicle (saline), divided into 3 equal portions for 3 consecutive days, -3,-2,-1 ((0.1 mg/kg VLPS/day), denoted by green arrows. All the 18 animals were operated for 2CLP on day 0. The results demonstrated that 80% of the vehicle-treated rats died within 48 hours, and the remaining 20% died during 2 additional days. Of the VLP-treated rats 25% died within 48 hours, while the other 75% survived.

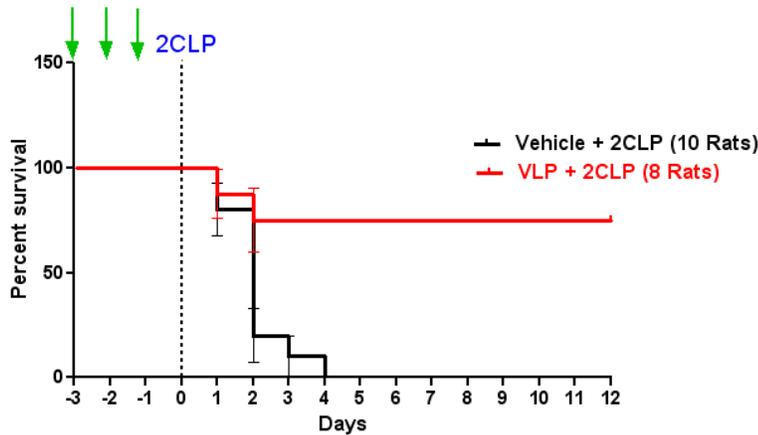


Figure 2. SV40 VLPs significantly increase survival of ARDS-model rats. Statistical analysis using Log-rank (Mantel-Cox) Test indicated that the results were significant. P = 0.0026

*** Milestones**

Milestone 1: Protocol for protective VLP administration for 2CLP induced ARDS.

Achieved on time.

Milestone 2: Data both on effective dose range and on VLP toxicity. Achieved on time.

Milestone 3: Proof of principle for therapeutic effect of VLP for 2CLP induced ARDS.

Achieved on time.

Major Task 8: disease progression and arrest

Subtask 1: Treat animals according to the protocol achieved in Task 7 (milestone 1) and harvest at different time point to follow disease progression and arrest.

The animals were randomly divided into the following 6 groups: Vehicle only, Vehicle+VLPs, Sham operation+vehicle, Sham+VLPs, 2CLP+vehicle and 2CLP+VLPs. All the groups were harvested on day 1 following the 2CLP operation, in order not to miss the many 2CLP rats that died during day 2 (see Fig. 2). Survivors of the 2CLP+VLPs group were harvested again on day 4 and on day 12, to follow recovery. The weight of the control rats, vehicle only group, was followed to day 4.

The data of the Sham and Sham+VLPs groups did not add insight into the process and were omitted from the analyses below.

Subtask 2: Collection of clinical data, plasma samples and analysis of clinical parameters and pathological examination of tissue sections.

1. Symptomes:

Within few hours following the 2CLP operation the rats, regardless whether they received VLPs or vehicle, began to appear less active, moving slowly and later becoming quiet. 24 hrs after the operation their activity lessened and some were completely inactive. Black circles appeared

around the eyes and sometimes also around the snout. Additional symptoms were piloerection and soft stools, progressing to diarrhea. Most of the vehicle-treated rats died within 48 hours. Many of the VLP-treated rats began showing signs of recovery, seen as slowly increased activity, at 24-48 hrs after the 2CLP operation. The general condition of both groups was reflected in their body weight, seen in Fig. 3 below.

2. Body Weight:

The animals were randomly divided into the following 6 groups: Vehicle only, Vehicle+VLPs, Sham operation+vehicle, Sham+VLPs, 2CLP+vehicle and 2CLP+VLPs. All the groups were harvested on day 1 following the 2CLP operation, in order not to miss the many 2CLP rats that died during day 2 (see Fig. 2). Survivors of the 2CLP+VLPs group were harvested again on day 4 and on day 12, to follow recovery. The weight of the control rats, vehicle only group, was followed to day 4.

Summary of body weight of the 4 rat groups along the experiment is depicted graphically in Fig. 3.

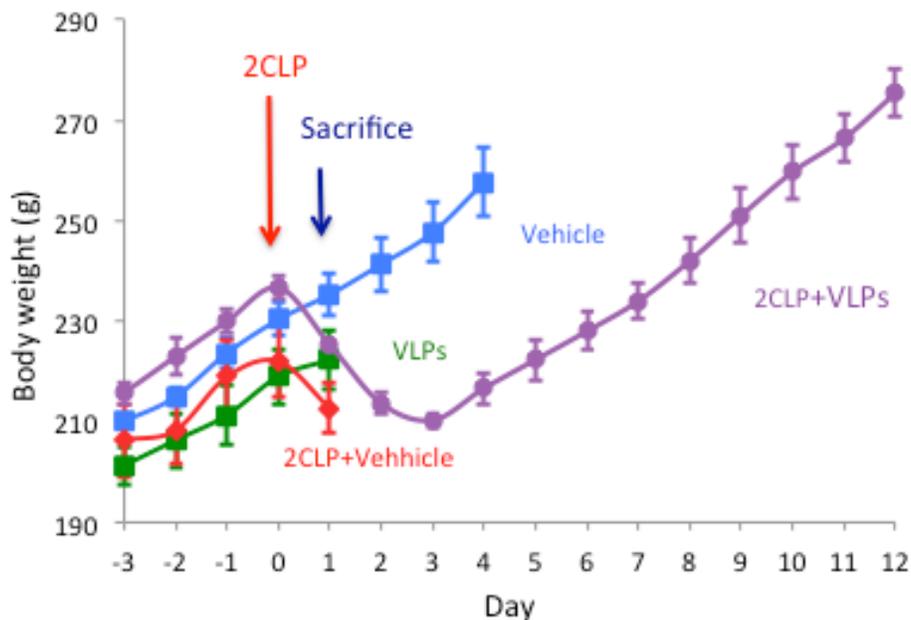


Figure 3. Changes in body weight for the 4 groups of rats. Both groups that underwent the 2CLP procedure (2CLP+Vehicle and 2CLP), on day 0, decreased in their body weight following the operation. The 2CLP+vehicle rats were sacrificed at 24 hours following the 2CLP operation, at imminent death. The weight of survivors that received VLPs started increasing after day 3 following the operation, and continued increasing at a rate similar to that of the control (vehicle) group, indicating a recovery process. The graphs show means and standard errors. Each group represents 3 rats.

