



The role of shed GP in Ebola virus pathogenicity

Beatriz Escudero Pérez

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The role of shed GP in Ebola virus pathogenicity

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La verdadera ciencia enseña, por encima de todo, a dudar y a ser ignorante.

Miguel de Unamuno (1864-1936)

“Don't let the noise of other's opinions drown out your own inner voice. And most important, have the courage to follow your heart and intuition. They somehow already know what you truly want to become. Everything else is secondary.”

Steve Jobs (1955-2011)

Abstract

Study of the role of Ebola virus shed GP

During Ebola virus (EBOV) infection several soluble glycoproteins are released in high amounts from infected cells but as yet still no clear role has been identified for these viral proteins. We hypothesized that the impairment of coagulation and vascular systems observed during EBOV infection could be, at least in part, due to these soluble glycoproteins.

Here, for the first time we identify the cellular targets of EBOV soluble protein shed GP and provide evidence that through its glycosylation, shed GP can activate non-infected dendritic cells and macrophages, inducing, through TLR4, the secretion of pro-inflammatory cytokines. We also demonstrate that shed GP activity is negated upon addition of Mannose-Binding sera Lectin (MBL), a molecule known to interact with sugar arrays present on the surface of different microorganisms. We further demonstrate that shed GP binds MBL and activates the MBL-associated proteases MASP-1 and MASP-2, implicated in the coagulation pathway. Moreover, shed GP is capable of competing with mannose for MBL binding. Importantly, we show that shed GP causes monocytes, macrophages and HUVECs to increase their expression of tissue factor (TF), which has been shown to be the primary molecule in endothelial cells that results in alterations in both permeability and procoagulant activity. Treatment of HUVECs with shed GP resulted in increased surface expression of the attachment factors ICAM and VCAM but also the release of soluble s-ICAM, s-VCAM, s-E-selectin s-TF and s-thrombomodulin. We have also revealed that shed GP activates permeability of HUVECs both directly and indirectly through cytokine release.

Overall, this study suggests that shed GP may be one of the principal factors responsible for the early stimulation of immune cells that then produce high amounts of proinflammatory cytokines. Moreover shed GP also activates MBL and therefore may play a critical role in the dysregulation of coagulation and permeability responses that, combined with massive virus replication and virus-induced cell damage, can lead to a septic shock-like syndrome and high mortality.

Keywords

Ebola virus

Soluble glycoproteins

Shed GP

Immune response

Coagulopathy

Permeability

Pathogenesis

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List of abbreviations

α	Anti- / Alpha
Δ	Delta
Ab	Antibody
AT	AntiThrombin
ATP	Adenosine TriPhosphate
AP-1	Activator Protein-1
APC	Antigen Presenting Cell
BEBOV	Bundibugyo EBOla Virus
BSL-4	BioSecurity Level 4
DNA	DeoxyRibonucleic Acid
°C	degrees Celsius
cDC	conventional Dendritic Cells
CIEBOV	Côte d'Ivoire EBOla Virus (=Taï Forest)
CK	CytoKines
CMV	CytoMegaloVirus
CRD	Carbohydrate-Recognition Domain
CTL	CytoToxic Lymphocytes
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
D-D	D-Dimers

DIC	Disseminated Intravascular Coagulation
DMEM	Dulbecco modified Eagle's medium
DRC	Democratic Republic of Congo
EBOV	EBOLA Virus
EC	Endothelial Cells
EHF	Ebola Haemorrhagic Fever
ELISA	Enzyme Linked ImmunoSorbent Assay
EPCR	Endothelial Protein C Receptor
FA	Fluorescence Assay
FBS	Fetal Bovine Serum
FDP	Fibrinogen Degradation Products
GFP	Green Fluorescence Protein
GP	Glycoprotein
HA	HemAgglutinin
HIV	Human Immunodeficiency Virus
HR1/HR2	Heptated Repeat 1/2
HS	High Shedding
*HS	deglycosylated High Shedding
HUVEC	Human umbilical vein endothelial cells
ICAM -1	Intercellular Adhesion Molecule-1 (=CD54)
IFA	ImmunoFluorescence Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IFL	Internal Fusion Loop
IFN	InterFeroN
IL	InterLeukine
IRADs	Innate Response Antagonist Domains
IRF-3/IRF-9	Interferon Regulatory Factor 3/9
ISG	Interferon Stimulated Genes
ISRE	Interferon Stimulated Response Elements
kDa	kilo Dalton
LBP	Lypopolysaccharide Binding Protein
LLOV	LLOviu Virus
LPS	Lipopolysaccharide
LS	Low Shedding
L-SECTin	Liver/Lymph node Sinusoidal Endothelial cell C-type lectin
L-SIGN	Liver/Lymph node-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin
MØ	Macrophages
MAL	Membrane Attack Complex
MASP	MBL Associated Serin Proteases
MARV	MARburg Virus
MBL	Mannose Binding Lectin
MDA-5	Melanoma Differentiation-Associated protein 5
MGL	Macrophage Galactose Lectin
MHC	Major Histocompatibility Complex

MLD	Mucin Like Domain
MOI	Multiplicity Of Infection
mRNA	messenger RiboNucleic Acid
NF-κB	Nuclear Factor-kappa B
NHP	Non-Human Primates
NK	Natural Killer cell
NO	Nitric Oxide
NP	NucleoProtein
NPC1	Niemann-Pick C1
PA	Plasminogen Activator
PAI-1	Plasminogen Activator Inhibitor-1
PAF	Platelet-Activating Factor
PAR	Protein-Activated Receptors
PCA	Protein C Activated
PCR	Polymerase Chain Reaction
pDC	plasmacytoid Dendritic Cells
PFA	ParaFormAlehyde
PKR	Protein Kinase R
qPCR	quantitative Polymerase Chain Reaction
RBD	Receptor-Binding Domain
REBOV	Reston EBOLA Virus
rNAP_{C2}	recombinant Nematode Anticoagulant Protein C2
RIG-1	Retinoic acid-Inducible Gene 1

RNA	RiboNucleic Acid
RNP	RiboNucleoProtein complex
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SARS	Severe Acute Respiratory Syndrome
SEBOV	Sudan EBOla Virus
sGP	secreted GlycoProtein
ssGP	small secreted GlycoProtein
SNP	Single Nucleotide Polymorphism
STAT-1	Signal Transducers and Activators of Transcription- 1
TACE	TNF- α converting enzyme
TAFI	Thrombin-Activatable Fibrinolysis Inhibitor
TAM	Tyro-3, Axl, and Mer families
TIM-1	T-cell Immunoglobulin Mucin Domain 1
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TLR	Toll Like Receptor
TM	TransMembrane
ThM	THromboModulin
TNF	Tumor Necrosis Factor
TRAIL	TNF Related Apoptosis-Inducing Ligand
PAMP	Pathogen Associated Molecular Patterns
PBL	Peripheral Blood Lymphocytes

PBMC	Peripheral Blood Mononucleated Cells
PC	Protein C
PECAM	Platelet Endothelial Cell Adhesion Molecule (=CD31)
pi	post infection
PRR	Pattern Recognition Receptor
t-PA	tissue-type Plasminogen Activator
u-PA	urokinase-type Plasminogen Activator
VCAM-1	Vascular Cell Adhesion Molecule- 1
VLP	Virus Like Particles
VP	Viral Protein
VSV	Vesicular Stomatitis Virus
WT	Wild Type
ZEBOV	Zaire EBOla Virus

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INTRODUCTION

1. Ebola virus hemorrhagic fever

1.1. Epidemiology

Ebola virus is the etiologic agent of a highly lethal hemorrhagic fever in humans and nonhuman primates (NHPs) with mortality rates of up to 90% in humans (Zampieri, Sullivan et al. 2007). Due to its high mortality rate, its potential for human-to-human transmission and the lack of an approved vaccine or therapy, the virus is classified as a category A pathogen requiring biosafety level 4 (BSL-4) containment.

Ebola virus belongs to the family *Filoviridae*, in the order Mononegavirales which includes *Rhabdoviridae* and *Paramyxoviridae*. The family consists of three genera: *Marburgvirus*, *Ebolavirus* and *Cuevavirus* (Kuhn, Becker et al. 2010).

Ebola haemorrhagic fever (EHF) is caused by five genetically distinct members of the *Filoviridae* family: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Côte d'Ivoire ebolavirus* (CIEBOV), *Bundibugyo ebolavirus* (BEBOV) and *Reston ebolavirus* (REBOV). The species vary in their pathogenicity in humans with ZEBOV being the most pathogenic (up to 90% case fatality rate). For Sudan and Bundibugyo Ebolavirus the mortality rate is lower, 53% and 34% respectively. The species Côte d'Ivoire is lethal for NHPs (Formenty, Boesch et al. 1999). Ebolavirus Reston is not pathogenic for humans (4 asymptomatic cases have been described), however, it is lethal for NHPs and pigs. All species are endemic in Africa except Ebolavirus Reston, which is endemic in the Philippines (Miranda, Ksiazek et al. 1999, Barrette, Metwally et al. 2009). A number of sporadic outbreaks have been reported since the initial discovery of EBOV in 1976 (**Table 1**) (Arthur 2002, Mahanty and Bray 2004, Baize, Pannetier et al. 2014).

Date	EBOV strain	Source of infection	Number of human cases	Fatality rate	Location
1976	ZEBOV	Unknown	318	88%	DRC
1976	SEBOV	Unknown	284	53%	Sudan
1977	ZEBOV	Unknown	1	100%	DRC (Tandala)
1979	SEBOV	Unknown	34	65%	Sudan
1989	REBOV	Imported macaques	-	-	USA (Virginia)
1990	REBOV	Imported macaques	-	-	USA (Pennsylvania)
1992	REBOV	Imported macaques	-	-	Italy
1994	CIEBOV	Dead chimp	1	0%	Côte d'Ivoire
1994-1995	ZEBOV	Unknown	49	59%	Gabon
1995	ZEBOV	Unknown	317	77%	DRC (Kiwit)
1996	REBOV	Imported macaques	-	-	USA (Texas)
1996	ZEBOV	Dead chimp	35	68%	Gabon (Mayibout)
1996	ZEBOV	Unknown	60	75%	Gabon (Booué)
2000-2001	SEBOV	Contact with NHP	425	53%	Ouganda (Gulu)
2001	ZEBOV	Contact with NHP	123	79%	Gabon/Congo
2002-2003	ZEBOV	Unknown	143	90%	Congo (Mbomo, Kéllé)
2003	ZEBOV	Unknown	35	83%	Congo (Mbomo)
2004	SEBOV	Unknown	17	42%	Sudan (Yambio)
2005	ZEBOV	Contact with bats	12	75%	Congo (Etoumbi, Mbomo)
2007	ZEBOV	Unknown	249	74%	DRC (Kasaï)
2007	BEBOV	Unknown	56	40%	Ouganda (Bundibugyo)
2008-2009	ZEBOV	Unknown	32	47%	DRC (Kasaï)
2011	SEBOV	Unknown	1	100%	Ouganda (Luwero)
2012	SEBOV	Unknown	24	71%	Ouganda (Kibaale)
2012	BEBOV	Unknown	52	48%	DRC (Haut-Uélé)
2014	ZEBOV	Unknown	604	63%	Guinea, Liberia, Sierra Leone

Table 1.- Ebola virus outbreaks in human beings. Adapted from (Arthur 2002, Mahanty and Bray 2004, Baize, Pannetier et al. 2014).

The geographical distribution of the different epidemics of filovirus hemorrhagic fevers corresponds to the equatorial zone of the globe (Figure 1). Ebola haemorrhagic fever remains a plague for the population of equatorial Africa, with an increase in the numbers of outbreaks and cases since 2000. Almost all human cases are due to the emergence or reemergence of Zaire Ebola virus in regions of Gabon, Republic of the Congo, DRC and very recently in west Africa, and of Sudan Ebola virus in Sudan and Uganda. Subsequently, Reston Ebola virus has been found in the Philippines on several occasions, including reports documenting infections in pigs (Barrette, Metwally et al. 2009). The emergence of Reston Ebola virus in pigs raises important concerns for public health,

agriculture, and food safety in the Philippines and could represent a serious issue for parts of Asia (Feldmann and Geisbert 2011).

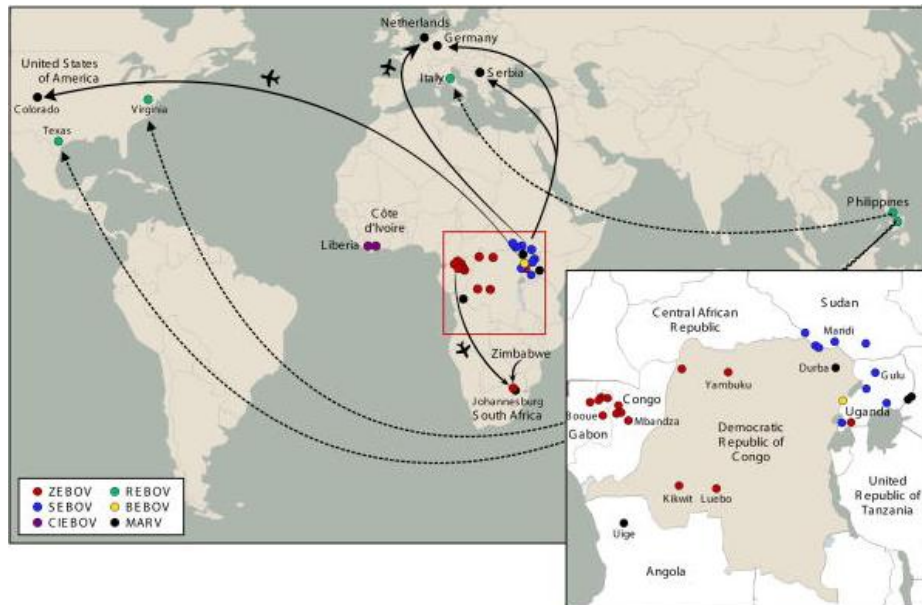


Figure 1.- Geographic distribution of filovirus outbreaks. (Prior to 2014)
Adapted from (Feldmann and Geisbert 2011).

A novel filovirus, provisionally named Lloviu virus (LLOV), was detected during the investigation of *Miniopterus schreibersii* die-offs in Cueva del Lloviu in southern Europe. LLOV is genetically distinct from other marburgviruses and ebolaviruses and is the first filovirus detected in Europe that was not imported from an endemic area in Africa (Negredo, Palacios et al. 2011). Phylogenetic analysis of LLOV indicates a common ancestor of all filoviruses, dating to at least 150,000 years ago (**Figure 2**). The discovery of LLOV in *M. schreibersii* is consistent with filovirus tropism for bats. However, unlike MARV and EBOV, where asymptomatic circulation is posited to be consistent with evolution to avirulence in this long-term host-parasite relationship, several observations suggest that in the case of LLOV, filovirus infection may be pathogenic; LLOV was found in the affected bat population but not in other healthy bats. In addition, RNA sequences were found in lung and spleen of the affected bat population. The discovery of a novel filovirus in western Europe, and the wide geographical distribution of the associated insectivorous bat is a significant concern. While the virus was detected in the north of Spain, simultaneous bat die-offs were also been observed in Portugal and France (Negredo, Palacios et al. 2011). Filoviruses had been suggested to show a geographically related

phylogeographic structure (Peterson, Bauer et al. 2004). Viruses from particular geographic areas cluster together phylogenetically, suggesting a stable host-parasite relationship wherein viruses are maintained in permanent local pools. The discovery of a novel filovirus in a distinct geographical niche suggests that the diversity and distribution of filoviruses should be studied further.

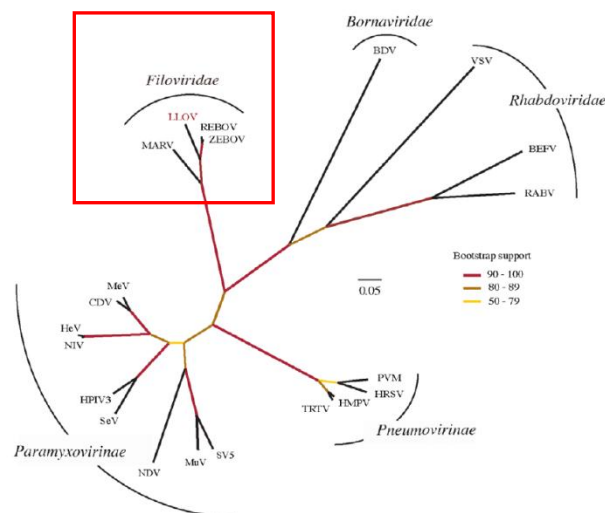


Figure 2.- Discovery of an ebolavirus-like filovirus in europe. Adapted from (Negredo, Palacios et al. 2011).

Recently, a study has shown that additional bat species not previously associated with EBOV such as *Epomorphorus gambianus*, *Nanycteris veldkampii* and *Eidolon helvum* contain antibodies against ZEBOV and REBOV (Hayman, Yu et al. 2012). This reinforces the hypothesis of bats being the possible natural reservoir of filoviruses.

Serological evidence of ebolavirus infection in several bat populations in China has also recently been reported (Yuan, Zhang et al. 2012). This, together with the Lloviu finding, suggests that filoviruses have a wider host range and geographical distribution than previously thought.

1.2. Ebola virus natural cycle

1.2.1. Ebola virus reservoir

Despite the discovery of this virus family almost 40 years ago, the natural reservoir of these lethal pathogens remains an enigma.

Several studies have been undertaken in order to try and identify the natural reservoir of the filoviruses. Since 1976 these studies have been based on the capture and analysis of plants and wild animals (vertebrates and invertebrates) suspected to be potential natural hosts of filoviruses.

In outbreaks where information is available, the human index cases have invariably had direct contact with gorillas, chimpanzees, antelope or bats. The search for a reservoir of EBOV has been very aggressive. Although great apes are generally involved in EHF outbreaks, NHPs are not thought to be natural reservoirs but, rather, susceptible hosts, based on the sudden sharp decline in populations of the great apes in Gabon and the Republic of Congo which coincided with EBOV outbreaks in humans (Pourrut, Kumulungui et al. 2005).