3. Laboratory blood tests:

Murine physiology is significantly different from that of humans. For example, difference in Toll-like receptor 4 (TLR4) from humans and mice lead to recognition of different lipopolysaccharide structures. Likewise there are species differences in innate immune responses, in the mononuclear phagocyte system, in chemokines and chemokine receptors and in nitric oxide pathways (Matute-Bello et al., 2008). Furthermore, published information on various clinical parameters is limited. Nevertheless, we followed a number of parameters as detailed below.

The human response to severe sepsis without treatment has not been studied. Inferring from data on severe human peritonitis which leads to early mortality we would have expected the following results in rats in the 2CLP group. We would have expected signs of impaired perfusion and reduced oxygen utilization leading to elevated lactate levels and increased urea and creatinine as early indices of these changes. Reduced hepatic perfusion could lead early hepatic damage seen by increased liver enzymes (SGOT and SGPT). We would have expected early activation of the immune response, or at a later stage an "immunological shutdown" signified either by an increase in neutrophil count or a severe decrease in the count. Early inflammation would have been followed by increased C-Reactive protein (CRP), although this increase may not be obvious at the very early stages of infection. Indiscriminate activation of coagulation (Disseminated intravascular coagulation- DIC) would lead to reduced platelet count and fibrinogen with a prolongation of INR.

The changes we actually found in the rat are not all in accordance with these expectations based upon human physiology. This is related both to the physiologic differences as delineated above as well as to the fact that the tests used in the current study were performed in Beckman Coulter counter calibrated for human parameters rather than murine.

The tests were performed on blood withdrawn during harvest of the different groups of rats. Vehicle+VLPs control was generally similar to the vehicle only (untreated) control and is therefore not included in the analyses, which comprised Untreated, 2CLP (vehicle-treated) harvested on day 1, 2CLP+VLPs harvested on day 1 and 2CLP+VLPs harvested on day 12. The results obtained for rats 4 days after the 2CLP-operation (2CLP+VLP group) were between the two other recovery time points, 1 and 12 days, and were omitted

The number of animals in each group was very small, 3-4, except for the untreated rats (vehicle only), which included up to 10. We therefore chose a nonparametric test, Kruskal–Wallis (Kruskal–Wallis, 2013), rather than ANOVA. The Kruskal–Wallis test relies on ranking the data according to values rather than assuming normal distribution. The analysis shows (Table 1, green row) that the treatments of the various experimental groups had a significant effect on each of the eight parameters tested; five of the comparisons were highly significant ($p < 0.01$). These were followed by *post-hoc* pairwise comparisons according to Conover 2-tailed test (Conover, 1999). Note that none of the parameters was significantly different between the untreated and the VLP-treated 2CLP groups harvested 1d after the insult. Interestingly, in the VLP-treated 2CLP survivors, most of the parameters returned to normal (untreated control) by day 12 post insult.

Table 1. The effect of treating various groups of rats on individual parameters (*p*-values)

Groups compared	Blood parameter		Fibrinogen	Urea	GPT	GOT	WBC	Platlets	RBC	Hgb
		<i>p</i> -value*								
Untreated vs 2CLP		0.0094	0.0003	0.2086	0.0067	0.0032	0.0217	0.039	0.006	0.0041
Untreated vs 2CLP+VLPs 1d		0.0097	0.0001	0.0027	0.0001	<0.0001	0.0182	0.1973	0.0011	0.0011
Untreated vs 2CLP+VLPs 12d		0.1627	0.4855	0.4855	0.0856	0.0696	0.9148	0.1863	0.0182	0.0172
2CLP vs 2CLP+VLPs 1d		0.4311	0.0752	0.0752	0.1508	0.4449	0.8687	0.8835	0.0276	0.0021
2CLP vs 2CLP+VLPs 12d		0.0235	0.0235	0.1176	0.3515	0.0592	0.0335	0.0068	0.911	0.5308
2CLP+VLPs 1d vs 2CLP+VLPs 12d		0.1686	0.0035	0.0035	0.0315	0.0141	0.0515	0.0077	0.455	0.2305

*As determined by a Kruskal–Wallis test.

All the other *p*-values are for the comparison of each pair of groups designated on the left, as determined by Conover's *post-hoc* pairwise-comparisons. The *p*-values are colored according to significance: $p > 0.05$, **0.05**; $p > 0.01$, **0.01**; all *p*-values are 2-tail.