In one study, short EBOV-specific genetic sequences were amplified from organs of six mice (*Mus setulosus* and *Praomys* sp) and a shrew (*Sylvisorex ollula*). However no firm conclusions as to the EBOV reservoir status of these animals can be drawn, given the lack of specific serologic responses, the lack of nucleotide specificities in the amplified viral sequences, the failure of virus isolation attempts and the non-reproducible nature of the results. Also several attempts were made to infect various plant and animal species, including rodents (mice, shrews, rats, guinea pigs, etc.), birds (mainly pigeons), reptiles (tortoises, snakes, geckos, frogs), molluscs (mainly snails), arthropods (spiders, cockroaches, ants, centipedes, mosquitoes and butterflies) and more than 33 plant varieties belonging to 24 species (tomatoes, cucumbers, wheat, cotton, lupin, corn, tobacco, etc.). All attempts to inoculate these species failed to yield evidence of virus replication (Pourrut, Kumulungui et al. 2005).

In contrast, EBOV infection of vertebrates and invertebrates gave the first evidence that both insectivorous and frugivorous bats can support the replication and circulation of EBOV (Swanepoel, Leman et al. 1996). This

evidence, along with reports of bat exposures for some of the Ebola index cases, directed research towards bats as potential reservoirs. Indeed, an ecological survey revealed the presence of ZEBOV-specific antibodies in six bat species caught in the field (*Epomops franqueti*, *Hypsignathus monstrosus*, *Myonycteris torquata*, *Micropteropus pusillus*, *Mops condylurus* and *Rousettus aegyptiacus*) (Pourrut, Kumulungui et al. 2005). Since 2000, trapping expeditions have been undertaken in areas close to outbreaks in order to identify the viral reservoir. In total, in one study 1,030 animals were captured and were tested for evidence of infection by Ebola virus. Immunoglobulin G (IgG) specific for Ebola virus was detected in serum from three different bat species: *Hypsignathus monstrosus*, *Epomops franqueti* and *Myonycteris torquata* (**Figure 3**). Viral nucleotide sequences, some of them clustering phylogenetically within the Zaire clade, were detected in liver and spleen in other bats from the same populations. Surprisingly, none of the IgG-positive animals was PCR-positive, and none of the PCR-positive animals was IgG-positive. Moreover it was not possible to isolate the virus itself (Leroy, Kumulungui et al. 2005).



Hypsignathus monstrosus



Epomops franqueti



Myonycteris torquata

Figure 3.- Possible Ebola virus reservoirs

The unsuccessful identification of ebolavirus-related genes in the samples is likely attributable to the often low-level of virus replication, the similarly transient

nature of the infection in bats or the sequence mis-match of the PCR primers used and the possibly divergent target sequence of the potential unknown ebolavirus genomes.

There are several possibilities to account for the failure to detect neutralizing antibodies. In general, bats seem to produce lower level of neutralizing antibodies in response to viral infection, possibly due to the lower affinity of the bat antibodies (Baker, Tachedjian et al. 2010). Alternatively, it is possible that one or more as-yet-unknown ebolaviruses are circulating among the bat populations sampled.

In addition, investigations are usually implemented retrospectively, several weeks or months after the index case has been infected from a putative reservoir. It is possible that by that time the putative reservoirs may have moved to another site. A surveillance system capable of early detection of Ebola cases could allow animal reservoir studies in 'real time', which is not always easy in remote places in African forests.

1.2.1.1. Bats as possible reservoir

Numerous publications in the past few years have reinforced the observation that bats carry a wide range of novel RNA and DNA viruses. One might hypothesize that they could play an important role in facilitating the dispersal of these viruses to different geographical locations and different hosts. The recent surge of interest in bats as a reservoir of viruses was driven by two factors. First, in the last 20 years, several high profile viral pathogens have been proven or hypothesized to have a bat origin (Hendra virus (Halpin, Young et al. 2000), Nipah virus (Yadav, Raut et al. 2012), Severe acute respiratory syndrome (SARS)(Field 2009),(Balboni, Battilani et al. 2012)) and MERS (Kupferschmidt 2013) .

The second driver for the recent surge in bat virus research has come from advances in modern molecular techniques which have presented opportunities for discovery of novel viruses, that were considered impossible or nonpractical just a decade ago. Using pan-virus-specific primers and next-generation

sequencing, it is now possible to detect and characterize novel viral sequences without the need for virus isolation by cell culture or the identification of virions by electron microscopy.

Bats are one of the longest living mammalian orders, and probably constitute the order with the most diversity between species. They originated early, around 50 million years ago, and the different species have changed relatively little over time compared to mammals of other taxa. The wide range of bat species could provide a large 'breeding ground' for viruses. Bat viruses may therefore have co-evolved with or adapted to bats over many millions of years. Besides, bats are the only mammalian species that can fly and some bat species can migrate hundreds of miles to their overwintering or hibernation sites. Thus, bats have more opportunities than terrestrial mammals to have direct or indirect contact with other animal species at different geographical locations, thereby enhancing the opportunity for interspecies virus transmission (Calisher, Childs et al. 2006).

Persistence in the absence of pathology or disease appears to be a common characteristic of bat viruses in their natural host population and this is also indicative of a highly evolved relationship (Taylor, Leach et al. 2010). It could be argued, therefore, that the most significant characteristic of viral infections in bats may not be the effectiveness of a highly evolved host immune response, but rather the absence of pathology as the result of an ancient and highly evolved viral survival strategy. For many RNA viruses such as those commonly infecting bats, accessory proteins and evolved secondary functions of other viral proteins play a key role in infection by blocking host innate immune defences, modulating cellular signaling pathways and re-directing normal cellular functions (Frieman and Baric 2008). Highly adapted viruses persistently infecting bat populations might also serve to protect bats at the species or population level from predators, in a sense acting as defensive 'biological weapons'. The best defensive weapons are those that do no harm to the host species and are released only when there is an imminent threat of danger and the emerging bat viruses satisfy these requirements. Henipaviruses are believed to persist in bat populations at a very low viral load and are totally harmless to their natural host. However, under stress, the viral load increases, facilitating transmission to other

animals. Such a mechanism might not be able to protect every individual animal in a population, but it would be an effective way to preserve the species.

In principle, such a symbiotic relationship with viruses would benefit any animal species and there is evidence that such relationships do exist in very different hosts including species ranging from humans to mice to fungi and bacteria. It is perhaps the long period of co-evolution and some unique selective pressures that have driven viral emergence and dominance in bats. Bats have a relatively low reproductive rate compared to other animals such as rodents and bats tend to live in very large and dense populations. These biological and behavioral characteristics may demand far more robust mechanisms to fight infection and predation in order to avoid extinction.

1.2.2. Ebola virus transmission

Filovirus transmission among humans occurs through mucosal surfaces, breaks and abrasions in the skin, or by parenteral introduction. Most human infections in outbreaks seem to occur by direct contact with infected patients (particularly in the late stages of infection) or cadavers, when viral loads are highest. Transmission can also happen through contaminated needle reuse following a 4 to 16 day incubation period (the period between infection and onset of symptoms).

Infectious virus particles or viral RNA have been detected in semen, genital secretions (Yu, Liao et al. 2006), and in skin of infected patients (Zaki, Shieh et al. 1999); they have also been isolated from body fluids, and nasal secretions of experimentally infected non-human primates (Geisbert, Young et al. 2003).

The natural reservoir of infection remains unknown, but the virus clearly has a zoonotic origin. As discussed above, infection cases of people having visited caves containing bats suggest a possible transmission of filoviruses from bats to humans. Despite the fact that this type of transmission has not been demonstrated, transmission between monkeys and humans has clearly been established. In most outbreaks, Ebola virus is introduced into human populations via the handling of infected animal carcasses. In these cases, the

first source of transmission is an animal found dead or hunted in the forest, followed by person-to-person transmission from the index case to family members or health-care staff. Animal-to-human transmission occurs when people come into contact with tissues and bodily fluids of infected animals, especially with infected nonhuman primates (Leroy, Rouquet et al. 2004). Virus transmission is also amplified during funerary rituals typical of some regions of Africa. Although proper cooking of foods should inactivate infectious Ebola virus, ingestion of contaminated food cannot wholly be ruled out as a possible route of exposure in natural infections. Notably, handling and consumption of freshly killed bats was associated with an outbreak of Zaire Ebola virus in Democratic Republic of Congo (Leroy, Epelboin et al. 2009).

Organ infectivity titres in non-human primates infected with Ebola virus are frequently in the range of 10^7 to 10^8 pfu/g; thus, exposure through the oral route could be associated with very high infectious doses. Transmission of Zaire Ebola virus to uninfected macaques housed in the same room as experimentally infected macaques has been reported; however, the study did not exclude the possibility that exposure had occurred from excreted virus that was aerosolized during cleaning of the cages rather than “true” primate-to-primate transmission (Jaax, Jahrling et al. 1995). In fact, studies have shown that filoviruses are relatively stable as aerosols, retain virulence after lyophilisation and can persist for long periods on contaminated surfaces (Belanov, Muntianov et al. 1996). Another study shown that Zaire Ebola virus is highly lethal when given orally to *Rhesus* monkeys causing a pathology close to the one observed through intraperitoneal infection and also leads to death of the animal (Johnson, Jaax et al. 1995). The possibility to generate aerosol containing filoviruses is a possible bioterrorism threat. This form of transmission has been observed with REVOB. In 1990 all monkeys from Reston animal facility were contaminated, including those who were separated from infected macaques, which could be explained by an aerosol inter-simian transmission. However, there is no evidence that primate-to-primate transmission by aerosol actually occurred (Jahrling, Geisbert et al. 1990).

Even though aerosolised filoviruses are highly infectious for non-human primates in the laboratory, transmission patterns during epidemics indicate that the virus does not spread naturally among human beings by the respiratory route, which suggests that it is not efficiently aerosolised by humans. Thus, aerogenic transmission of filovirus has still not been demonstrated in humans.

In human beings, the route of infection seems to affect the disease course and outcome. The mean incubation period for cases of Zaire Ebola virus infection known to be due to injection is 3-6 days, versus 5-9 days for contact exposures. Moreover, the case-fatality rate in the 1976 outbreak of Zaire Ebola virus was 100% (85 of 85) in cases associated with injection compared with about 80% (119 of 149) in cases of known contact exposure. For nonhuman primates infected with Zaire Ebola virus the disease course seems to progress faster in animals exposed by intramuscular or intraperitoneal injection than in animals exposed by aerosol droplets (Geisbert, Daddario-Dicaprio et al. 2008).

Currently there is no treatment or vaccine approved to use in humans against Ebola virus.

1.3. Clinical aspects of Ebola virus haemorrhagic fever

Ebola viruses (EBOV) are among the most pathogenic viruses known to humans with a high case fatality rate of up to 90% that is rarely observed in viral diseases. EBOV hemorrhagic fever (EHF) is often characterized by the sudden onset of fever, weakness, muscle pain, headache, and a sore throat, which are followed by vomiting, diarrhea, rash, kidney and liver dysfunction, and often internal and external bleeding (Sadek, Khan et al. 1999).

After an incubation period of 1–21 days, patients become abruptly ill. The signs and symptoms evolve in three successive phases, which sometimes overlap (**Figure 4**). The first phase is characterized by nonspecific symptoms; the second by multiple organ involvement; and the third, terminal phase, by recovery or death, depending on both host and viral factors.

Phase I

Symptoms typically begin with a flu-like syndrome with abrupt-onset fever (39–40°C), chills, violent headaches and generalized fatigue with myalgia. Sometimes, myalgia with concomitant neck muscle tension occurs, mimicking meningitis.

Phase II

The second phase begins with severe visceral disorders on day 2–4 after symptom onset, and lasts for 7–10 days. During this phase several symptoms occur: gastrointestinal problems (stomach pain, vomiting, watery diarrhoea), respiratory disorders (throat and chest pain, cough) and neurological manifestations (prostration, confusion, delirium), indicating systemic viral dissemination and multi-organ failure. Haemorrhagic manifestations vary in severity and location, and often include skin rashes, nosebleeds, melaena, haematemesis and bleeding at venepuncture sites. There is also a loss of appetite leading to weight loss and marked dehydration.

Patients have “a ghostly appearance” with deep-set eyes, an expressionless face, and extreme lethargy. Respiratory symptoms appear at the same time, with violent sore throat accompanied by chest pain and dry cough, resulting from pharyngeal damage. Swallowing is hindered, further aggravating nutritional status. A non-itchy rash appears between the second and seventh day, followed by fine scaling. Bleeding is frequent, with petechiae at various sites, an ocular burning sensation, reddening, melena, haematemesis, haematuria, epistaxis, haemoptysis, and bleeding at puncture sites. Ultimately, the nervous system can be involved, leading to behavioural disorders (aggressiveness, confusion, delirium), paresthesia, hyperesthesia, and seizures.

Phase III

The terminal phase results in recovery or death. Tachypnea may occur with hiccups, followed by anuria. Death occurs after 2–3 days due to plasmatic shock with capillary extravasation and multiorgan failure. Survivors experience a lengthy period (one month or more) of painful convalescence with intense

fatigue, loss of appetite, profound prostration, weight loss, and migratory arthralgia.

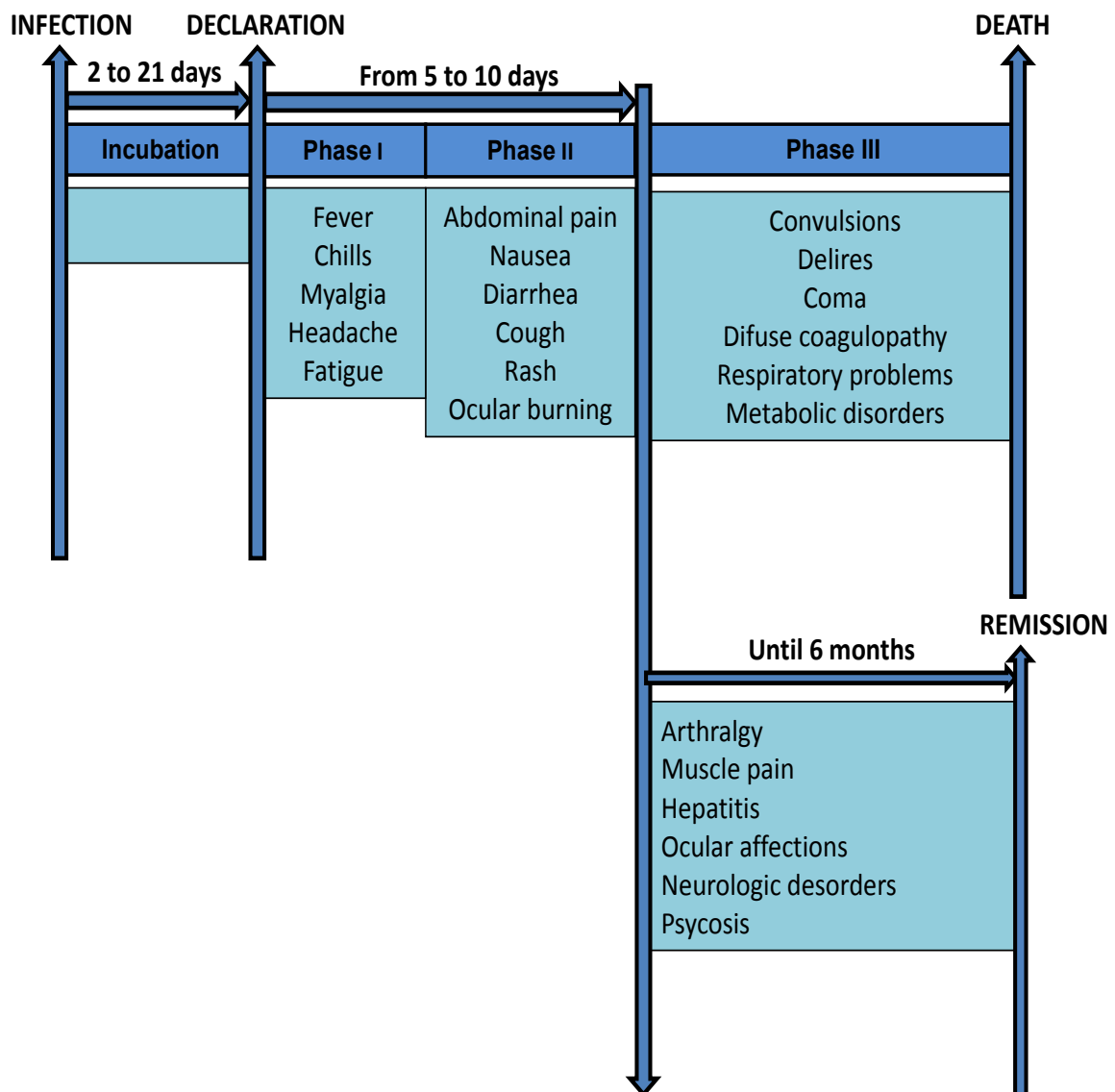


Figure 4.- Phases of EBOV hemorrhagic fever (EHF)

Filovirus infection triggers the development of coagulopathy. Haemorrhaging can be severe but is only present in fewer than half of patients. In those individuals who survive the illness, signs of coagulopathy generally remain limited to conjunctival haemorrhages, easy bruising, and failure of venepuncture

sites to clot. By contrast, many fatally infected individuals have blood in the urine and faeces, and some bleed massively from the gastrointestinal tract during the terminal phase of illness, consistent with disseminated intravascular coagulation (DIC). The condition of these patients continues to decline, and the persistence of severe nausea and vomiting and the development of prostration, tachypnoea, anuria, delirium, or coma signal the onset of irreversible shock (Kortepeter, Bausch et al. 2011).

Biological parameters include mild and early leucopenia, associated with lymphopenia, thrombocytopenia ($< 100\,000$ platelets/mm³), hyperproteinaemia, and marked transaminase elevation. The prothrombin time is always prolonged and fibrin split products are often detected, indicating disseminated intravascular coagulation (Leroy, Gonzalez et al. 2011).