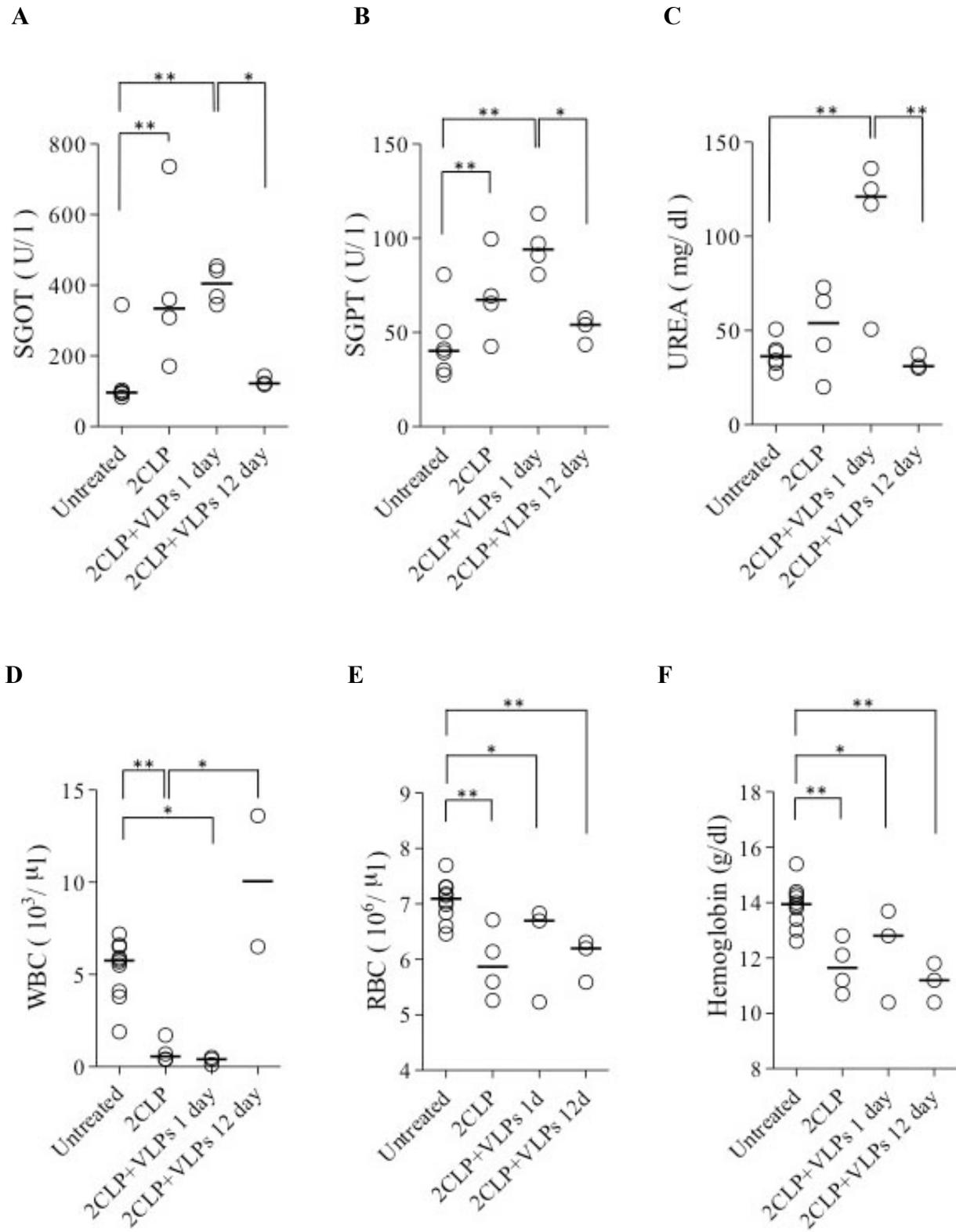


Fig. 4. Scatter plots for blood parameters.
A. SGOT; **B.** SGPT; **C.** Urea; **D.** WBC; **E.** RBC; **F.** Hemoglobin..

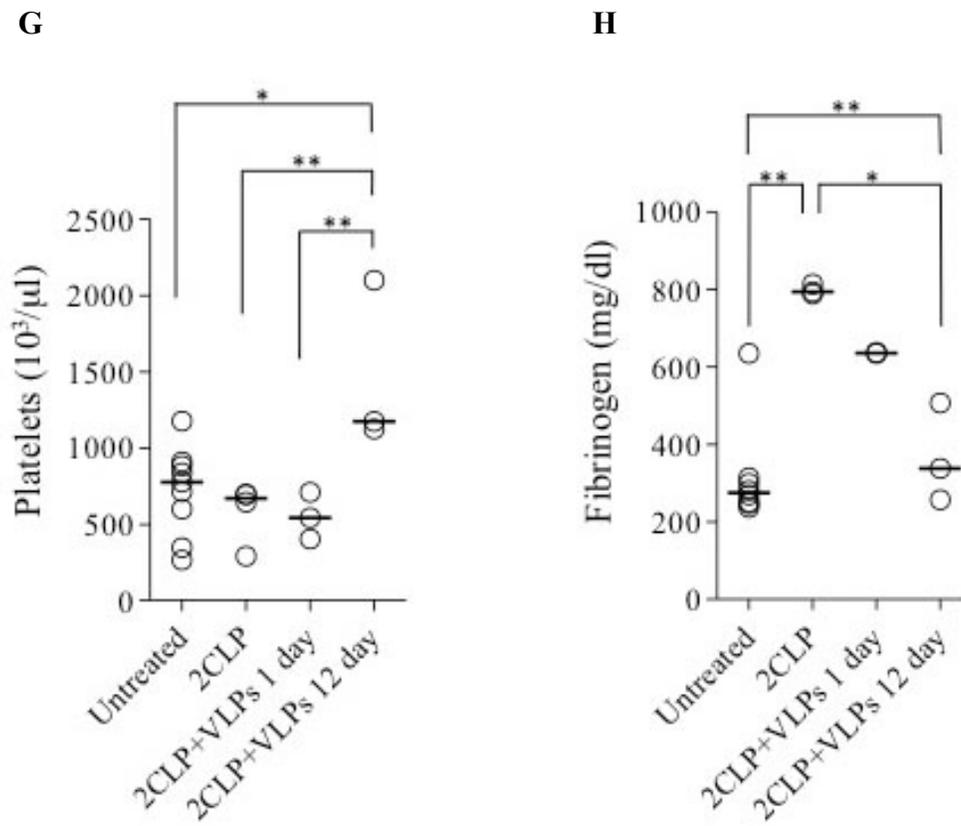


Fig. 4. Scatter plots for blood parameters. G. Platelets; H. Fibrinogen.

Liver function: Liver function and damage were measured by bilirubin (levels did not change after 24 hours (data omitted), and the liver enzymes SGOT and SGPT. These increased significantly following injury both in treated and untreated animals and returned to baseline values within 12 days in surviving treated animals.

Kidney function was assessed by creatinine, which did not change and therefore omitted. This is consistent with a previous report (Kim et al., 2006) that showed that BUN and creatinine were not affected by 2CLP in Male Sprague-Dawley rats. Urea increased only in the VLP treated animals but returned to normal after 12 days. An isolated increase in urea without a concomitant increase in creatinine could suggest either dehydration or increased urea production due to increased protein metabolism.

The hematological parameters found in the control untreated animals are consistent with previously reported values for this rat strain (Petterino and Argentino-Storino, 2006).

White blood count decreased significantly one day following 2CLP in both treated and untreated animals. This decrease could be a sign of severe neutropenia, that can be associated with severe sepsis in humans, or on the other hand, be related to the automated count technique that is calibrated to human blood. The WBC count increased to normal with recovery at 12 days in the VLP-2CLP group. Hemoglobin and RBC count decreased following the 2CLP whether

treated or not. Platelet count was not affected by 2CLP in the early stages but increased significantly 12 days after the 2CLP in the treated animals. This finding can be explained by the ongoing local infective process expected after non-lethal bowel perforation.

Fibrinogen levels were elevated early after insult but returned to normal after 12 days in survivors.

CRP levels did not change after injury, neither did lactate levels. Their results are omitted. INR appeared prolonged; however due to technical problems only partial results are available at this time. These tests are continuing and will be included in the final manuscript.