Fatal illness is associated with high and increasing amounts of virus in the bloodstream. By contrast, patients who survive infection begin to show a decrease in amounts of circulating virus and clinical improvement around day 6–11. In most cases, this improvement coincides with the appearance of ebolavirus-specific antibodies. Patients with non-fatal or asymptomatic disease increase specific IgM and IgG responses that seem to be associated with a temporary, early and strong inflammatory response, including interleukin β , interleukin 6, and tumour necrosis factor α (TNF α). However, whether this is the mechanism for protection from fatal disease remains to be proven (Feldmann and Geisbert 2011). The humoral response can result in the formation of antigen-antibody complexes, and some recovering patients develop acute arthralgia and other symptoms consistent with immune complex disease. Although virus disappears quickly from the bloodstream of survivors, it may persist in particular immunologically privileged sites and has been recovered from the semen of a few patients weeks after the acute illness. Some survivors are unable to recall the most severe period of illness. Thus, convalescence is extended and often associated with sequelae which may include orchitis, recurrent hepatitis, transverse myelitis, and uveitis. Psychiatric sequelae include confusion, anxiety, and restless and aggressive behaviour (Nkoghe, Leroy et al. 2012).

1.4.Diagnostic techniques to identify Ebola virus

Ebola haemorrhagic fever can be suspected in acute febrile patients with a history of travel to an endemic area, if they present with fever and constitutional symptoms. Identification might be difficult because severe and acute febrile diseases can have a wide range of causes in areas endemic for Ebola virus, with the most prominent being malaria and typhoid fever followed by others such as shigellosis, meningococcal septicaemia, plague, leptospirosis, anthrax, relapsing fever, typhus, murine typhus, yellow fever, Chikungunya fever, and fulminant viral hepatitis. Laboratory diagnosis for viral haemorrhagic fevers is generally done in national and international reference centres, which are generally contacted immediately on suspicion for advice on sampling, sample preparation and sample transport. Laboratory diagnosis of Ebola virus is achieved in two ways: measurement of host-specific immune responses to infection and detection of viral particles, or particle components in infected individuals.

The development of sensitive diagnostic assays capable of detecting EBOV in human and nonhuman primates is important for identifying outbreaks and supporting ensuing epidemiologic investigations.

Several diagnostic assays for Ebola infection are currently used (**Table 2**). Virus isolation, the most definitive but not necessarily the most sensitive method, requires high level biocontainment, and regardless is too slow for outbreak detection and management (Leroy, Baize et al. 2000). Similarly electron microscopy and histological techniques are sensitive methods particularly reliable for post-mortem diagnosis but require sophisticated equipment and reagents (Zaki, Shieh et al. 1999).

Several serological techniques have been developed to diagnose EBOV infection. Nowadays, RT-PCR and antigen detection ELISA are the primary assays to diagnose an acute infection. Viral antigen and nucleic acid can be detected in blood from day 3 up to 7–16 days after onset of symptoms. For antibody detection the most generally used assays are direct IgG and IgM ELISAs and IgM capture ELISA. IgM antibodies can appear as early as 2 days

post onset of symptoms and disappear between 30 and 168 days after infection. IgG-specific antibodies develop between day 6 and 18 after onset and persist for many years. An IgM or rising IgG titre constitutes a strong presumptive diagnosis. Decreasing IgM, or increasing IgG titres (four-fold), or both, in successive paired serum samples are highly suggestive of a recent infection. ELISAs show a good measure of sensitivity and specificity and are able to detect all of the EBOV species. Antigen-capture diagnostic assays together with nested reverse-transcription (RT)–polymerase chain reaction (PCR) have been developed and used in previous outbreaks. ELISA is the best test for identifying patients because of its rapidity and robustness; it is particularly likely to be positive in the more severely ill patients. RT-PCR is usually more sensitive but also more subject to artifacts and contamination. Both methods have proved to be very effective as field diagnostic tools for the detection of EBOV antigen and nucleic acids in patient serum, plasma, and whole blood (Onyango, Opoka et al. 2007).

Moreover these assays can only be done on materials that have been rendered non-infectious. An efficient way to inactivate the virus for antigen and antibody detection is the use of gamma irradiation or heat inactivation. Similarly, nucleic acid can be amplified by purification of the virus, such as RNA from materials treated with guanidinium isothiocyanate - a chemical chaotrope that denatures the proteins of the virus and renders the sample non-infectious.

Repeated Ebola virus outbreaks in several countries of equatorial Africa have occurred in recent years. Often these outbreaks occur in remote sites where advanced medical support systems are scarce and timely diagnostic services are very difficult to provide. Provision of basic on-site diagnostics (including confounding differential diagnosis, truly portable real-time thermocyclers and simple serological assays appropriate for field use) could help with the management of patients specifically and with the outbreak in general.

Test	Element detected	Samples	Advantages	Disadvantages
RT-PCR	Viral ARN	Blood, serum, tissues	• Fast, sensitive ,specific • Use of inactivated material	• Requires special equipment • False positives and negatives
Antibody capture by ELISA	Viral antigens	Blood, serum, tissues	• Fast, sensitive • Use of inactivated material	• Requires special equipment
ELISA	Specific antibodies (IgM or IgG)	Serum	• sensitive ,specific	• Longer than IFA • No antibodies in fatal cases
Immuno-histochemistry	Viral antigens	Tissues (skin, liver)	• Specific • Use of inactivated and fixed material	• Long • Requires special equipment
Fluorescent assay (FA)	Viral antigens	Tissues (liver)	• Fast	• Subjective interpretation
Indirect immunofluorescence (IFA)	Specific antibodies	Serum	• Easy	• Low sensitivity • False positives • Subjective interpretation
Electronic microscopy	Viral particles	Blood, tissues	• Unique morphology of Filovirus	• Low sensitivity • Requires special equipment
Immunoblot	Viral proteins	Serum	• Specific	• Sometimes difficult interpretation
Virus isolation	Viral particles	Blood, tissues	• Virus available for subsequent studies	• Requires special equipment and BSL-4 conditions

Table 2.-Techniques to analyse and detect Ebola virus infection.

1.5.Ebola virus animal models

Development of animal models is critical to understanding Ebola virus pathogenesis. Several animal models have been developed in order to study pathophysiology, vaccines and therapeutics of Ebola hemorrhagic fever including mice, guinea pigs, hamsters and NHPs (Connolly, Steele et al. 1999, Bente, Gren et al. 2009, Bradfute, Warfield et al. 2012). However, they do not all show the same pathological features when infected by Ebola virus (**Table 3**).

-Mouse model: development of mice, in contrast to guinea pigs and NHP models, has been unsuccessful due to the fact that serial passages of wild-type EBOV in mice are needed for adaptation. The adapted virus resulted in 8 amino acid changes in both coding and non-coding regions of the virus genome compared to the original wild-type virus; nucleotide substitutions leading to amino acid changes were found in VP35, VP24, NP and L polymerase (Ebihara, Takada et al. 2006). Moreover, the route of viral inoculation is determinant to observe or not infection: intraperitoneal inoculation causes lethal infection whereas subcutaneous inoculation does not cause a

symptomatic illness (Gibb, Bray et al. 2001). This phenomenon is not observed in NHP models, which are susceptible to wild-type infection. Infected mice become acutely ill with symptoms of ruffled fur, reduced activity and loss of weight. However, mice infected with mouse-adapted filovirus show differences when compared to infection in human disease or NHP models in terms of a lack of severe coagulation disorder and fibrin deposition; in addition, the cytokine profile observed during infection is not the same as in humans and NHP. Nevertheless, mouse models can be useful tools for studying basic aspects of replication, pathogenesis, and immune responses and also serve to evaluate candidate vaccines and therapeutic agents (Nakayama and Saijo 2013).

-Guinea pig model: Infection of guinea pigs with wild-type EBOV causes only a transient febrile illness; Ebola virus needs to be serially passaged in guinea pigs to acquire the ability to cause lethal infection in this model (Ryabchikova, Strelets et al. 1996). The lack of virulence of wild-type virus in guinea pigs is associated with an inability of the virus to replicate/or be released from macrophages and hepatocytes. Guinea pigs inoculated with guinea pig-adapted virus showed similar symptoms to those reported in humans and NHP: fever, anorexia, dehydration, lymphopenia, fibrin deposition and a decrease in platelet count during infection (Nakayama and Saijo 2013). Guinea pig adapted virus resulted in 8 nucleotide changes that lead to 5 amino acids changes found in NP and L (single mutations) and VP24 (with three substitutions) (Volchkov, Chepurinov et al. 2000). Guinea pig models are useful tools for studying basic aspects of replication, pathogenesis and coagulation disorders. The guinea pig provides a more stringent evaluation of vaccine efficacy and is more predictive of success in NHP model than the mouse model. Unfortunately, significantly fewer tools are available for immunological research using this animal model.

-Syrian Golden hamster model: this model is based on infection of adult hamsters with mouse-adapted Ebola virus and shows an uniformly lethal outcome. In contrast to other rodent models, hamsters accurately reproduce the severe coagulation abnormalities associated with Ebola virus infection, including fibrin deposition, thrombocytopenia and reduction of Protein C levels.

Importantly, this model also shows cytokine dysregulation and suppression of IFN type I responses, which are important for EBOV pathogenesis (Ebihara, Zivcec et al. 2013). Differently from guinea pigs, in the hamster model prolonged thrombin time (TT) and severe lymphopenia is observed. However, as with guinea pigs, immunological tools are limited.

-Non-human primate (NHP) model: African green monkeys, cynomolgus macaques and hamadryas baboons have been employed as models for EBOV infection (Baskerville, Bowen et al. 1978, Fisher-Hoch, Platt et al. 1985, Jaax, Davis et al. 1996). The NHP model has been proven to be valuable in providing new information regarding filoviral pathogenesis; the differences in the disease pathology observed in other models (**Table 3**) means that the NHP model remains the most useful and closest to haemorrhagic fever observed in humans despite the practical, and especially ethical, considerations that lead to the restriction of experiments (Geisbert, Young et al. 2003). NHP is the best model for vaccine and drug testing, since it shows both the characteristic clinical and pathological features seen in human EBOV infection. It should be taken into account that genetic differences, even among the same animal species, and the origin of the species may influence disease presentation and progression.

	Mouse	Guinea pig	Hamster	NHP	Human
Virus adaptation	Adapted	Adapted	Adapted	Wild-type	Wild-type
Viremia	High	High	High	High	High
Virulence	High	High	High	High	High
Weight loss	Severe	Severe	No	Severe	Severe
Rash	No	No	No	Yes	Yes
Thrombocytopenia	Yes	Yes	Yes	Yes	Yes
Lymphocyte apoptosis	Yes	Limited	Yes	Yes	Yes
Cytokine response	Yes	Yes	Yes	Yes	Yes
PT	Remained	Increased	Increased	Increased	Increased
PTT/aPTT	Remained	Increased	Increased	Increased	Increased
TT	ND	ND	Increased	Increased	ND
Fibrin deposition in organs	Little	Moderate	Little	Abundant	Observed
Protein C activity	ND	ND	Decreased	Decreased	ND

Abbreviation; PT, prothrombin time; PTT, partial thromboplastin time; APTT, activated partial thromboplastin time; TT, thrombin time ; ND, no data

Table 3.- Comparison of pathological features of different animal models of Ebola virus infection. Adapted from (Nakayama and Saijo 2013).

1.6.The fight against Ebola virus

1.6.1.Treatments

In order to fight against an illness two strategies are generally followed: (i) fight the virus by directly inhibiting its entry into cells or its replication (antiviral treatment) or (ii) fight the inappropriate immune response of the host (symptomatic treatment).

For the moment, the only treatment provided to patients during outbreaks is palliative to limit dehydration and the development of certain symptoms (nausea, fever and pain principally) and control of secondary infections. However, several treatments have been tested in animal models (**Table 4**). Some treatments also target coagulation impairment by using the anticoagulant protein derivative from nematodes (rNAP_{C2}) or recombinant protein C, both having given encouraging results in infected monkeys (Geisbert, Hensley et al. 2003). Contrary to ribavirin, which has not shown to be very efficient as an EBOV infection treatment (Moss and Wilson 1992, Gunther, Asper et al. 2004), the administration of oligonucleotides targeting viral mRNAs seems to be a promising technique (Warren, Warfield et al. 2010).

Therapy trials injecting EBOV immunized serums have given controversial results in hospitals and NHPs. In fact, during Ebola virus infection, infected patients develop a very weak antibody response after serum administration thus suggesting that administration of serum did not provide protection. Concentrated immune serum produced in horses seemed very efficient in baboons and guinea pigs infected with Ebola virus. However, their effectiveness has not been proved in Rhesus monkeys or mice models (Jahrling, Geisbert et al. 1996). During the outbreak in 1995 in the Democratic Republic of the Congo blood transfusions from convalescent patients containing IgG against Ebola virus improved the survival of EBOV infected patients when compared to non-treated/transfused patients (Mupapa, Massamba et al. 1999). However, the real involvement of these transfusions in the illness has not been proved and is probably negligible. The use of monoclonal antibodies against EBOV-GP has been shown to be efficient in EBOV infected mice (Wilson, Hevey et al. 2000).

The most promising results so far were obtained when siRNA was used and a 100% survival rate in NHPs was achieved when animals were treated daily for 7 days and the first treatment started 30 minutes after challenge (Lodmell, Parnell et al. 2002).

Treatment	Mechanism/target viral protein	Species tested	Strategy			Challenge virus	Survival rate (%)	References
			Dose	Route	Dose schedule			
rNAPc2	Blocks TF: FvIIa mediated activation of factor X	NHP	30 µg/kg bw	sc	10 mpi and administration daily for 14 days	EBOV	33	
					24 hpi and administration daily for 8 days		33	
APC	Anti-thrombotic: cleaves and inhibits coagulation cofactors FVIIIa and Fva	NHP	2 mg/m ² /h	iv	30–60 mpi and administration for 7 days	EBOV	18	
PMO	Targets viral mRNA to block transcription	Mouse	500 µg	ip	twice at 24 h and 4 h before infection	mouse-adapted EBOV	100	
					single dose at 24 hpi	GP-adapted EBOV	100	
		Guinea pig			single dose 24 h before infection		> 25	
					single dose 24 hpi		25–50	
					single dose 96 hpi		50–75	
		NHP	12.5–200 mg	im	2 day before challenge and administration for 9 days	EBOV	75	
PMO plus	EBOV VP24 and VP35 (AVI-6002)	NHP	40 mg/kg bw	sc and ip	30–60 mpi and administration daily for 10 or 14 days	EBOV	63	
			28 or 40 mg/kg bw	iv	30–60 mpi and administration daily for 14 dpi		60	
			4 mg/kg bw				0	
			16 mg/kg bw				20	
siRNA	Targets viral mRNA to block transcription	guinea pig	PEI-mixed, 8 mg/kg	ip	3 h before infection and 24, 48 and 96 hpi	GP-adapted EBOV	25	
					1, 24, 48, 72, 96, 120 and 144 hpi		60	
							100	
		NHP	SNALP-formulated, 2 mg/kg	iv	30 mpi, 1, 3 and 5 dpi	EBOV	66	
					30 mpi, 1, 2, 3, 4, 5 and 6 dpi		100	

^aTwo of five guinea pigs received the siRNAs using the SNALP delivery systems died but the death could not be attributed to viral replication.

Abbreviation: PMO, Phosphorodiamidate morpholino oligomers; bw, bodyweight; sc, subcutaneously; iv, intravenously; ip, intraperitoneally; im, intramuscularly; mpi, minutes post-infection; hpi, hours post-infection; dpi, days post-infection.

Table 4.- Efficacy of post-exposure treatment in animal models of Ebola virus infection. Adapted from (Nakayama and Saijo 2013)

1.6.2.Vaccines

Successful vaccination engenders a protective immune response capable of protecting the animal against a lethal viral infection. It seems that both types of response, cellular and humoral, are required in order to clear Ebola virus (Sullivan, Martin et al. 2009).

Several studies done in mice and later also in NHP have shown that protection against Ebola virus infections seems to correlate with a humoral immune response (Konduru, Bradfute et al. 2011). However, this is not always the case, and it happens that a high titer of antibodies does not result in a protective immune response (Warfield and Olinger 2011); inversely, sometimes protective responses do not induce neutralizing antibodies (Sullivan, Martin et al. 2009). Finally, according to Sullivan, there is a correlation between the antibody titer (IgG) and survival (Sullivan, Martin et al. 2009), and according to Warfield, CTL responses play an essential role in controlling viral replication and clearance of the virus (Warfield and Olinger 2011). Altogether data suggests that an effective vaccine against Ebola virus infection should be able to induce a complete immune response including the humoral immune response and specific immune cellular response (CD4⁺, CD8⁺ and CTL).

At the present time, there are no vaccines authorized against Ebola virus. Several vaccination strategies have been generated and several candidate vaccines are still being tested on animal models (**Table 5**).

Various studies were first made with inactivated viral particles showing that guinea pigs could be partially protected against Ebola virus infection (Lupton, Lambert et al. 1980). It was then also shown that it was possible to obtain protective immunity during baboon infection using viral particles previously fixed with paraformaldehyde, whereas, conversely Chepurinov's team did not manage to induce an immunological response strong enough to offer protection in NHPs vaccinated with gamma-irradiation inactivated virus (Geisbert, Bausch et al. 2010).

For a long time, the idea of using attenuated virus as a candidate of vaccine remained in the state of "theory" due to the high risk that virus could mutate and

become pathogenic for humans (Vanderzanden, Bray et al. 1998). In the hope of amplifying humoral and cellular immune responses in animal models, encapsulated liposomes were used as a tool to release inactivated particles but the strategy failed to protect NHPs against a lethal challenge (Geisbert, Pushko et al. 2002). Nowadays, all these strategies have been abandoned. Virus-like-particles expressing GP, NP and VP40 have also been tested but this approach remains difficult and expensive.

Research has focused henceforth on the use of recombinant viral vectors expressing one or several Ebola viral proteins, principally EBOV glycoprotein. Viral vectors concern non-pathogenic or non-replicative recombinant virus. Several viral vectors have been used in order to generate a vaccine against Ebola virus: alphavirus (Venezuelan equine encephalitis virus), vesicular stomatitis virus (Garbutt, Liebscher et al. 2004), parainfluenza human virus type 3 (Bukreyev, Rollin et al. 2007) or human adenovirus serotype 5 (Sullivan, Geisbert et al. 2003). The attenuated recombinant VSV expressing EBOV-GP was shown to be protective against 50% of EBOV infected guinea pigs and 100% of mice 24 hours after lethal challenge and 50% of NHP treated 30 minutes after a lethal challenge (Feldmann, Jones et al. 2007).

Since viral glycoprotein GP is the only viral protein exposed on the surface of the virus, it would seem to be the principal target of the host's immune system. However, difficulties related to development of vaccines increase with viral divergence. In fact, the amino acid sequence between glycoproteins of EBOV-Zaire and EBOV-Sudan shows only 50% homology. These antigenic differences are responsible for a lack of cross immune protection between different species of Filovirus.

System	% protection, number of vaccinations, time required for vaccination scheme ^{a)}			Remarks
	NHPs	guinea pigs	mice	
Non-replicating vaccines				
inactivated ebolavirus	10% ^{b)} , 3, 11w	64%, 3, 21w	100%, 2, 4w	
replicons	0%, 3, 15w	100%, 3, 22w	100%, 2, 8w	
DNA	n/a	100%, 3, 19w	100%, 2, 8w	safe and immunogenic in human phase 1 clinical trials
DNA + Ad5	100%, 4, 32w	n/a	n/a	
Ad5	100%, 1, 4w	100%, 1, 4w	100%, 1, 3w	safe and immunogenic in human phase 1 clinical trials; problems with preexisting immunity, but this can be overcome by multiple vaccinations
baculovirus-derived GP	n/a	50%, 3, 11w	n/a	
recombinant GP-Fc fusion protein	n/a	n/a	83%, 4, 11w	
GP-immunocomplexes	n/a	n/a	80%, 4, 12w	
VLPs	100%, 3, 15w	n/a	100%, 2, 7w	
rEBOVΔ VP30	n/a	100%, 2, 9w	100%, 2, 11w	
inactivated rRABV (BSNP333-GP)	n/a	n/a	100%, 1, 11w	only inactivated vaccine shown to protect after a single vaccination; extensive experiences with this vaccine platform for wildlife vaccination
Replicating vaccines				
rVSV/Δ G/GP	100%, 1, 4w ^{c)}	100%, 1, 3w ^{c)}	100%, 1, 1d ^{c)}	capable of post-exposure protection
rHPIV3/GP	100%, 2, 10w	100%, 1, 4w	n/a	potential problems with preexisting immunity
rHPIV3/Δ HN-F/GP	n/a	100%, 1, 4w	n/a	no problems with preexisting immunity
rRABV (BNSPΔ G-GP)	n/a	n/a	100%, 1, 11w	
rCMV	n/a	n/a	100%, 2, 10w	disseminating vaccine

^{a)} abbreviations: w = weeks, d = days, n/a = no data from challenge experiments available

^{b)} no protection was observed in 8 cynomolgus macaques; however, 1 out of 2 rhesus macaques was protected

^{c)} postexposure vaccination protects 50% of NHPs and 83% of guinea pigs if given 0.5 to 1 hour post infection, and 100% of mice as late as 24 hours post infection

Table 5.- Overview of Ebola virus vaccines. Adapted from (Hoenen, Groseth et al. 2012)

2. Molecular biology of Ebola virus

2.1. Morphology and genome organisation

The morphology of EBOV is unique among viruses. The virion is pleomorphic, producing 'U'-shaped, '6'-shaped, or circular forms but the predominant forms of the virion most frequently seen by electron microscope are long tubular structures about 14 000 nm long and 80 nm wide. These characteristic filamentous particles give the virus family its name (Kiley, Bowen et al. 1982).

The filovirus genome consists of a single 19 kb strand of negative-sense RNA. Transcription begins at the 3' end and produces seven individual mRNAs: nucleoprotein (NP), virion protein 35 (VP35), VP40, glycoprotein (GP), VP30, VP24, and RNA-dependent RNA polymerase (L) (**Figure 5A**). Intergenic regions contain initiation and termination signals. Leader and Trailer are non-coding regions which are very important during transcription, replication and encapsidation. The central core of the virion is composed of the ribonucleoprotein complex (RNP), which consists of the genomic RNA molecule encapsulated by NP and linked to the inner matrix proteins VP30 and VP35 and the L polymerase. This complex is involved in transcription and replication. The other three proteins, GP, VP24 and VP40 are membrane-associated. The matrix proteins VP40 and VP24 are linked to the ribonucleoprotein complex at the inner surface of the lipid bilayer of the viral envelope, which is derived from the host cell. VP40 and VP24 are involved in viral nucleocapsid formation, viral budding or assembly, and host range determination. The viral envelope contains only the glycoprotein GP, organized as trimeric spikes (**Figure 5B and C**).

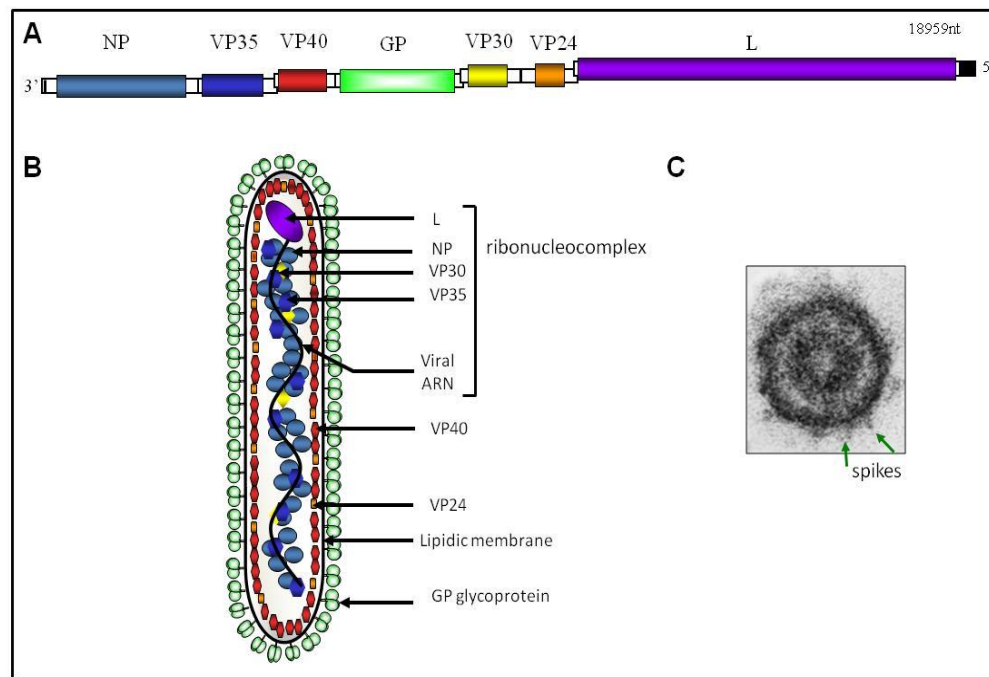


Figure 5.- Ebola virus morphology and organisation.- (A) genome organisation of Ebola virus; **(B)** Protein organisation in viral particles and **(C)** Transversal cut of a virion; Spikes are indicated with green arrows.

EBOV differs from MARV and from other members of the Mononegavirales in that infected cells secrete large amounts of a non-structural glycoproteins: shed GP and sGP, this last being the primary product of the GP gene, and which is expressed from non-edited mRNA species (Volchkov, Volchkova et al. 1998).

The linear arrangement of the seven viral genes and the replication strategy in infected cells resemble those of the better characterised rhabdoviruses and paramyxoviruses. Because the genome cannot be transcribed or copied by host cell enzymes, all of the components required for viral replication must be carried within the virion (Mahanty and Bray 2004).

2.2.EBOV proteins and their role during pathogenesis

2.2.1.Nucleoprotein (NP)

The first reading frame in EBOV genome will allow the transcription of an mRNA which codes for the viral nucleoprotein (NP). It is the most abundant protein of the RNP complex and it is responsible for genomic RNA and antigenome encapsidation after transcription and replication, respectively. Associated to RNA, it represents the essential link of RNP that will allow L polymerase, RNA and the other viral proteins of the nucleocapsid to interact (Becker, Rinne et al. 1998). Similarly to other enveloped, negative stranded RNA virus, the amino-terminal domain of NP protein, rich in hydrophobic amino acids, is responsible for the association between NP and viral genomic RNA. The carboxy terminal domain, which is less conserved, is very rich in negatively charged amino acids and its hydrophobicity indicates a possible implication in protein-protein interactions, especially during viral assembly (Sanchez, Kiley et al. 1992).

EBOV NP protein contains 739 amino acids with 7 phosphorylated domains rich in serine and threonine distributed in the carboxy terminal domain. Phosphorylation of these residues seems to be essential for NP activity; in fact, in viral particles NP is only present in a phosphorylated form (Becker, Huppertz et al. 1994).

In the absence of other viral proteins, NP is capable of auto-assembly and of generating RNP-like helicoidal structures. However, these structures show a larger diameter and a more irregular organisation compared to regular RNPs. This indicates the requirement of the viral proteins in order to assemble mature RNPs (Mavrakis, Kolesnikova et al. 2002).

2.2.2.VP35

The VP35 gene position in EBOV genome corresponds to that of phosphoprotein P in Paramyxoviruses and Rhabdoviruses. Even if no significant sequence homology with P protein in other *Mononegavirales* has been identified, a functional analogy has been observed (Muhlberger, Lotfering et al. 1998).

VP35 is known to bind NP protein and to condense RNPs, obtaining a regular helicoidal structure during mature RNP assembly (Becker, Rinne et al. 1998). VP35 also interacts with viral polymerase L; in fact, VP35 is the cofactor that allows, in association with NP, recruitment of the polymerase to viral genomic RNA and antigenomes in order to carry out transcription and replication (Muhlberger, Lotfering et al. 1998).

VP35 has also been shown to be an antagonist of the type I interferon response. It is capable of inhibiting IFN α/β in infected cells by blocking phosphorylation of IRF-3 regulation factor (Basler, Mikulasova et al. 2003) and protein kinase PKR (Feng, Cerveny et al. 2007).

2.2.3.VP40

EBOV VP40 has 326 amino acids and is the protein responsible for assembly and virus budding. The matrix protein VP40 is the most abundant protein in virions; it covers the inner part of the viral membrane and it allows the cohesion between RNP and the viral envelope. Like VP35, this protein does not share homology when compared to other matrix proteins in *Mononegavirales*, however, a functional analogy has been identified (Timmins, Ruigrok et al. 2004).

Ebola virus VP40 is sufficient for virus assembly and budding from the plasma membrane (Timmins, Scianimanico et al. 2001). However, the addition of GP protein increases VP40-induced viral production (Licata, Johnson et al. 2004).

Interestingly, EBOV VP40 contains two overlapping late domains that interact with Tsg101, a component of the export complex in endosomes, which plays a

role during the late state of viral budding (Hoenen, Groseth et al. 2006). The efficiency of viral production is considerably reduced when these two domains are altered (Harty, Brown et al. 2000). However, the interaction between late domains and endosomal cellular proteins is not essential for EBOV replication, it only implies a reduction in viral titer (Neumann, Ebihara et al. 2005).

2.2.4.GP

In EBOV, four variants of the envelope glycoprotein are synthesized as a result of transcriptional stuttering or posttranslational processing (**Figure 6A**): the surface glycoprotein GP and three soluble glycoproteins (sGP, shed GP and ssGP). In fact, Ebola virus GP gene contains two overlapping reading frames. The majority of mRNAs are transcribed from the first reading frame leading to the synthesis of soluble glycoprotein sGP (or secreted GP). The expression of Ebola virus GP requires an editing phenomenon during transcription. About 25% of transcripts from GP gene will result from the addition of an extra Adenosin (A) in the editing site, already containing 7A; this will lead to the synthesis of mRNA transcribed from the totality of the two reading frames and leads to the production of surface GP, which has 767 amino acids (Figure 6A).

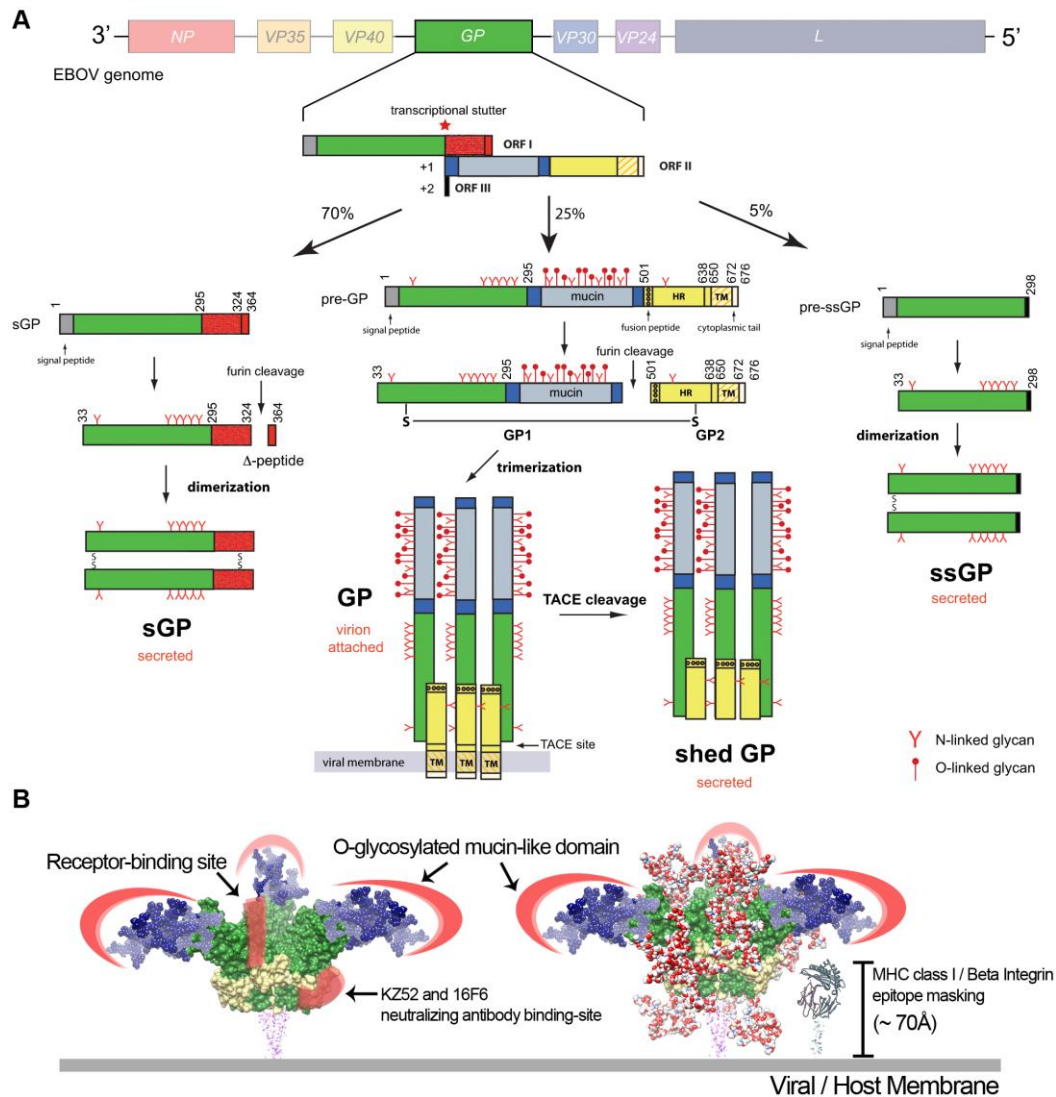


Figure 6. Ebola virus glycoprotein synthesis and conformation. (A) Processing of EBOV glycoproteins. The EBOV genome contains seven genes but nine proteins are produced due to editing of the GP gene. The GP gene primary transcript encodes for a secreted GP (pre-sGP). Furin cleavage of pre-sGP produces mature sGP and a secreted Δ -peptide. Transcriptional stuttering results in the production of the envelope-attached GP and a small, secreted GP (ssGP). GP is cleaved by furin to form a disulfide-linked GP1 (green)-GP2 (yellow) heterodimer, which then assembles as trimers on the virus surface. Cleavage at the membrane-proximal external region by the tumor necrosis factor- α converting enzyme (TACE) releases shed GP. **(B)** Molecular representation of EBOV GP subunits are shown in green (GP1) and yellow (GP2). Complex-type N-linked glycans are modelled onto the EBOV GP surface as red spheres revealing a heavy glycan layer that buries much of the GP

surface, including the receptor binding site; only a small patch at the base of the GP is accessible (KZ52/16F6 antibody-binding site). The O-linked glycosylated mucin-like domain (blue) is thought to form a structure that provides another glycan layer of protection to the virus. Adapted from (Cook and Lee 2013).

GP is a type I transmembrane protein which is synthesized as a single polypeptide chain (pre-GP) that will be processed by several post-translational modifications: (i) cleavage of the signal peptide and N-glycosylation of the protein in the endoplasmic reticulum (Volchkov, Becker et al. 1995); (ii) acylation and oligomerisation in the pre-Golgi compartment (Ito, Watanabe et al. 2001); (iii) O-glycosylation and N-glycosylation in the Golgi apparatus (Feldmann, Nichol et al. 1994). Pre-GP precursor is also cleaved in the trans-Golgi compartment at arginine residues in the sequence RRTRR₅₀₁ by furin proteases. This will give the extracellular subunit GP1 (501 amino acids) and the transmembrane subunit GP2 (175 amino acids) (Volchkov, Feldmann et al. 1998, Neumann, Geisbert et al. 2007). These subunits remain together through non-covalent interactions and through a disulfide bond between cysteine residues at position 53 and 609. Finally, the GP1,2 protein is directed as a trimeric form through lipid rafts in the plasma membrane. GP1 contains four different domains: base, receptor-binding domain (RBD), glycan cap, and mucin-like domain (MLD) (**Figure7**).