4. Lung pathology:

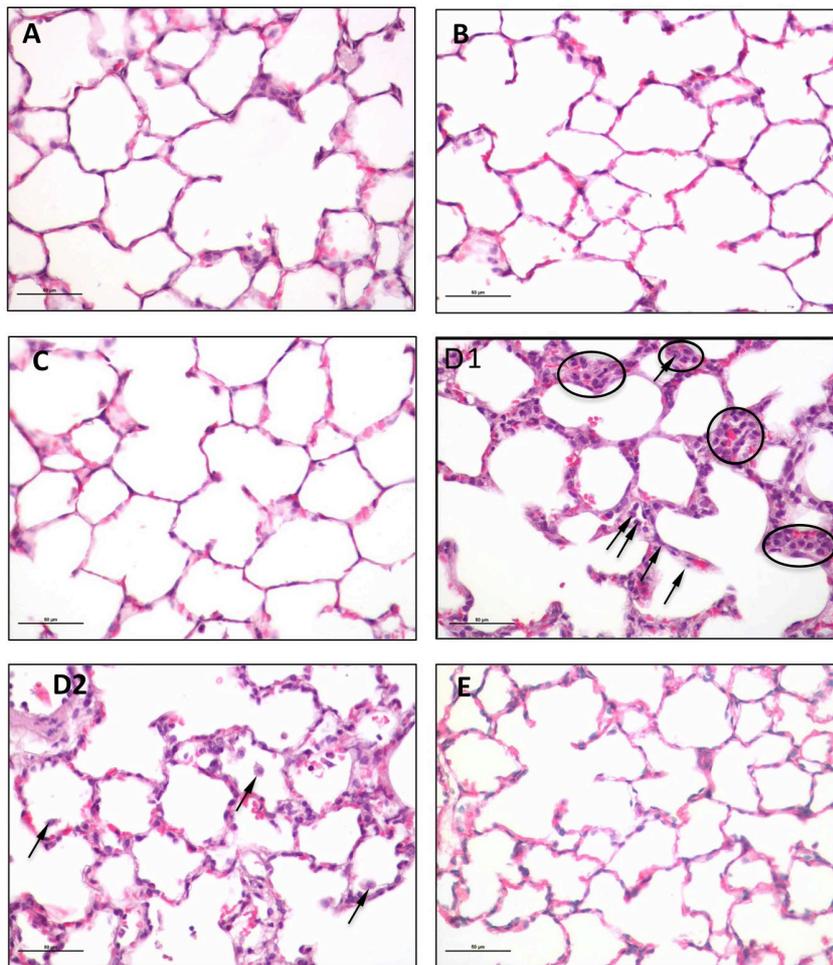


Fig. 5. Lung histology. The left lung was fixed following inflation and stained with H&E for histological study. A. Vehicle only control; B. Vehicle-treated and 2CLP-operated, harvested 24 hrs post insult; C. VLP-treated and 2CLP-operated harvested 24 hrs post insult; D1,2. VLP-treated and 2CLP-operated harvested 4 days post insult; E. VLP-treated and 2CLP-operated harvested 12 days post insult. Magnification x400.

Lungs of the sacrificed rats were harvested and the left lung was fixed for histological studies. As seen in Fig. 4 at 24 hours following the 2CLP-operation, both the vehicle-treated (B) and the VLP-treated rats (C) showed normal lung structure. Pathology was seen only in the rats harvested 4 days after the insult (D1, D2), when the VLP-treated animals began recovering from the severe sepsis. Fig. D1 shows mild septal thickening (surrounded by circles), associated with an increase in the number of histiocytic cells, indicated by arrows. A few neutrophils are seen in the thickened septae. Neutrophils were also observed in the lumen of blood vessels (not shown). Alveolar histiocytes/macrophages are visible in D2, indicated by arrows. On day 12 after the 2CLP-operation lungs of the recovered VLP-treated rats appeared normal.

Consistent with previous reports (reviewed in (Matute-Bello et al., 2008)) our histological study in the Sprague-Dawley rat presents a picture that is different from typical human ARDS.

In summary, The 2CLP sepsis model used in the present study leads to significant liver and lung injury and rapid death of the animals. VLP-treatment ameliorated the clinical condition sufficiently to allow the animals to overcome the crisis and begin recovery within 72 hours.

* **Milestones**

Milestone 1: Detailed description of disease progression in the 2CLP model and arrest by VLPs. Accomplished on time.

Milestone 2: Fixed and frozen tissue for Aim 4 –studies on the protective mechanism of VLPs in the 2CLP-induced model. Accomplished on time and used in Aim 4, described below.

Specific Aim 3. Development of a simplified reagent (“lead compound”).

VLPs cannot be produced in *E. coli*, because the different intra-cellular environment of the bacteria is unfavorable for VLP assembly. We routinely express them in insect cells, using the baculovirus expression system, which is cumbersome and costly. The aim of this part was to develop VP1 derivatives that do not assemble into capsid, and that can be produced in *E. coli*.

Major Task 9: Construction of plasmids

4 plasmids were designed and constructed. All included a 5' HIS-tag followed by TEV site for easy purification.

1. VP1, the complete VP1 protein.
2. VP1ΔC, in which the complete C-arm, which connects the pentamers in the capsid, is deleted (60 amino acids).
3. Core14, in which in addition to the complete C-arm deletion, is also deleted for 14 amino acids of the N-arm.
4. Core20, in which in addition to the complete C-arm deletion, is also deleted for 20 amino acids of the N-arm.

The rationale behind the cloning of two Core derivatives was based on the presence of a small loop at amino acids 15-20 with no obvious role in the capsid. We were uncertain whether the loop would be required for stabilizing the N-arm with the 20 amino acids deletion. Our data however showed that both Core derivatives had similar expression and stability.

Major Task 10: Expression and production of the VP1 derivatives

E. coli BL21 was transformed with the 4 plasmids, and optimal conditions for growth and IPTG induction were established. The protocol included lysis in a microfluidizer, concentration of the lysate by ultrafiltration and affinity chromatography on Ni-NTA column. The eluted fractions were tested for protein size, purity and concentration by SDS-polyacrylamide gel electrophoresis and OD 280/260.

The candidate lead compounds were tested in 2CLP rats at different doses as shown in Table 2.

Table 2. Survival of 2CLP rats following treatment with VP1 derivatives.

Derivative	Survivors at different doses			
	0.3 mg	0.6 mg/kg	1.2 mg/kg	2.4 mg/kg
VLPs	3/4			
VP1ΔC	0/2	0/2	0/2	1/2
Core14		0/4	1/4	1/4
Core20		0/4	0/4	1/4
VP1			1/4	2/4

The results demonstrated that efficacy of the derivatives was significantly lower than that of VLPs, produced in insect cells. Interestingly most effective was VP1, and perhaps also VP1ΔC. The other two derivatives were even less effective.