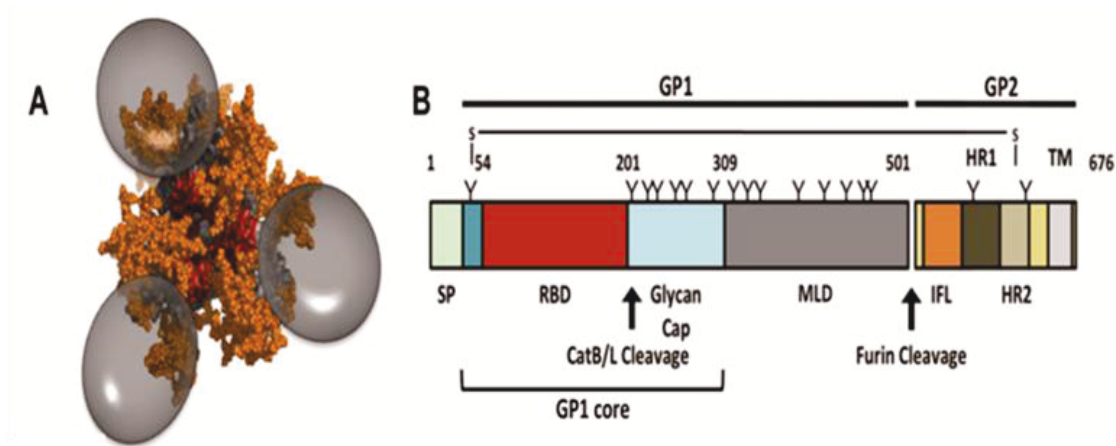


Figure 7.- Schematic representation of Ebola virus GP. (A) Molecular model of EBOV GP1,2 from a top-down view. N-glycans are shown in orange, GP is shown in light gray, RBD is shown in red, and MLD structure that has not yet been solved is represented as a gray sphere. **(B)** Linear model of EBOV GP.

The disulfide bond between GP1 and GP2 is indicated, as well as the locations of N-linked glycans (marked with “Y”s) in the GP1 and GP2 domains, and the known protease cleavage sites are indicated. SP, signal peptide; RBD, receptor-binding domain; MLD, mucin-like domain; IFL, internal fusion loop; HR1 and HR2, heptad repeats 1 and 2; TM, transmembrane domain (Lennemann, Rhein et al. 2014).

The N-terminal region of GP1 (residues 57 to 149) has been defined as a receptor-binding domain (RBD). Although Ebola viruses are antigenically distinct, the RBD is highly conserved, with an average of 87% amino acid identity. This conservation suggests an absence of selective pressures driving diversification of this region of the protein. In contrast, the glycan cap and MLD have extensive sequence diversity between different Ebola virus species. GP2 contains the hydrophobic fusion peptide (residues 524-539) and heptad repeats that mediate membrane fusion (Dube, Brecher et al. 2009, Lee and Saphire 2009). GP2 also contains a sequence of 26 hydrophobic amino acids that shares 40% similarity with the immunosuppressive domains of some retrovirus; however, the role of this domain in mature GP during infection is still not known. In the carboxy-terminal extremity, residues from 651-672 have been characterised as a protein anchor to the plasma membrane on the surface of cells. Finally, a short cytoplasmic tail containing 4 amino acids has also been identified (Volchkov, Volchkova et al. 1998) (**Figure 6A**).

Surface GP is heavily N- and O- glycosylated. Depending on the EBOV species, envelope GP contains 11–18 N-linked glycan sites. Both the glycan cap and the MLD are highly glycosylated, resulting in the majority of the protein being masked by glycans. The glycan cap contains only N-linked glycan sites and, even if the glycan cap is generally poorly conserved, these sites are well conserved among Ebola viruses, suggesting functional significance. In contrast, the 150-residue unstructured MLD is highly variable between the different filoviruses and include both N- and O-linked glycans (Jeffers, Sanders et al. 2002) (**Figure 6B**). The N-linked glycans are a heterogeneous mixture of high-mannose, hybrid, and complex oligosaccharides, while the O-linked glycans are

primarily smaller trisaccharide structures that contain varying amounts of sialic acids (Ritchie, Harvey et al. 2010).

High glycosylation of Ebola virus surface GP masks essential epitopes on EBOV GP (**Figure 6B**) and creates an unfavorable environment for the interaction of otherwise neutralizing antibodies. No antibodies have been identified that target the receptor-binding site, however a number of neutralizing antibodies have been generated against the more variable mucin-like domain (Wilson, Hevey et al. 2000). The mucin-like domain is not required for pseudotyped EBOV entry in Vero E6 cells (Kaletsky, Simmons et al. 2007, Hood, Abraham et al. 2010). However, the mucin-like domain could play a more important role in filovirus entry into natural target cells such as endothelial cells (ECs), hepatocytes, and antigen-presenting cells. In mice, removal of the the mucin-like domain of EBOV GP leads to the production of cross-species antibodies directed at the conserved glycoprotein core structure (Hood, Abraham et al. 2010). A small non-glycosylated area near the base of the EBOV GP core is available for antibody recognition (**Figure 6B**). This region is very conserved in both Zaire and Sudan EBOV species, and the neutralizing antibodies KZ52 and 16F6-1 bind to this hotspot (Dias, Kuehne et al. 2011).

Several studies have shown that EBOV glycoprotein GP1,2 is responsible of the cytopathic effects observed in EBOV infected cells. In fact, expression of GP1,2 causes cell rounding in different cell lines, including human embryonic kidney cells (293T), monkey kidney cells (VeroE6), macrophages and endothelial cells (Chan, Ma et al. 2000). EBOV mucin-like domain of surface GP also has the unique ability to provide a “glycan umbrella” that shields surface epitopes and inhibits surface protein recognition by masking the function of host cellular proteins that are important in response to viral pathogens (Reynard, Borowiak et al. 2009). Indeed, transient expression of EBOV GP results in low detectable levels of various cell surface proteins such as major histocompatibility complex (MHC) class I proteins and β -integrins. Initially, it was thought that EBOV GP downregulated expression or degraded these proteins from the cell surface (Simmons, Wool-Lewis et al. 2002). However, MHC class I and β -integrins are not removed from the cell surface but masked by a “glycan-umbrella” (Reynard,

Borowiak et al. 2009, Francica, Varela-Rohena et al. 2010) (**Figure 6B**). This represents a novel mechanism of disrupting immune function that does not involve downregulation or degradation of surface proteins.

Ebola virus GP protein is responsible for viral tropism. The use of retroviral vectors has shown that GP1 subunit is responsible for viral attachment to the surface of target cells (Takada, Robison et al. 1997). After binding, internalisation and trafficking of EBOV particles into late endosomes, surface GP in the virion is cleaved in two steps, first by cathepsin L (into 20kDa species) and further by cathepsin B (into 19 kDa species) at the β 13- β 14 loop of GP1 (Schornberg, Matsuyama et al. 2006, Dube, Brecher et al. 2009, Hood, Abraham et al. 2010). This cleavage implies the removal of approximately 60% of GP1 amino acids including the mucin-like domain, glycan cap and a part of RBD (Manicassamy, Wang et al. 2005, Mpanju, Towner et al. 2006). Low pH is required to facilitate conformational changes in GP and allow fusion to endosome membrane (Gregory, Harada et al. 2011). Once the hydrophobic fusion peptide in GP2 is exposed, it binds to the membrane and forms a rod-shaped intermediate (prehairpin) which will connect the viral and target membranes. Then, it brings the viral and the target membranes into close contact eventually causing them to merge (Harrison 2008, White, Delos et al. 2008).

The sequencing of viral isolates recovered from successive outbreaks of Zaire ebolavirus has shown a high genetic stability over time. Ebola virus GP gene isolated in 1995 differed by less than 2% from that of the 1976 virus, even though they were collected at sites some 3000 km apart. Samples recovered during subsequent epidemics in Gabon have shown similar close homology. Because viral RNA-dependent RNA polymerases typically have a high error rate, such lack of variation appears unusual (Mahanty and Bray 2004).

2.2.5.Ebola virus soluble glycoproteins

Many enveloped viruses, including EBOV, Lassa, respiratory syncytial and herpes simplex produce free glycoproteins that act as either “antibody sinks” or decoys of host immunity (Bukreyev, Yang et al. 2008, Branco, Grove et al. 2010, Cook and Lee 2013). The shedding or secretion of soluble viral glycoproteins exemplifies a viral strategy of humoral misdirection.

2.2.5.1.Secreted GP

EBOV-infected cells secrete two glycoproteins (secreted GP and shed GP) into an infected person's sera in very high amounts (Sanchez, Yang et al. 1998, Dolnik, Volchkova et al. 2004). 70% of GP gene transcripts code for secreted GP (sGP), which is a dimeric 110kDa protein that has 364 amino acids (**Figure 6A**). Whereas the first 295 amino acids of sGP are identical to the GP1 subunit of surface GP, due to transcriptional stuttering the 69 amino acids forming the carboxy-terminal region are unique and form different disulfide linkages leading to a homodimeric rather than a trimeric assembly. As a result, sGP lacks regions found in GP that have been shown to be important in the neutralization of the virus (Dias, Kuehne et al. 2011). sGP protein is synthesized as a precursor: pre-sGP, which is the post-translationally modified, similarly to surface GP, leading to mature sGP protein; its post-translational modifications include: cleavage of signal peptide, N-glycosylation and oligomerization in the endoplasmic reticulum and then, glycosylation and post-translational cleavage in the Golgi (Volchkova, Feldmann et al. 1998). Mature sGP and pre-sGP precursor are anti-parallel-orientated or parallel homodimers linked by two disulfide bridges between cysteines 53 and 306 (Volchkova, Feldmann et al. 1998, Falzarano, Krokhn et al. 2006). The cleavage of sGP by furin leads to the release of a small highly glycosylated peptide of about 10 to 14kDa: Δ -peptide (Volchkova, Klenk et al. 1999). It has been shown that Δ -peptide could have a role in viral replication by its ability to inhibit entry of retroviruses pseudotyped with Marburg virus GP as well as Marburg virus and Ebola virus infection in a dose-dependent manner (Radoshitzky, Warfield et al. 2011). Several roles have

been shown for sGP: first, sGP synthesis allows a reduction in surface GP production thus limiting its cytotoxicity for cells; this would allow therefore better replication and propagation of Ebola virus (Volchkov, Volchkova et al. 2001). In addition, it has been shown that sGP has a protective role on vascular endothelium integrity; in fact, sGP would inhibit the effects of TNF- α on endothelial barrier permeability thus being a regulation factor of the inflammatory response in endothelial cells (Wahl-Jensen, Afanasieva et al. 2005). Moreover, secretion of sGP protein by infected cells suggests that sGP could be a molecular lure that would allow the virus to escape from the immunosurveillance system (Sanchez, Ksiazek et al. 1999). Indeed, it has been demonstrated that sGP subverts immune responses by acting as a decoy antibody; in fact, sGP induces a host antibody response that focuses on epitopes it shares with GP1,2 thereby allowing it to bind and compete for anti-GP1,2 antibodies. Distinct from previously proposed “decoy” mechanisms in which secreted glycoprotein simply passively absorbs anti-glycoprotein antibodies, it has been demonstrated that sGP can also subvert an existing anti-GP1,2 immune response that was only weakly cross-reactive with sGP (Mohan, Li et al. 2012).

2.2.5.2. Shed GP

Cleavage at the membrane proximal external region by the tumor necrosis factor- α converting enzyme (TACE) releases the trimeric glycoprotein, termed shed GP (**Figure 6A**). In general, the process of shedding affects different cellular proteins both structurally and functionally; this is the case for a range of secreted cellular proteins including receptors, precursors of cytokines, growth factors, cell adhesion molecules, etc. (Edwards, Handsley et al. 2008). Cleavage usually occurs close to the extracellular face of the membrane, providing a mechanism for down-regulating the protein at the cell surface or releasing physiologically active proteins (Hooper, Karran et al. 1997). It should be mentioned that a classical consensus cleavage motif for the metalloprotease TACE does not exist (Ehlers, Schwager et al. 1996). However, there are certain preferences for amino acids at positions P1', P1, P2, P3 and P5 within the

sequences surrounding cleavage sites. Indeed TACE was shown to have strong preference for hydrophobic residues at P1' position, for alanine in P1 and P2 positions and for proline in P3 or P5 positions (**Figure 8**) (Caescu, Jeschke et al. 2009). Therefore three parameters seem to be important for TACE cleavage efficiency: the amino acid sequence, the amino acid conformation and the distance between the cleavage site and the transmembrane anchor.

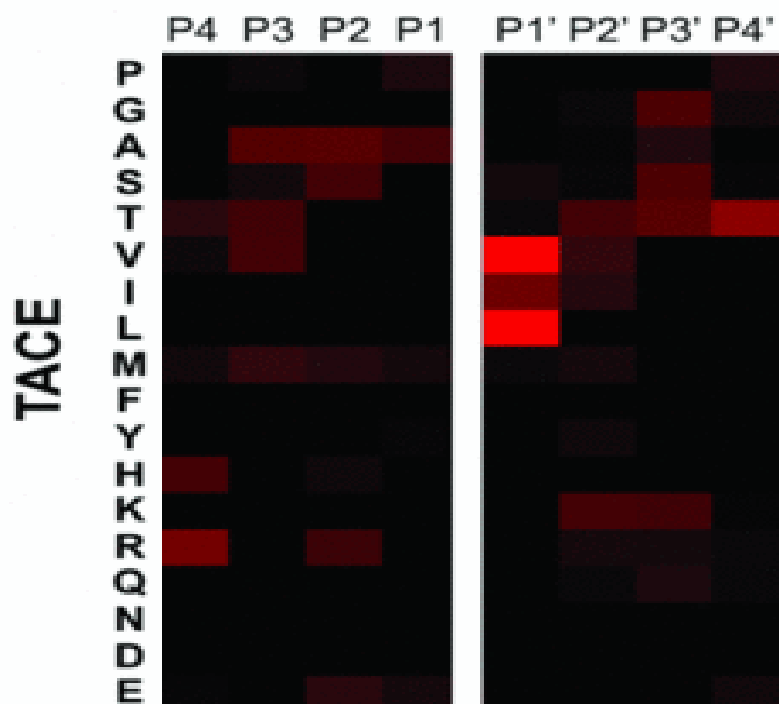


Figure 8.- TACE cleavage site selectivity.- A peptide library was used to determine preferences at the positions upstream and downstream of the cleavage site. Data were normalized such that a value of 1 (black) corresponds to the average quantity per amino acid in a given sequencing cycle and would indicate no selectivity, while residues having a value greater than 1 (red) are positively selected by the protease. Adapted from (Caescu, Jeschke et al. 2009)

Overall, shed GP's structure is practically identical to that of surface GP. It has also been demonstrated that shed GP and surface GP have the same glycosylation pattern by treatment with Endo H or PNGase F. Furthermore, it has been showed that shed GP is present in significant amounts in the blood of virus-infected animals and that, in a guinea pig model, it efficiently blocks the activity of KZ52, an Ebola virus-neutralizing antibody. It has also been

hypothesized that the release of surface GP in a soluble form could be another mechanism, besides sGP production, to reduce cellular cytotoxicity (Dolnik, Volchkova et al. 2004).

Besides its neutralising antibody function, no other role has yet been found for shed GP in other studies (Wahl-Jensen, Kurz et al. 2005). However, due to its similar structure to surface GP and the high quantity in which it is released in infected animals and humans, it is possible that other roles for this protein have still not been identified.

2.2.5.3.Small secreted GP

In 5% of transcripts, the insertion of two adenosines produces another editing product of the GP gene of Ebola virus: small secreted glycoprotein (ssGP) (**Figure 6A**), which shares 295 amino-terminal amino acid residues with sGP. It is secreted from cells in a monomeric form due to the lack of the carboxyl-terminal part (present in sGP), including a cysteine at position 306. The role of this 298-residue secreted ssGP protein is still unknown (Volchkova, Feldmann et al. 1998).

2.2.6.VP30

Contrary to other *Mononegavirales*, EBOV RNP contains a fourth protein: VP30. It has 288 amino acids and its amino-terminal region contains six serines and one threonine suitable to be phosphorylated by cellular kinases and phosphatases (Modrof, Muhlberger et al. 2002).

The use of a minigenome system has shown that VP30 is an essential transcription factor. In fact, non-phosphorylated VP30 has a role as a transcription factor of Ebola virus but it cannot bind NP protein; when it is completely phosphorylated, only its association function to the nucleoprotein is kept. In contrast, when one or two serine residues are phosphorylated, VP30 maintains both activities: as an assembly and as a transcription factor. Particularly, phosphorylation of serine residues at position 40 and 42 is

essential for VP30 binding to NP protein, whereas a Zinc finger structure in the amino-terminal region is required for its transcriptional function (Modrof, Muhlberger et al. 2002).

2.2.7.VP24

The sixth gene of Filovirus genome codes for VP24 protein, which has 253 amino acids and shares 35% of homology with Marburg VP24 (Bukreyev, Belanov et al. 1995). Its high content of hydrophobic amino acids and the detection of its incorporation into virions has led to its definition as a secondary matrix protein (Elliott, Kiley et al. 1985). EBOV VP24 has been shown to have biochemical and functional properties as a matrix protein, thus suggesting a role in assembly and budding of virus. However, the absence of budding in viral pseudo particles in the presence of VP24 alone and its absence in EBOV VLPs represent two arguments against its possible role as a strict matrix protein.

It has also been shown that VP24 is an interferon antagonist. It is capable of blocking STAT-1 translocation by binding to karyopherin $\alpha 1$ (an importin), a mechanism that has as a consequence the blocking of an antiviral response (Reid, Leung et al. 2006), as will be explained below in the section detailing Ebolavirus pathogenesis. Recently it has also been shown that it can directly bind STAT-1 (Zhang, Bornholdt et al. 2012).

Interestingly, Ebola virus infection of guinea pigs with wild-type EBOV virus results in an asymptomatic illness. However, sequential passaging of wild-type virus in guinea pigs can lead to the generation of highly pathogenic variants of the virus. These viruses present mutations in their genome; for instance, variant 8mc has, after 8 passages in guinea pigs, 5 mutations: 1 in L polymerase, one in NP and 3 in VP24 (Volchkov, Chepurnov et al. 2000). By generating several recombinant virus containing different combinations of these mutations, it has been demonstrated that mutations in VP24 were sufficient to acquire virulence (Mateo, Carbonnelle et al. 2011).

2.2.8. L polymerase

EBOV L protein is the largest (2212 amino acids, 260 KDa) but the least abundant protein in virions and infected cells. It contains three conserved domains: A (putative binding domain), B (RNA recognition domain and formation of phosphodiester bridges) and C (ATP and tri-phosphate ribonucleotide binding domain) (Muhlberger, Sanchez et al. 1992, Volchkov, Volchkova et al. 1999). L protein is the RNA- polymerase RNA dependent which contains all of the enzymatic activities necessary to synthesize viral RNA and antigenomes during transcription and replication, respectively; however, it is not functional without the other nucleocapsid proteins.

2.3.Ebola virus replication cycle

EBOV replication cycle is similar to the other Mononegavirales. Host cell entry is the first essential step in EBOV infection. EBOV has a very broad cell tropism including primary targets such as monocytes, macrophages and dendritic cells and secondary targets including hepatocytes, adrenal cortical cells, fibroblasts and endothelial cells. EBOV binds to multiple attachment factors, notable among which are:

- **C-type lectins.** The highly glycosylated GP of EBOV contains a set of N- and O-linked glycans that, depending on their structure, can be recognised in a calcium-dependent manner by different C-type lectins including: DC-SIGN and macrophage galactose lectin, MGL, on macrophages and immature DCs, and L-SIGN and LSECtin on endothelial cells in liver and lymph nodes (Alvarez, Lasala et al. 2002, Simmons, Reeves et al. 2003, Takada, Fujioka et al. 2004, Gramberg, Hofmann et al. 2005). It has also been shown that MBL (Mannose binding lectin) enhances EBOV infection at low complement levels (Brudner, Karpel et al. 2013). However other studies have shown that MBL blocks EBOV infection (Ji, Olinger et al. 2005, Michelow, Dong et al. 2010, Michelow, Lear et al. 2011). Interestingly, very recently it has been shown that removal of N-

glycans in GP1 subunit does not affect Ebola virus pseudovirion (VSV-GP) entry into murine peritoneal macrophages (Lennemann, Rhein et al. 2014).

- **Niemann-Pick C1** (NPC1) is an endosomal and lysosomal membrane protein which is known to be a cholesterol transporter. Mutations in *NPC1* gene result in cholesterol accumulation that leads to altered protein and lipid trafficking (Carstea, Morris et al. 1997). Cathepsins B and L process EBOV GP thus exposing its RBR domain which leads to binding to NPC1 (Carette, Raaben et al. 2011, Cote, Misasi et al. 2011, Shoemaker, Schornberg et al. 2013). It is considered as the intracellular receptor for EBOV since it is the only molecule that has been shown to be essential for EBOV entry after uptake into the cell and proteolytic processing of EBOV-GP.

- **TAM family** includes tyrosine kinase receptors Tyro3, Axl and Mer (TAM), which play important roles in different cellular process such as cell proliferation, cell adhesion and cytokine release (Linger, Keating et al. 2008). Axl, which is highly expressed on macrophages, has been shown to be an entry factor during EBOV infection (Shimojima, Takada et al. 2006, Brindley, Hunt et al. 2011). Even if all studies failed to demonstrate an interaction between EBOV-GP and Axl, it was clearly shown that Axl augmented EBOV pseudovirion internalisation suggesting that endosomal uptake of filoviruses is facilitated by Axl.

- **β 1 integrins** are expressed in a wide range of cells and are responsible for very diverse functions (cell-cell adhesion, migration, differentiation, proliferation...). They are implicated in EBOV-GP pseudotyped VSV entry (Takada, Watanabe et al. 2000, Schornberg, Shoemaker et al. 2009). However, direct binding of EBOV has still not been demonstrated.

- **T-cell immunoglobulin mucin domain 1 (TIM-1)** is expressed on the surface of activated T-cells, epithelial cells, renal tissue and some cell lines such as Huh7 (liver cell line) and Vero cells (monkey kidney cell line) (Hofmann-Winkler, Kaup et al. 2012). TIM-1 has been shown to promote EBOV infection of certain cells lines and soluble TIM-1 inhibits virus entry into permissive cells (Kondratowicz, Lennemann et al. 2011). However, monocytes, macrophages

and DCs do not express TIM-1 meaning that their cognate receptor has yet to be identified.

-Toll-Like receptor 4 (TLR4) is principally expressed on polymorphonuclear cells, monocytes, macrophages and DCs (Muzio, Bosisio et al. 2000). It is responsible of binding of LPS through LBP (lipopolysaccharide-binding protein) that will transfer LPS to CD14, which, in turn, will transfer LPS to TLR4-MD2 heterodimers (Triantafilou and Triantafilou 2002). TLR4 has been shown to bind GP-EBOV VLPs (Okumura, Pitha et al. 2010). Moreover, EBOV-VLPs stimulate the expression of proinflammatory cytokines but also type I interferons (IFNs) in murine DCs (Ayithan, Bradfute et al. 2014).

The filovirus replication cycle is depicted in **Figure 9**. EBOV binds to the previously described attachment factors on the cell surface **(1)** and enters target cells either by clathrin/caveolae endocytosis (Bhattacharyya, Warfield et al. 2010, Aleksandrowicz, Marzi et al. 2011) or by macropinocytosis (Nanbo, Imai et al. 2010, Saeed, Kolokoltsov et al. 2010), depending on the type of target cell involved and the viral system used to study viral entry. Virions are then trafficked through early and late endosomes where, under acid pH, cathepsin L and B will process EBOV-GP thus exposing RBR domain by proteolysis in acidified endocytic vesicles leading to EBOV-GP binding to NPC1 receptor and subsequent release of genetic material into the cytoplasm **(2)**. There, the nucleocapsid serves as a template for both transcription **(3)** and replication **(4)**. During transcription, the seven viral genes are transcribed from 3'-5' by the viral polymerase L into 7 monocistronic mRNAs, all capped and polyadenylated, which will be translated into viral proteins by cellular ribosomes. Transcription occurs in a sequential gradient from 3'-5', inducing a strong expression of NP protein and weaker for L polymerase.

During replication, RNA antigenomes (positive-sense mRNA) are generated and encapsidated. These antigenomes are used for the synthesis of negative mRNA for the new viral progeny, which will accumulate in inclusion bodies. In inclusion bodies, viral proteins and nucleoproteins get assembled to form

nucleocapsids which will be transported to the plasma membrane **(5)**, where nucleocapsids will be wrapped with the cellular membrane. Virions will then bud mostly in lipid rafts (microdomains in the plasma membrane containing sphingolipids and cholesterol) **(6)**.

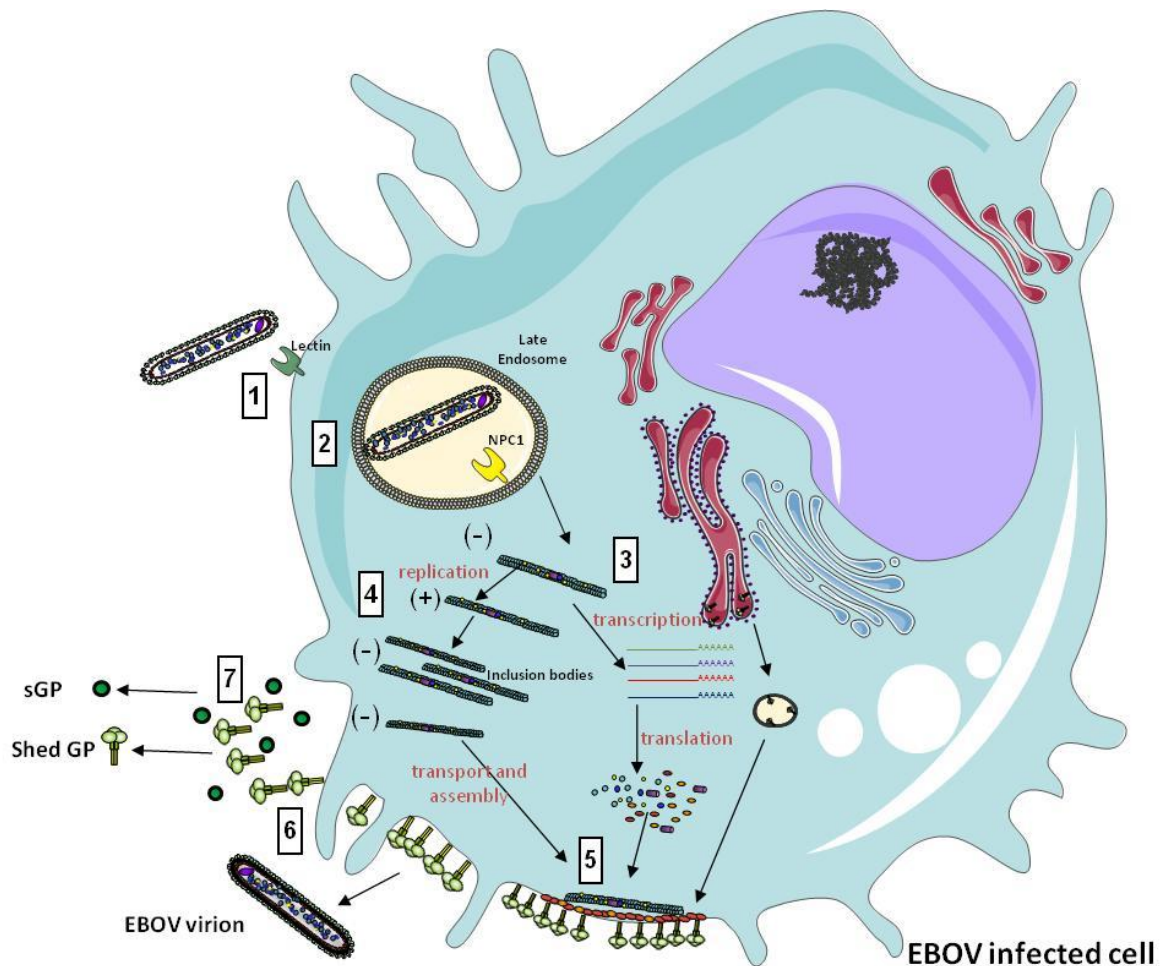


Figure 9.- Illustration of Ebola virus infection cycle. Filoviruses enter the cell by receptor-mediated endocytosis or macropinocytosis (1). After fusion of the viral and cellular membrane (2), the nucleocapsid is released into the cytoplasm and serves as a template for transcription (3) and replication (4). The replicated RNA is encapsidated by the nucleocapsid proteins. The newly synthesized nucleocapsids and viral proteins are transported to the sites of viral release (5), where budding takes place (6). Soluble glycoproteins sGP and shed GP are also released from infected cells (7).

3. Ebola virus pathogenesis

Ebola virus pathogenesis is different depending on the viral species. Besides that, they have in common that the high lethality of Ebola virus is due to a combination of the deleterious effects of high viral titers and direct viral damage and a nonspecific and abnormally sustained innate immune response.

3.1. Host immune response to Ebola virus infection

3.1.1. Innate immune response

Innate immunity is the first line of defense against infectious agents and pathogens. It begins very early during infection and is functional for several days. The innate immune response acts in two different ways:

1.- The specific recognition of the “non-self”: it involves pattern recognition receptors (PRR), which will recognise “non-self” patterns on the surface of pathogens (Pathogen associated molecular patterns: PAMPs). Recognition of the “non-self” also induces the intervention of pro-inflammatory cytokines and chemokines and starts the antiviral cell function by inducing the recruitment of innate immune cells and apoptosis of the infected cell.

2.- Induction and organisation of the antigen-specific immune response involving T cells and antibodies (Ab).

During Ebola infection, both processes are affected.

3.1.1.1. Ebola virus increases the cytokine response

Ebola virus has a broad cell tropism, infecting a wide range of cell types. In-situ hybridisation and electron microscope analyses of tissues from patients with fatal disease or from experimentally infected non-human primates show that monocytes, macrophages, dendritic cells (DCs), endothelial cells, fibroblasts, hepatocytes, adrenal cortical cells, and several types of epithelial cells all lend support to replication of Ebola virus (Zaki, Shieh et al. 1999).

Studies in non-human primates experimentally infected with Zaire Ebola virus suggest that monocytes, macrophages, and dendritic cells are the early and preferred replication sites for this virus (Geisbert, Hensley et al. 2003). These

antigen presenting cells (APCs) seem to have a role in the dissemination of the virus by supporting viral replication but also by spreading the virus from the initial infection site to regional lymph nodes, probably through the lymphatic system, and to the liver and spleen through the blood. Monocytes, macrophages, and dendritic cells infected with Ebola virus migrate out of the spleen and lymph nodes to other tissues including the liver and kidneys, thus amplifying and disseminating the infection (**figure 10**).

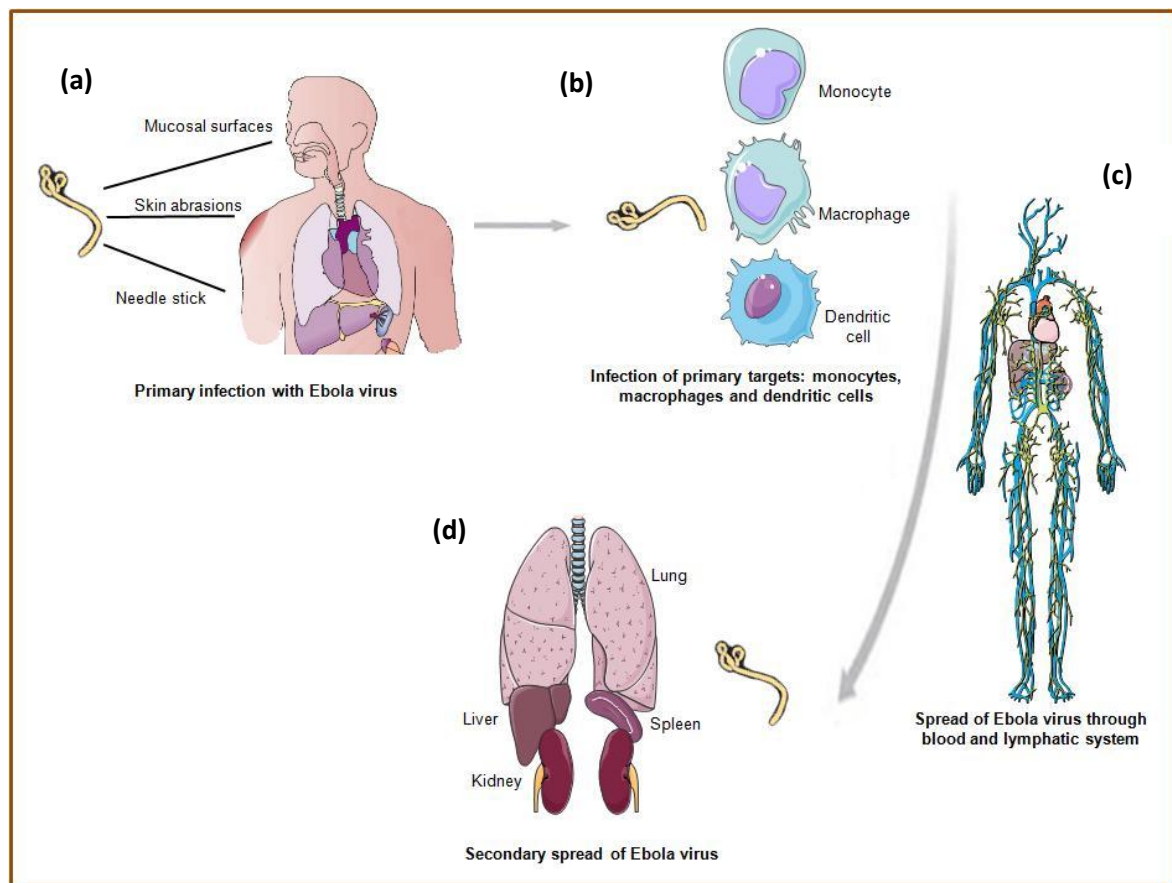


Figure 10.- Ebola virus infection and spreading. (a) Ebola virus infects subjects through contact with body fluids or secretions from an infected patient. Entry can occur through abrasions in the skin, accidental needle sticks, contact with infected bushmeat or across mucosal surfaces. Early targets for replication are monocytes, macrophages and dendritic cells which, through the lymphatic

(in green) and blood (in blue) systems **(c)** will spread Ebola virus to different organs including lungs, liver, spleen and kidneys **(d)**.

Monocytes can present antigens and also, especially under inflammatory conditions, go to tissues to differentiate into macrophages or dendritic cells (Geissmann, Manz et al. 2010). Macrophages are considered highly phagocytic, they contain a large number of lysosomes and vacuoles important for breaking down ingested antigens, and they are found mostly in tissues. Infection of monocytes and macrophages leads to an aberrant release of proinflammatory cytokines and chemokines including interleukin-1b (IL-1b), tumour necrosis factor- α (TNF α), IL-2, IL-6, IL-8, IL-15, IL-16, IL-1 receptor antagonist, soluble TNF receptor, IL-10, growth regulated oncogene- α , CCL3, CCL4, CXCL10, monocyte chemoattractant protein-1 (MCP-1), regulated upon activation normal T cell expressed and secreted (RANTES), eotaxin and reactive oxygen and nitrogen species such as Nitric Oxide (NO). Increased blood concentrations of nitric oxide in patients were associated with mortality. Abnormal production of nitric oxide has been associated with several pathological disorders including apoptosis of bystander lymphocytes, tissue damage, and loss of vascular integrity, which might contribute to virus-induced shock observed during the latter stages of Ebola virus infection. Nitric oxide is an important mediator of hypotension, and hypotension is a prominent finding in most viral haemorrhagic fevers including those caused by Ebola virus.