* Milestones

Milestone 1. VP1-reagents for testing in animals – achievement was delayed.

Milestone 2. Protocols for production and storage of the VP1-reagents - achievement was delayed.

Milestone 3. Proof of principle for therapeutic effect of the selected VP1-derivative.

Although some therapeutic effect of the VP1-derivatives was observed, it was lower than the efficacy obtained with VLPs. We concluded that either production and folding in the bacterial cell reduces the efficacy of the derivatives or that the complete capsid is needed for the higher therapeutic value of the VLPs.

In the future we plan to examine the possibility that using a different method for production and purification of VP1 and VP1ΔC, such as adding a leader peptide that will serve to excrete the product to the bacterial periplasm or to the medium. These approaches were demonstrated by others to enhance correct folding of eukaryotic protein produced in bacteria. Another possibility is to produce VLPs in yeast, which is also compatible with drug production

Specific Aim 4: The mechanism of ARDS attenuation by VLPs in the 2CLP model

Our plan was to perform RNAseq on tissue cultured cells in order to obtain a quick overview on potential pathways induced by the VLPs in ameliorating sepsis and sepsis-induced ARDS. The rationale was to validate these initial data by protein studies. We selected BEAS-2B, a normal lung cell for these studies. The data was not satisfactory, and we decided to move on to lung RNA.

Major Task 11: Analysis of animal tissue samples: to be done at different time-schedule for each ARDS model.

RNA was extracted from the right lung and purified for RNAseq. Sequences of the libraries, prepared by the Core Facilities of the Medical School, were sent to the Bioinformatics Unit for analysis. Pathway analyses were performed by Ingenuity software (IPA). (<https://www.qiagenbioinformatics.com/>).

The main findings obtained from RNA extracted from lungs harvested 24 hours after the 2CLP insult indicated that most of the predicted pathways were common to the rats treated with VLPs and those treated with vehicle only (designated VO). Since there was almost no difference between VLPs only control and vehicle control, both CLP+VO and CLP+VLP were compared to vehicle control. We attribute these common pathways to the 2CLP insult. Those are listed in Table 1. Only pathways with probability value $p < 0.05\%$ ($-\log(p\text{-value}) > 1.3$) are included.

Each data point is an average of RNA extracted from lungs of 3 animals. The data was evaluated for statistical reliability before further analysis.

Canonical pathways common to CLP+VO vs VO and CLP + VLP vs VLP	CLP+VO vs VO		CLP+VLP vs VO	
	$-\log p\text{-value}$	Z-score	$-\log p\text{-value}$	Z-score
Acute Phase Response Signaling	7.34	3.889	8.05	4.004
iNOS Signaling	3.66	3.464	4.38	3.606
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	2.91	3.182	4	4.564
TREM1 Signaling	4.07	3.13	5.84	2.711
Toll-like Receptor Signaling	1.95	2.714	2.85	1.941
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	1.7	2.668	2.71	4.025
IL-8 Signaling	4.05	2.667	3.21	2.959
IL-6 Signaling	4.14	2.353	5.25	3.024
Type I Diabetes Mellitus Signaling	4.76	2.294	6.54	2.746
Colorectal Cancer Metastasis Signaling	3.4	2.214	3.53	2.846
Antioxidant Action of Vitamin C	2.02	-2.5	2.16	-3

Table 3: Canonical Pathway affected with and without VLP-treatment. The data is arranged according to Z-score values of CLP+VO vs VO. Z-scores, which help in predicting the overall activation or the repression state of a canonical pathway, were calculated by Ingenuity IPA (Ingenuity Pathways Analysis). The Table presents only pathways with Z-scores greater than 2 or smaller than -2, which may be considered significantly activated or repressed, respectively. The many pathways that were not included had either Z-scores $2 > -2$, or, in many cases, Z-score could not be calculated due to lack of information on the pathway. Probability of significance was computed based on Benjamini and Hochberg false discovery rate (FDR), using FDR controlling procedures 0.05 (Benjamini and Hochberg, 1995). Only pathways with p-values < 0.05 , or $-\log(B\text{-H } p\text{-value}) > 1.3$, are included in the table.