Normally, the release of proinflammatory cytokines recruits other monocytes, macrophages or dendritic cells, all of which are sensitive to Ebola virus infection; however, during Ebola virus infection the inflammatory response will be uncontrolled and will lead to what is called a “cytokine storm”. Overall, this ‘cytokine storm’, particularly prominent in the terminal stages of the disease in humans and non-human primates, has dramatic consequences. Virus-induced expression of these mediators results in an immunological imbalance that partly contributes to the progression of disease. In addition, some of these mediators may induce the expression of adhesion molecules on the surface of endothelial cells, allowing neutrophils and monocytes to invade sites of infection; this results in the release of further inflammatory cytokines, such as TNF- α ,

chemokines and other species such as nitric oxide that will in turn mobilise immature circulating neutrophils and will also facilitate the exit of inflammatory molecules and proteins from the blood that will contribute to vasodilatation and increased vascular permeability (causing tissue damage) (Bray and Geisbert 2005). In addition, infected monocytes and macrophages express cell surface tissue factor (TF), which may be involved in the development of coagulopathies and in the induction of disseminated intravascular coagulation (DIC).

Proinflammatory responses recorded in fatal cases of Ebola haemorrhagic fever are dysregulated, whereas early and well regulated inflammatory responses have been associated with recovery (Baize, Leroy et al. 2002).

Like monocytes and macrophages, immature dendritic cells are targets of Ebola virus. Dendritic cells are among the most effective antigen-presenting cells of the immune system. They express a large number of MHCII and are considered relatively less phagocytic than macrophages but they are highly stimulatory for T cells by secreting important interleukins and cytokines that provide a critical link between innate and adaptive immune responses to many pathogens (Martinez, Leung et al. 2012). DCs infected with Ebola virus are severely compromised in these critical functions. Human myeloid DCs infected with live virus fail to secrete the normal profile of proinflammatory cytokines and costimulatory molecules (Mahanty, Hutchinson et al. 2003, Bosio, Moore et al. 2004). These cells do not become mature or activated and are unable to upregulate major histocompatibility complex (MHC) molecules and thus to stimulate T cells. By contrast, treatment with noninfectious Ebola virus-like particles (VLPs) activates DCs and stimulates a robust inflammatory response (Bosio, Moore et al. 2004).

Inhibition of DC function with live or inactivated virus, but not with VLPs, indicates that the suppression of DC function and maturation is likely to be due to the presence of viral proteins or genomic material not present in the VLPs. Further studies are needed to clarify the detrimental effects of Ebola virus infection on other subpopulations of DCs, in particular on plasmacytoid DCs, which are important in antiviral interferon responses. The consequences of nonfunctional DCs include a diminished ability to stimulate humoral or cell-

mediated immune responses, which may contribute to the lack of control of viral replication.

It appears that there is a difference in the cytokine profile observed according to Filovirus species. Different expression of TNF- α and IFN- γ has been observed between Ebola Zaire, Sudan and Bundibugyo during human infections, suggesting a different immune response depending on Ebola virus species which could explain the differences in the virulence and the mortality rate between the different Ebola virus species (Hutchinson and Rollin 2007, Gupta, MacNeil et al. 2012).

3.1.1.2. Ebola virus inhibits the interferon response

The interferon (IFN) system represents a major element of the innate immune response against viral infection (Mordstein, Neugebauer et al. 2010). We can distinguish three types of IFN: IFN type I, which includes IFN α and IFN β and produced by all cell types, IFN type II which is only represented by IFN γ and is produced by dendritic cells, lymphocytes T and Natural killer cells and type III IFN, which is represented by IFN- λ and is principally produced by endothelial cells. Type I interferons (IFNs) are recognized as an early and powerful host defense against virus infection. Virus infection generally results in the synthesis and secretion of the type I alpha/beta interferons (IFN- α/β). Once secreted, IFN- α/β acts in an autocrine or paracrine manner by binding the ubiquitously expressed IFN- α/β receptor (IFNAR) (**Figure 11**). Receptor binding activates the Jak-STAT signaling cascade by recruiting the Janus kinases (JAK1 and TyK2). JAKs phosphorylate STAT1 and STAT2, which then dimerize and interact with IFN regulatory factor 9 (IRF9), forming a complex named ISGF3. ISGF3 binds to IFN-stimulated response elements (ISRE) in the promoters of IFN-stimulated genes (ISG) to regulate their expression (Jewell, Cline et al. 2010).

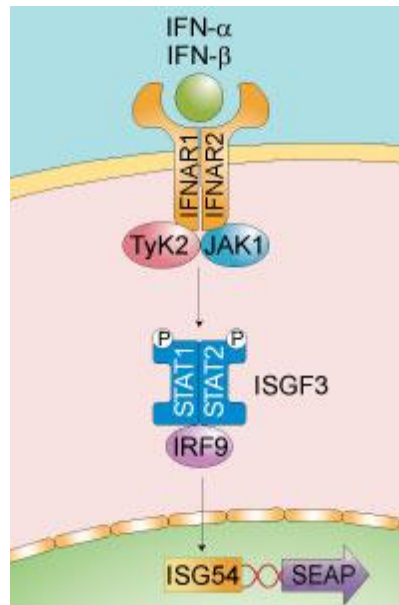


Figure 11.- IFN pathway.- IFN type I binds receptor IFNAR thus activating Jak-STAT signaling cascade and upregulating IFN-stimulated genes.

Induction of type I (α/β) IFN gene expression is tightly regulated. Synthesis of IFNs depends on the recognition of Pathogen Associated Molecular Motifs (PAMPs) by Pathogen Recognition Receptors (PRRs). The IFN pathway is biphasic: the first phase is called the IFN independent pathway (**Figure 12A**) and consists of the synthesis of IFN after viral recognition. These IFNs are then secreted and act in an autocrine and paracrine fashion by inducing the second phase of IFNs synthesis: the IFN dependent pathway (**Figure 12B**).

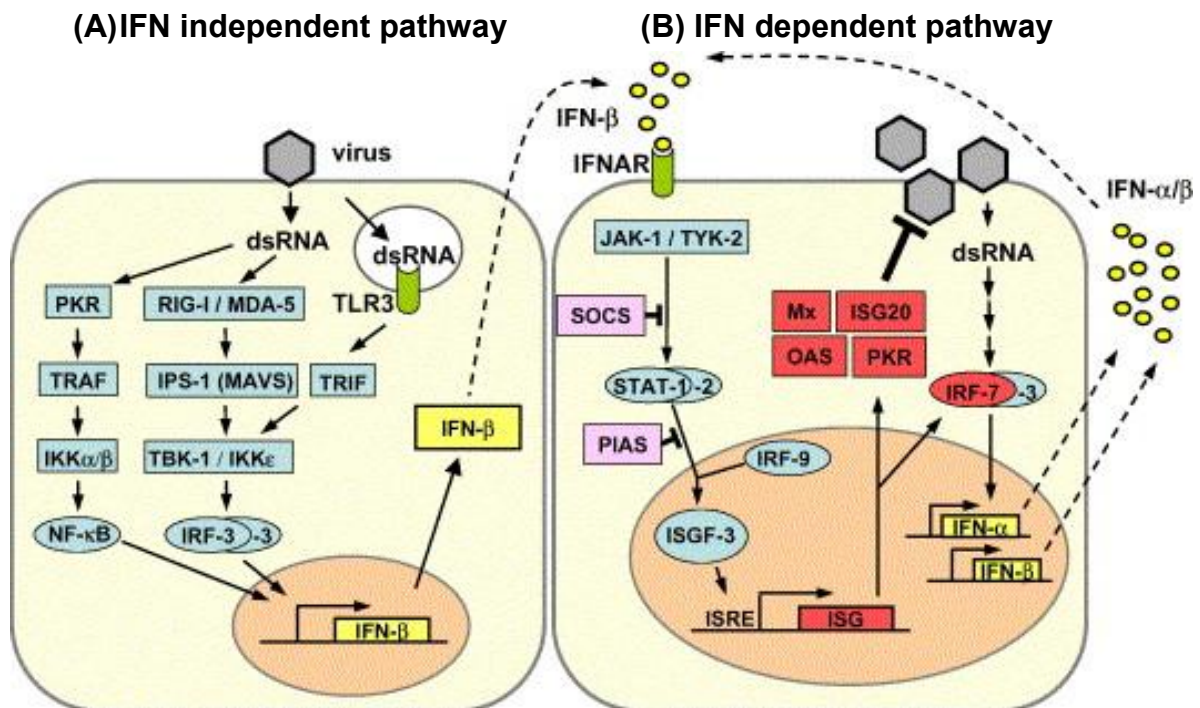


Figure 12.- Type I IFN induction, signaling and action. During the IFN independent pathway, Protein Kinase R (PKR) and helicases RIG-1 and MDA-5 can recognize dsARN synthesized during viral replication but also the 5'-triphosphate extremities of some viral genomes (Yoneyama, Kikuchi et al. 2004). The recognition of viral components in the cellular cytoplasm by these intracellular sensors leads to activation of the transcription factors NF- κ B, IRF-3 and AP-1. The cooperative action of these factors is required for full activation of the IFN- β promoter. A second signaling pathway involves endosomal TLR-3 and TRIF.

During the IFN dependent pathway, newly synthesized IFN- β binds to the type I IFN receptor (IFNAR) and activates the expression of numerous ISGs via the JAK/STAT pathway. IRF-7 amplifies the IFN response by inducing the expression of several IFN- β subtypes. SOCS and PIAS are negative regulators of the JAK-STAT pathway (Haller, Kochs et al. 2006).

Recent findings suggest that cells make use of two major but distinct cellular signal transduction pathways to sense viruses and activate their type I IFN genes. Most cells of the body including fibroblasts, hepatocytes and

conventional dendritic cells (cDCs) use the so-called classical pathway. They have intracellular sensors that, upon infection, detect viral components in the cytoplasm and activate the main interferon regulatory transcription factors IRF-3 and NF- κ B which in turn transactivate IFN- β gene expression. Infected cells secrete mainly IFN- β as an initial response to infection but switch to IFN- α during the subsequent amplification phase of the IFN response.

In contrast, plasmacytoid dendritic cells (pDCs) use Toll-like receptors (TLRs) expressed on the cell surface or in endosomes to sense extracellular or engulfed viral material. TLR signaling in pDCs primarily involves the adaptor protein MyD88 and activates IRF-7 which is constitutively expressed in pDCs and serves as a master regulator of IFN- α/β gene expression. This cell type predominantly secretes high levels of IFN- α and represents the so-called natural interferon producing cells of the body. Both pathways play important roles during infection (Haller, Kochs et al. 2006).

The absence of interferon production and also the ability of Ebola virus to also block cellular responses to exogenously added IFN likely helps to sustain virus replication in cells, promoting cytokine secretion by macrophages. Due to the fact that signaling pathways that trigger IFN production and also the pathways activated by exogenously added IFN are both implicated in DC maturation, the mechanisms that filoviruses use to block these pathways could contribute to the inhibition of DC maturation and function, thereby promoting the suppression of adaptative immune responses (Martinez, Leung et al. 2012).

Interferon production is blocked in macrophages, peripheral blood mononuclear cells and DCs by Ebola virus infection *in vitro* and *in vivo* (Bosio, Aman et al. 2003, Mahanty, Gupta et al. 2003). In addition, the expression of interferon-stimulated genes important in the type-I interferon response is decreased in Ebola virus-infected cells (Kash, Muhlberger et al. 2006). Like some other viruses, Ebola encodes specific viral proteins that antagonize the interferon response. Two virally encoded proteins, VP24 and VP35, have been shown to interfere with the induction of the interferon response (Basler, Mikulasova et al. 2003, Reid, Leung et al. 2006). VP35 blocks the IFN independent pathway and VP24 blocks the IFN dependent pathway (**Figure 13**). Ebola VP35 blocks the

activity of the interferon regulatory factor 3 (IRF-3), thus decreasing interferon responses (Basler, Mikulasova et al. 2003). More recently, VP35 was shown to counteract the activity of the double-stranded RNA–dependent protein kinase (PKR) (Feng, Cervený et al. 2007). Translocation of phosphorylated STAT1 to the nucleus is necessary for transcriptional activation of multiple genes induced by type I interferon (IFN-I). This translocation is triggered by its interaction with karyopherin $\alpha 1$ (KPN $\alpha 1$) (Najjar and Fagard 2010). EBOV VP24 protein interacts with KPN $\alpha 1$ thereby preventing translocation of STAT-I (Reid, Valmas et al. 2007), and also interacts with heterogeneous nuclear ribonuclear protein complex C1/C2 (hnRNP C1/C2) partially altering its nuclear transport (Shabman, Gulcicek et al. 2011). VP24 also blocks the phosphorylation of p38 (Halfmann, Neumann et al. 2011), which triggers the phosphorylation of transcription factors mediating the IFN response (Reid, Leung et al. 2006). It has recently been shown that DC maturation caused by GP or other components of EBOV particles is suppressed by Innate Response Antagonist Domains (IRADs) located in VP35 and, to a lesser degree, VP24 (Lubaki, Ilinykh et al. 2013).

(A) VP35 inhibition of IFN independent pathway (B) VP24 inhibition of IFN dependent pathway

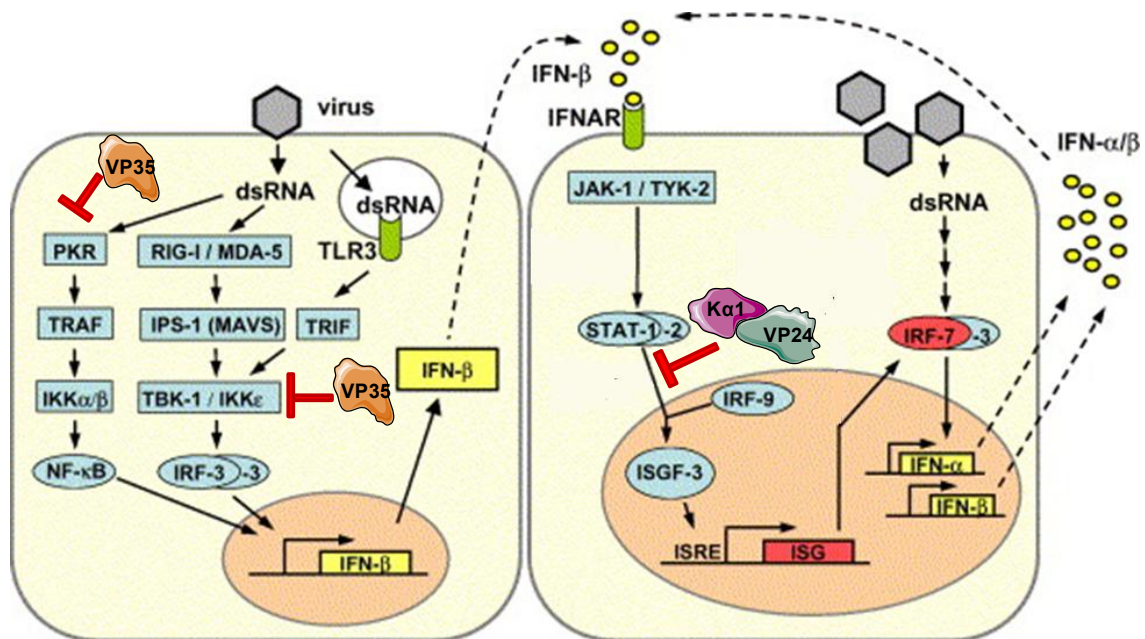


Figure 13.- Function of Ebola virus-encoded IFN-antagonist proteins. (A) EBOV VP35 inhibits IFN- α/β production by interacting with the IRF-3 kinases IKK- ϵ and TBK-1. This prevents efficient phosphorylation of IRF-3, blocking activation of the IFN- β promoter. **(B)** EBOV VP24 prevents nuclear accumulation of activated, tyrosine-phosphorylated STAT1 through an interaction with NPI-1 family karyopherin- α proteins, which otherwise import dimerized STAT1 into the nucleus.

In combination, these studies suggest that virus-induced inhibition of the interferon pathway not only decreases interferon-stimulated gene transcription to prevent an antiviral response state, but also contributes to lower numbers of mature and activated myeloid DCs, which in turn hinders the activation of the adaptive immune response.

3.1.2. Adaptative immune response

DCs play an important role in activating cellular adaptative immune responses. DCs present pathogen-derived antigens to T_H lymphocytes through major histocompatibility complex II (MHCII), which are present on the surface of APCs, and this will stimulate lymphocytes and polarize the immune response towards a cellular TH₁ response (T lymphocytes CD4⁺ and T lymphocytes CD8⁺) or towards a humoral TH₂ response (B lymphocytes, antibodies).

3.2.2.1. Cellular immune response

Patients who succumb to Ebola virus infection show little evidence of an activated adaptive immune response. Adaptive immunity is severely compromised not only because of a lack of functional DCs and the decrease of APCs due to infection, but also because lymphocytes undergo massive apoptosis in infected humans and nonhuman primates (phenomena known as lymphopenia) (Baize, Leroy et al. 1999, Gupta, Spiropoulou et al. 2007). Indeed, although lymphocytes are not targets of the virus, substantial numbers - except for B lymphocytes - undergo apoptosis during the illness (Geisbert, Hensley et al. 2000); as a result, CD4⁺ and CD8⁺ T lymphocytes and NK cells (Reed, Hensley et al. 2004) are considerably reduced in fatal human and nonhuman primate infections before death (Baize, Leroy et al. 1999, Reed, Hensley et al. 2004). Lymphocyte apoptosis is also a common manifestation of other viral hemorrhagic fevers and is frequently observed during septic shock (Geisbert and Jahrling 2004).