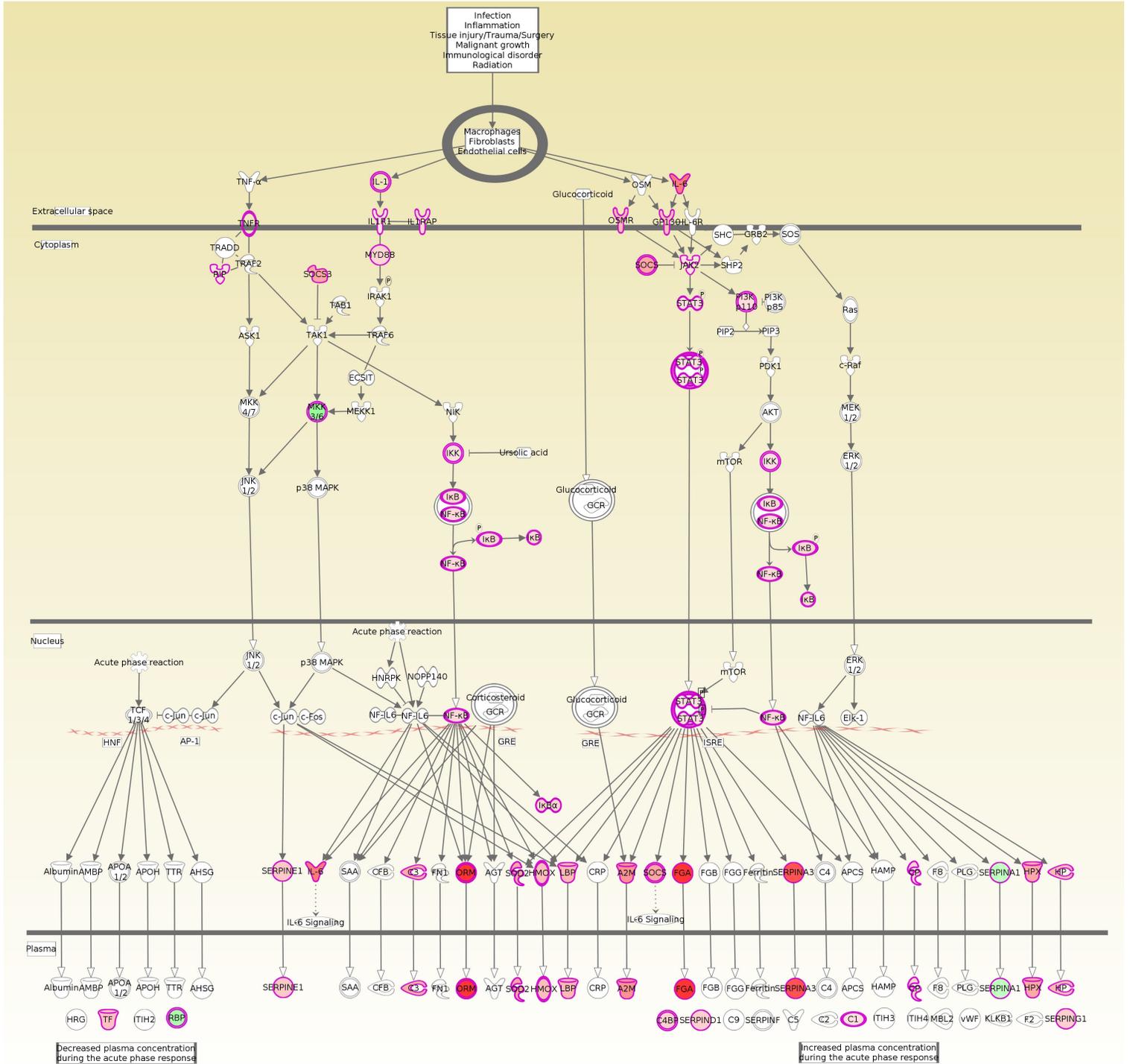


Fig. 6. Acute Phase Response Signaling pathway. Arranged by ingenuity. Red designates an increase and Green – decrease in the molecule. The shape of the molecule indicates its type.

The pathways presented in Table 3 are part of sepsis signaling networks induced by infection, consistent with their presence in both groups that underwent the 2CLP procedure, whether or not treated by VLPs. Receptors engaged in recognizing bacteria or viruses, including Toll-like receptor, serve as sensors of the infection (Akira et al., 2006). Those receptors function in conjunction with TREM-1, the signaling receptor expressed on neutrophils and monocytes (Arts et al., 2013). TREM-1 activates the cascade that leads to production of cytokines (interleukin-8 and -6) and chemokines (Akira et al., 2006), which induce the innate immune cascade leading to inflammation (Newton and Dixit, 2012). The acute phase response is a signaling network activated by the organism to restore homeostasis, by limiting the inflammatory effect of cytokines (Moshage, 1997). On the other hand, the inflammatory response induced by cytokines leads to leukocyte infiltration to the infected site, and is associated with activation of a key enzyme in macrophage inflammatory response, iNOS (inducible nitric oxide synthase), which functions in production of nitric oxide and reactive oxygen species (Newton and Dixit, 2012). These pathways also participate in a number of diseases, in particular diabetes and cancer. Finally, vitamin C, a powerful antioxidant, is an anti-inflammatory agent. The reduction in its pathway is therefore not surprising (Ceriello et al., 2013).

Remarkably, our analysis demonstrates that the VLP treatment induced pathways that were not induced by the vehicle. These canonical pathways are of great interest, as they might indicate the mechanism (or mechanisms) underlying the therapeutic effect of VLPs (Fig. 6A).

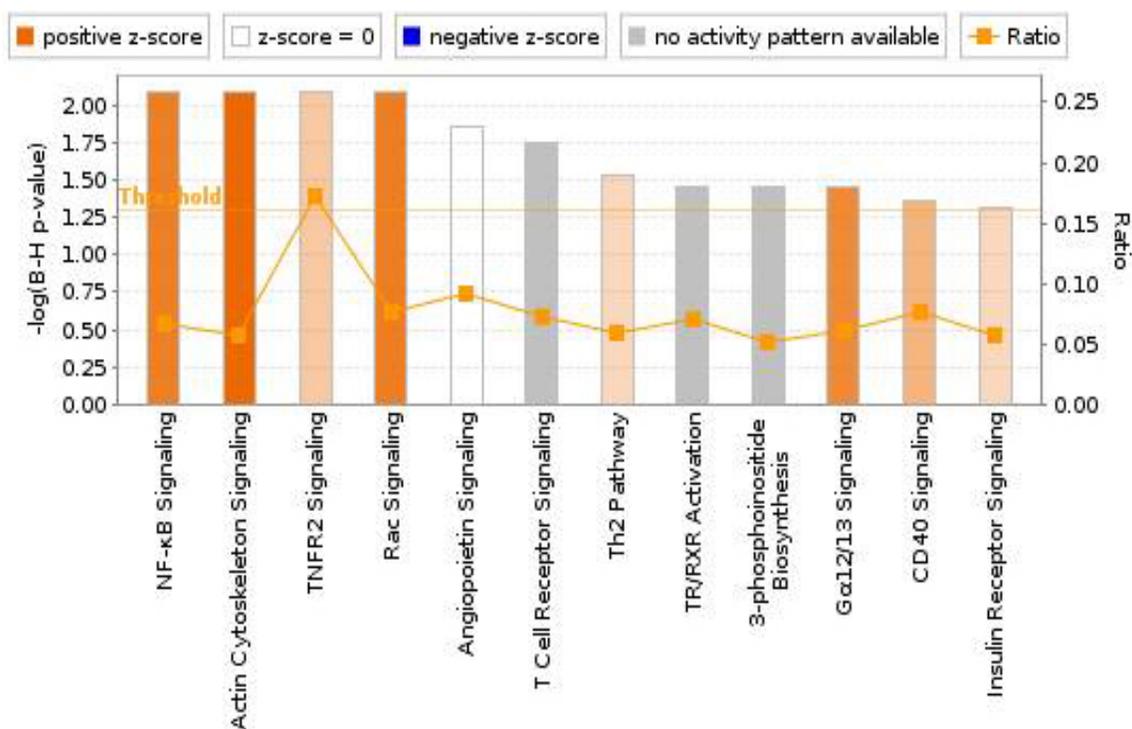


Fig. 6. Pathway induced in the VLP-treated 2CLP animals and not in untreated 2CLP animals. Only pathways with $-\log(\text{B-H p-value}) < 1.3$ are shown. Dark orange designates pathways with Z-scores > 2 ; light orange – pathways with Z-scores < 2 ; Pathways whose Z-scores could not be computed because of lack of information are colored gray; white indicates that the pathway did not increase compared to the control rats that did not undergo 2CLP insult.

Nf-κB, Actin cytoskeleton and Rac and Gα12/13 are canonical signaling pathways that increase in the VLP-treated septic rats (Fig. 6), with Z-scores > 2 . Nf-κB high level activation during

inflammation is considered to be fatal (Natoli et al., 2011). The increase in Nf- κ B, a central inflammatory stimulus (Natoli et al., 2011), in rats that were treated with VLPs in comparison to vehicle-treated rats, appears to be paradoxical. Detailed inspection of the data showed that in fact, this pathway is also elevated in the vehicle-treated rats, but with a low Z-score, 1.3, suggesting a mild increase in comparison with the VLP-treated rats (Z-score of 2.31). Since the untreated rats were almost dying at that time point, and as Nf- κ B is an early inflammation signal, we interpret the results to suggest that in those rats the Nf- κ B signal is beyond its peak at that time. Similarly the G α 12/13 signaling pathway, which functions in endothelial cell permeability and is increased during early inflammatory stage (Gavard and Gutkind, 2008), appears to be beyond its peak in the untreated rats. Its Z-score in those rats is \sim 0, suggesting that at that time point the pathway returned to normal level.

Finally, the two other pathways that increase in VLP-treated rats provide a glimpse into the therapeutic mechanism of VLPs. The actin cytoskeleton pathway functions in stabilizing the endothelial barrier that is compromised during sepsis allowing blood cell infiltration (Schlegel and Waschke, 2014). Rac1, activated by cAMP and other factors, is at the hub of the network, mediated enforcement of adherens junctions and strengthening of the cortical actin cytoskeleton. Additional support to its anti-inflammatory role is the findings that inflammation interferes with the Rac network (Schlegel and Waschke, 2014).

Our findings suggest that stabilization of the endothelial barrier is a critical early step induced by VLPs in counteracting inflammation.

* **Milestones**

Milestone 1: Data of signaling pathways induced during ARDS development in the 2CLP.

Milestone achieved.

Milestone 2: Data on beneficial signaling pathways induced by VLPs. Data achieved. Validation of the data is to be continued.

Major Task 12: Summary of the findings and drawing conclusions

This short 18-month project yielded a large amount of data. This annual report provides an almost complete summary of the findings. However some experiments (Milestone 2 above) are still in progress. These experiments will be completed and a comprehensive manuscript will be submitted to publication in a prestigious journal.

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What opportunities for training and professional development has the project provided?

2 postdocs and 1 PhD student worked on the project. All 3 were trained in the subject matter, contributing to their professional development.

How were the results disseminated to communities of interest?

- A. The results were presented orally in two Scientific Conferences, and raised much interest in both:
1. **The DNA Tumor Virus Meeting**, Montreal (Quebec) Canada, July 18 -23, 2016
 2. **Virus Structure and Assembly, FASEB Science Research Conference**, July 24-29, 2016 Steamboat Springs, Colorado.
- B. We are in the process of writing a complete manuscript for a high impact journal.

What do you plan to do during the next reporting period to accomplish the goals?

This is a final report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

1. Proof of concept of the efficacy of SV40 VLPs in attenuating ARDS, preventing death and facilitating recovery of a significant proportion of the 2CLP model ARDS rats.
2. Some understanding of the pathophysiology of severe sepsis and ARDS in rats.
3. Initial findings on the mechanism of therapeutic activity of VLPs.

These studies revealed that SV40 VLPs may be effective not only against ARDS, but also for treating sepsis and trauma.

What was the impact on other disciplines?

The findings of this study also impact the field of virus-host interactions.

What was the impact on technology transfer?

This project provides a crucial step in the way to apply SV40 capsid elements to human treatment. The next step in this process is experiments in large animals, such as pigs, under conditions recapitulating Intensive Care Units in hospitals. Development of methods to produce a capsid-derived reagent in *E. coli* is also necessary. In addition, efficacy of administration of the capsid-derived reagent when sepsis is first suspected or diagnosed will also affect the range of applications of this potential therapeutic agent.

What was the impact on society beyond science and technology?

We anticipate a significant impact on human health, in particular in the field of sepsis and trauma.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

We originally planned to also use a toxic ARDS model, induced by E. coli liposaccharide (LPS). However we found that the disease induced by LPS was too mild for testing for the effect of VLPs in a reasonable number of animals. While all the rats showed signs of illness around 24 hours, they almost all recovered afterwards. We therefore stopped those efforts and proceeded directly with the 2CLP model (see fig. 1).

The change was approved by our officer Dr. Usamah S Kayyali, in his letter of May 18, 2016.

Actual or anticipated problems or delays and actions or plans to resolve them

We did not achieve a satisfactory lead compound, produced in bacteria, for drug development. A major reason may be that a complete capsid is needed for full activities. We therefore plan to produce full capsids either in E. coli or in yeast. Future studies will be performed in close collaboration with experts in the field of protein production.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Manuscript in preparation:

Title: **Signaling pathways elicited by SV40 harnessed for therapy of critical clinical conditions.**

Oral Presentations at scientific meeting:

1. **The DNA Tumor Virus Meeting**, Montreal (Quebec) Canada, July 18 -23, 2016.
2. **Virus Structure and Assembly**, FASEB Science Research Conference, July 24-29, 2016 Steamboat Springs, Colorado.
3. **Seminar at the Hebrew University Medical School**, January 17, 2017.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Participants

Name:	Prof. Ariella Oppenheim
Project Role:	PI
Researcher Identifier:	None
Nearest person month worked:	6
Contribution to Project:	Directed the project
Funding Support:	This project.

Name:	Dr. ArieH Eden
Project Role:	Consultant
Researcher Identifier:	None
Nearest person month worked:	2

Contribution to Project: Clinical consultation
Funding Support: None

Name: Dr. Rohit Srivastava
Project Role: Postdoc Researcher
Researcher Identifier: None
Nearest person month worked: 18
Contribution to Project: Performs and directs the animal experimentations. Assists in summarizing data.
Funding Support: This project and BSF grant 2013041

Name: Dr. Shashi Gandhi
Project Role: Postdoc Researcher
Researcher Identifier: None
Nearest person month worked: 18
Contribution to Project: Production and purification of VLPs. Cloning, production and purification of VP1 derivatives.
Assists in animal experimentations and summarizing data.
Funding Support: This project and BSF grant 2013041

Name: Mahdi Khatib
Project Role: PhD Student
Researcher Identifier: None
Nearest person month worked: 6
Contribution to Project: Assists in animal experimentations and summarizing data.
Funding Support: This project and BSF grant 2013041

Name: Mrs. Carol Levi
Project Role: Laboratory Technician
Researcher Identifier: None
Nearest person month worked: 12
Contribution to Project: Assisting in animal experimentations.
Funding Support: This project

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS:

Quad Chart, Attached as Appendix 2.

9. APPENDICES:

Appendix 1: Abstracts of oral presentation at two Scientific Conferences during July 2016.

Appendix 2: Quad Chart.

Appendix 1.

I. Oral Presentation at:

The DNA Tumor Virus Meeting, Montreal (Quebec) Canada, July 18 -23, 2016

Signaling pathways elicited by SV40 harnessed for therapy of critical clinical conditions.

Rohit Srivastava¹, Shashi Gandhi¹, Carol Levi¹, Orly Ben-nun-Shaul¹, Arieh Eden² and Ariella Oppenheim¹

¹Hebrew University-Hadassah Medical School, Jerusalem, and ²Carmel Medical Center, Haifa, Israel.

Our research on cellular signaling elicited by SV40 showed that immediately following adsorption the virus induces a complex, robustly balanced network. The network comprises host response to the viral attack and virus induced signaling that overcomes host response. It includes Ca⁺⁺ signaling, caspases, stress response (chaperones), Akt-1 survival pathway and anti-apoptotic signals. These signals are required for SV40 cell entry during early steps, prior to its nuclear entry and viral T-antigen expression, before T-antigen takes over control of viral DNA synthesis. Not surprisingly, the same (or similar) signaling pathways are induced by SV40 VLPs, composed exclusively of recombinant VP1, without any genetic material.

Using a mouse model for toxic Acute Kidney Injury (AKI), we demonstrated that systemic administration of SV40 VLPs significantly increased survival from 12% to >60%. We further showed that AKI attenuation was achieved by upregulation of Hsp70 and activation of Akt-1 survival pathway. Presently we investigate whether SV40 VLPs might also ameliorate sepsis.

Sepsis syndrome is characterized by multiple organ injury due to uncontrolled inflammatory response and leads to significant morbidity and mortality even with current medical advances. The treatment of sepsis is limited to control the infection source, antibiotics, and supportive therapy for failing organs. Over the past few decades multiple attempts have been made to control the immunological response to sepsis. However, while successful in small animal studies, these interventions failed in clinical trials. We reasoned that the failure was because the interventions were aimed at single targets within the immune response. Our approach, on the other hand, is to target multiple endogenous signaling pathways, shaped by evolutionary forces of virus-host interactions

Using a rat model for sepsis, we have recently found that systemic delivery of VLPs dramatically increased survival, from nil to 75%. The mechanisms underlying sepsis attenuation by VLPs is currently under investigation. The implications of this study to the development of a therapeutic mode to treat sepsis and other critical clinical conditions will be discussed.

Supported by the US Army, contract No. W81XWH-15-1-0125

II. Oral Presentation at:

Virus Structure and Assembly, FASEB Science Research Conference, July 24-29, 2016, Steamboat Springs, Colorado.

Signaling pathways elicited by SV40 harnessed for therapy of critical clinical conditions.

Rohit Srivastava¹, Shashi Gandhi¹, Carol Levi¹, Orly Ben-nun-Shaul¹, Arieh Eden² and Ariella Oppenheim¹

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Using a rat model for sepsis, we have recently found that systemic delivery of VLPs dramatically increased survival, from nil to 75%. The mechanisms underlying sepsis attenuation by VLPs is currently under investigation. The implications of this study to the development of a therapeutic mode to treat sepsis and other critical clinical conditions will be discussed.

Supported by the US Army, contract No. W81XWH-15-1-0125

PR140862: Potential application of viral empty capsids for the treatment of Acute Lung Injury/ Acute Respiratory Distress Syndrome

PI: Prof. Ariella Oppenheim, Hebrew University of Jerusalem

Budget: \$ 197,224.00

Topic Area: Respiratory Health

Mechanism: Discovery Award

Research Area(s): **Award Status:** Open; POP: 01-JUL-2015 TO 31-DEC-2016

Study Goals:

The overall goal is to test the hypothesis that SV40 VLPs are capable of ameliorating ARDS. The hypothesis was to be tested in the two pre-clinical rat models. The study also aimed to provide information with regard to dose, kinetics and pharmacokinetics of the VLPs in this proposed medical application.

Specific Aims:

1. To test the efficacy of VLPs in the treatment of ARDS in the endotoxin (LPS) model through assessment of lung pathology, the inflammatory state, oxidative stress and damage to alveolar and endothelial cell in VLP treated in comparison to untreated ARDS rats.
2. To test the efficacy of VLPs in the treatment of ARDS in the 2CLP model, similarly to Aim 1.
3. To begin developing a simplified reagent ("lead compound").
4. To obtain an insight into the mechanism of VLP attenuation of ARDS, by investigating signaling pathways elicited by VLPs.

Key Accomplishments:

Specific aim 1: Studies on the endotoxin (LPS) model were discontinued because we were not able to achieve 50-80% death in 48 hrs. Studies on this model were discontinued. VLPs stocks for animal experiments were prepared.

Specific aim 2: Efficacy of VLPs in treatment of the 2CLP-ARDS rat model. The 2CLP model was established. Efficacy studies demonstrated survival of 75% of the VLP-treated rats while none of the untreated rats survived. Detailed studies on disease progression and arrest indicated that 24 hours post-2CLP injury both treated and untreated groups were severely ill. While the condition of the untreated rats continued to deteriorate, the VLP-treated rats stopped deteriorating and began recovery. By 12 days they were fully recovered.

Specific aim 3: Four different VP-1 derivatives were constructed and produced in *E. coli*, and tested for efficacy as potential lead-compound. They all reduced mortality of untreated 2CLP rat, however at a significantly higher dose than VLPs. This aim will be continued in future studies.

Specific aim 4: RNA was extracted from lung tissue of treated and untreated 2CLP rats. RNAseq experiments indicated the many central pathways that are involved in 2CLP-induced sepsis, including Acute Phase Response Signaling, immune-response, cytokine dependent pathways, damage to endothelial cells, coagulation signaling, inflammation and disease-related signaling. The first pathway induced by VLPs in treated rats is stabilization of blood cells via RAC-1 and Gα12/13 signaling.

Key Outcomes:

Proof of principle for therapeutic effect of VLP for 2CLP induced severe sepsis and ARDS.

Demonstration of very high efficacy of VLPs in treatment of 2CLP in rats.

Description of the disease clinical progress and its arrest by VLPs via symptoms, body weight, blood parameters and lung histology.

Obtaining an insight into the biological pathways that are involved in sepsis-induced ARDS and VLP-therapeutic mechanisms in rats.