Studies with lymphocytes *in vitro* indicate that several molecules involved in triggering apoptosis are present on these cell populations, including TNF related apoptosis-inducing ligand (TRAIL) and FasL (Gupta, Spiropoulou et al. 2007). However, the mechanisms responsible for this apoptosis are still under investigation. It may be that inflammatory mediators and other factors, such as the pro-apoptotic soluble factor nitric oxide (NO) secreted by infected macrophages, are capable of inducing the observed lymphocyte apoptosis. Alternatively, impaired DC function and an overall immunosuppressive state may contribute to the phenomenon. Also, the fact that a part of monocytes,

macrophages and DCs die during infection, could reduce antigen presentation to lymphocytes. Yet another possibility is that cell death is actively triggered by direct interactions between lymphocytes and Ebola virus or soluble Ebola virus proteins. The recognition of an immunosuppressive motif in the carboxyl-terminal region of the virus glycoprotein lends support to the notion that virus particles or proteins might partly contribute to the dysfunction or the loss of lymphocytes, or both (Zampieri, Sullivan et al. 2007).

The importance of early responses involving cells of the innate immune system and/or a rapid adaptive antibody response is highlighted by a study showing protection of nonhuman primates with post-exposure vaccine administration (Feldmann, Jones et al. 2007). However, massive apoptosis of lymphocytes does not seem to be a major factor of Ebola virus pathogenesis in mice; in fact, transgenic mice highly expressing the anti-apoptotic factor bcl-2 showed that, inhibition of lymphocyte apoptosis did not protect animals against a lethal challenge with mouse adapted Ebola virus (Bradfute, Swanson et al. 2010). This would suggest that lymphocyte apoptosis would be a secondary effect due to cytokine overexpression rather than a direct consequence of infection. In contradiction to this, it has previously been shown that individuals having survived Sudan Ebola virus infection had an increased CD8⁺ cellular profile when compared to patients that had not survived (Baize, Leroy et al. 1999). However, another study showed that it was possible to protect naive mice against mouse adapted Ebola virus infection by transferring purified CD8⁺ lymphocytes (Bradfute, Warfield et al. 2008).

3.2.2.2. Humoral immune response

During fatal Ebola virus infection, antibody production is normally weak or even absent, suggesting that antibody titer might be correlated with protection to EBOV infection. A humoral immune response is sometimes generated during lethal EBOV infection, meaning that antibodies produced by survivors were more efficient than the ones synthesized by the patients who did not survive. Such a humoral immune response seems to be long lasting: 20 patients having survived Zaire Ebola virus infection from 3 different outbreaks in Gabon, still

had IgG antibodies against Zaire Ebola virus 11 years after infection (Wauquier, Becquart et al. 2009).

A neutralising antibody was isolated from a survivor's bone marrow during an outbreak of Zaire Ebola virus in 1995. This antibody, named "KZ52", is capable of neutralising 90% of viral particles (Maruyama, Rodriguez et al. 1999). It specifically recognizes a 15 amino acid long sequence of GP; it neutralises the virus by preventing fusion loop rearrangement thus precluding the fusion of viral and cellular membrane in the endosome. Moreover, it appears that KZ52 fixation to its epitope, generates a steric bulk that would hide the binding domain from the cellular receptor (Lee, Fusco et al. 2008). Treatment with this antibody has been tested in NHP but it did not protect animals neither before, nor after exposure to Ebola virus; this had put into question the idea of using antibodies to treat infected patients (Oswald, Geisbert et al. 2007).

However, a more recent study has shown that it was possible to protect NHP against a lethal infection by passive transfer of virus-specific polyclonal antibodies, obtained from vaccinated monkeys. Treatments were delayed 48 h, with additional treatments on days 4 and 8 post-exposure; the delayed treatments protected EBOV-challenged NHPs (Dye, Herbert et al. 2012). These studies clearly demonstrate that post-exposure antibody treatments can protect NHPs (probably depending on the antibody quality, quantity and the moment of administration) and open avenues for filovirus therapies for human use.

Although filoviruses are among the most virulent and fatal pathogens known, as mentioned before, some patients infected with Ebola virus recover from infection. Identifying the differences in the immune response between fatal and nonfatal cases is important for future development of effective therapies and vaccines. Specific differences in clinical presentation and immune responses have been noted in those who succumb contrasted with those who recover from Ebola virus infection (**Table 6**) (Zampieri, Sullivan et al. 2007).

Non-lethal infection	Lethal infection
Prominent CD8 ⁺ activation	No CD8 ⁺ activation
Above-normal numbers of T cells	Below-normal numbers of T cells
10 ⁷ viral genome copies/ml serum	10 ¹⁰ viral genome copies/ml serum
Detectable antibodies in blood at onset of symptoms	No detectable antibodies in blood at onset of symptoms
Low Nitric Oxide (NO)	High NO

Table 6.- Correlative differences between survivors and non survivors of Ebola virus infection. Adapted from (Zampieri, Sullivan et al. 2007).

This comparison shows that the development of an antigen specific cell-mediated immune response correlates with clearance of the virus. Studies demonstrating antigen-specific cellular immune responses in vaccinated nonhuman primates that survived infectious Ebola virus challenge support this finding (Sullivan, Sanchez et al. 2000, Sullivan, Geisbert et al. 2003). Based on these considerations, it is possible that an early and robust, but transient, innate immune response and the subsequent activation of adaptive immune response are necessary to protect against fatal infection.

3.1.3.Coagulation impairment

In vertebrates, the role of the coagulation cascade is to repair damage and protect the cardiovascular system. Coagulation prevents blood loss through haemostatic mechanisms and limits the spread of systemic infection by encapsulation of pathogens.

Ebola haemorrhagic fever is characterized by coagulative disorders and haemorrhages. These defects in blood coagulation and fibrinolysis are manifested as petechiae, ecchymoses, mucosal haemorrhages, congestion, and uncontrolled bleeding at venepuncture sites. Massive loss of blood is infrequent and, when present, is mainly limited to the gastrointestinal tract. Even

in these cases, the amount of blood that is lost is not substantial enough to cause death (Feldmann and Geisbert 2011).

Thrombocytopenia, consumption of clotting factors, deposition of fibrin and increased concentrations of fibrin degradation products are other indicators of the coagulopathy that characterises Ebola virus infection. The mechanisms responsible for triggering the coagulation disorders that typify Ebola haemorrhagic fever are not wholly understood. Besides the fact that Ebola virus replicates in endothelial cells, their infection does not extensively disrupt the vascular endothelium in cynomolgus monkeys. Moreover, endothelial cell infection *in vivo* was only seen at late stages of disease, after the onset of hemorrhagic abnormalities that characterize Ebola virus fever (Geisbert, Hensley et al. 2003), meaning that other non viral factors such as cytokines could be the cause of endothelial barrier disruption. This is in agreement with the fact that this disruption was shown to be not associated with the presence of intracytoplasmic Ebola virus antigens (Geisbert, Young et al. 2003). This data is consistent with the histological observations in fatal human cases from initial outbreaks in 1976, where no vascular lesions were identified (FA 1978). Although it may be a relatively late event in Ebola virus haemorrhagic fever, and not a direct contributor to the hemorrhagic diathesis, late-stage endothelial cell infection may induce other host cell changes that play important roles in the disease and outcome of the infection.

Several studies suggest that the coagulation abnormalities caused during Ebola haemorrhagic fever are generally consistent with disseminated intravascular coagulation (DIC) (Levi 2007). Furthermore, histological and biochemical evidence of disseminated intravascular coagulation during Ebola virus infection in several non-human primate species have been shown (Bray, Hatfill et al. 2001, Geisbert, Young et al. 2003).

Coagulation is initiated by tissue factor , a structural member of the cytokine receptor family (Edgington, Ruf et al. 1991). Microbial mediators directly induce rapid upregulation of TF in cells of the innate immune system, principally monocytes and macrophages, whereas endothelial cell expression of TF appears to come later, dependent on secondary mediators and restricted to

selective vascular beds. TF triggers coagulation by binding the enzyme coagulation factor VIIa and the substrate factor X. Then, Xa is generated and leads to thrombin formation, fibrin deposition and platelet activation, processes that are tightly controlled by anticoagulant mechanisms of the protein C pathway (**Figure 14**) (Ruf 2004).

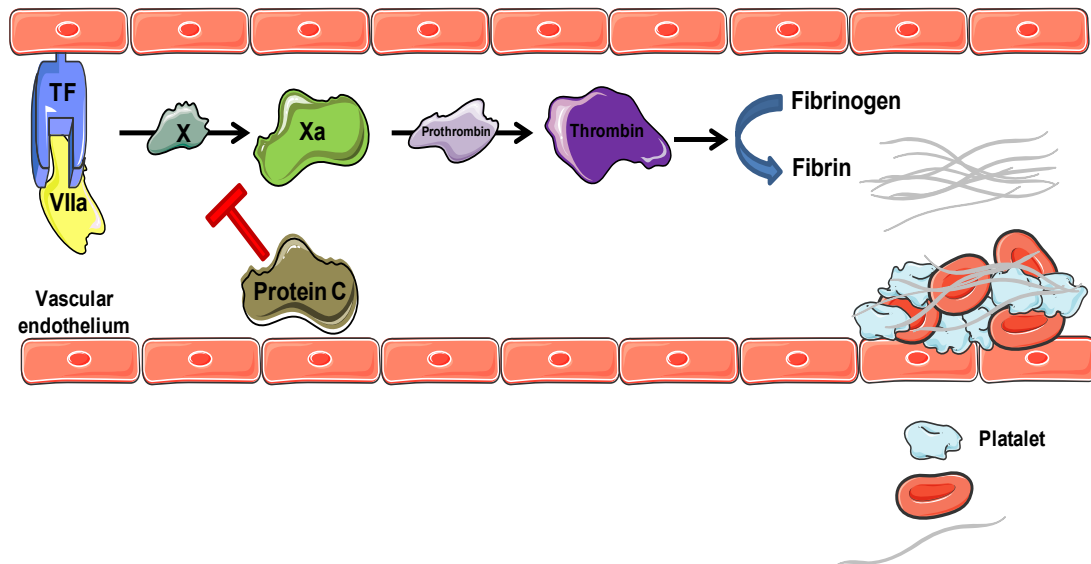


Figure 14.- Tissue Factor coagulation pathway Tissue factor expression is enhanced leading to increased production of prothrombin that is converted to thrombin, and that in turn generates fibrin from fibrinogen thus generating clotting.

Results from several studies strongly suggest that expression or release of tissue factor from monocytes and macrophages infected with Ebola virus are key factors that induce the development of coagulation irregularities reported in Ebola haemorrhagic fever (Geisbert, Young et al. 2003). Overexpression of tissue factor, a glycoprotein of 47kDa that functions as the primary cellular initiator of the coagulation protease cascades, is a leading cause of DIC and thrombosis-related organ failure. During DIC, the tissue factor (TF)-dependent pathway is the dominant route to thrombin generation that leads to fibrin deposition (**Figure 15**).

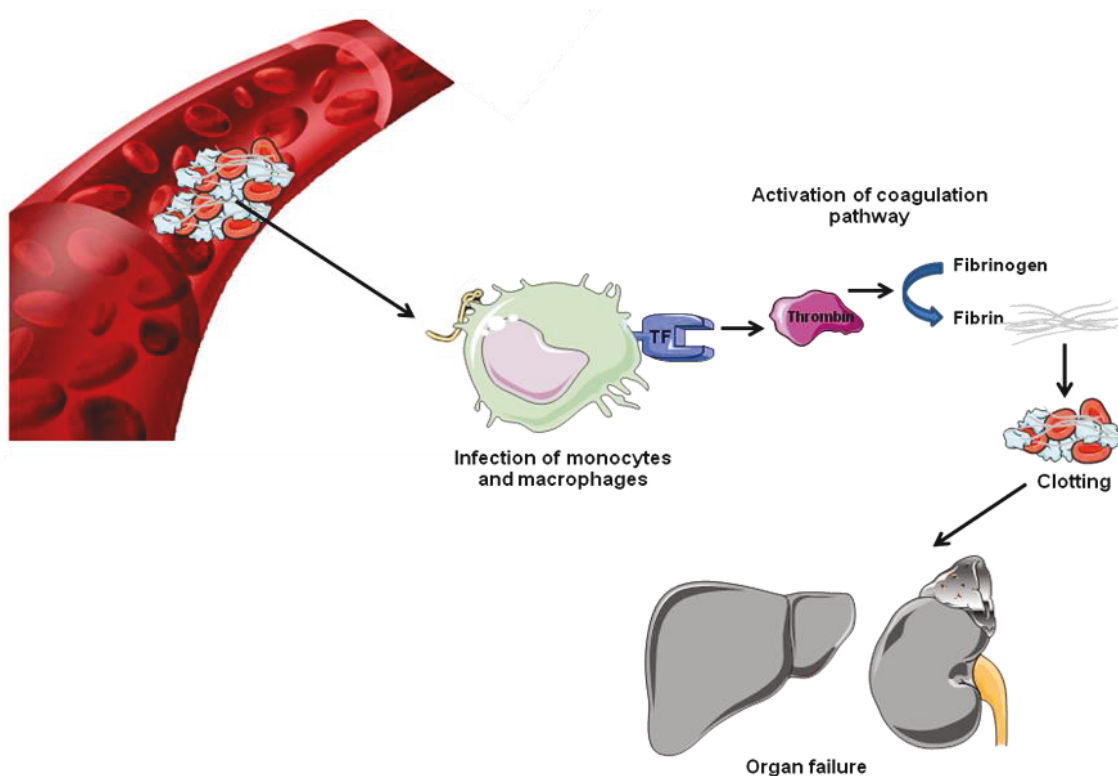


Figure 15.- Expression of Tissue Factor can lead to Disseminated Intravascular Coagulation syndrome. Ebola virus infection of monocytes and macrophages will induce the expression of Tissue Factor (TF) that will initiate the coagulation pathway, leading to clotting. The resulting consumption of clotting factor will cause abnormal bleeding, but it will also disrupt the blood flow to organs thus contributing to organ failure.

However, coagulopathy noted during Ebola haemorrhagic fever could be caused by several factors, especially during the later stages of disease. For example, rapid reductions in plasma concentrations of the natural anticoagulant protein C were recorded during the course of Zaire Ebola virus infection of cynomolgus monkeys (Geisbert, Young et al. 2003). During inflammatory states, cytokines decrease endothelial cell expression of thrombomodulin, in turn decreasing activation of protein C. Importantly, hepatic filovirus infection results in hepatocellular necrosis that leads to decreased synthesis of coagulation and other plasma proteins but also elevations in aspartate aminotransferase, alanine aminotransferase, γ -glutamyltransferase, alkaline phosphatase, and lactate dehydrogenase concentrations (enzymes involved in

metabolism and stored in hepatocytes and released into the blood when hepatocytes are acutely damaged).

Adrenocortical infection and necrosis have also been reported in humans and non-human primates infected with Ebola virus (Geisbert, Hensley et al. 2003). The adrenal cortex plays an important role in the control of blood pressure homeostasis. Impaired secretion of enzymes that synthesis steroids leads to hypotension and sodium loss with hypovolaemia, which are important elements that have been reported in nearly all cases of Ebola haemorrhagic fever. Impairment of adrenocortical function by Ebola virus infection could therefore significantly contribute to the shock that typifies the late stages of Ebola haemorrhagic fever.

Together, the data so far suggest that an impaired and ineffective host response leads to high concentrations of virus and proinflammatory mediators in the late stages of disease, which is important in the pathogenesis of haemorrhage and shock. The prevailing hypothesis at this time is that infection and activation of antigen-presenting cells is fundamental to the development of Ebola haemorrhagic fever. The release of proinflammatory cytokines, chemokines, and other mediators, like tissue factor, from antigen presenting cells and other cells, causes impairment of the vascular and coagulation systems leading to multiorgan failure and a syndrome that in some ways resembles septic shock (Feldmann and Geisbert 2011).

3.1.3.1. Disseminated Intravascular Coagulation (DIC)

Coagulation abnormalities caused during Ebola haemorrhagic fever are generally consistent with disseminated intravascular coagulation (DIC) (Levi 2007). Disseminated Intravascular Coagulation (DIC) is a syndrome characterized by systemic activation of the blood coagulation system, which results in the generation and deposition of fibrin, leading to microvascular thrombi (**Figure 16A**) in various organs and contributing to the development of multi-organ failure. Moreover, clotting can lead to an increased consumption of platelets and of some clotting factors which may induce severe bleeding

complications (**Figure 16B**) (Levi and Ten Cate 1999). These changes, which may occur rapidly or at a more chronic rhythm, can induce abnormal bleeding, small-vessel obstruction and single or multiple organ dysfunction. Hence, a patient with DIC can present simultaneously thrombotic and bleeding problems.

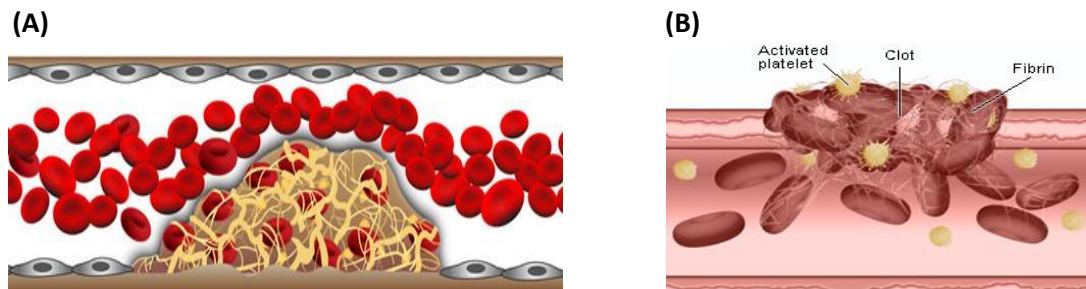


Figure 16.- Disseminated Intravascular Coagulation. (A) Deposition of fibrin leads to clotting **(B)** Clotting implies consumption of coagulation factors thus inducing abnormal bleeding. Both will contribute to the development of multiorgan failure.

In general, there are two major pathways that may cause DIC :

- (i) A systemic inflammatory response, leading to activation of the cytokine network and subsequent activation of coagulation (such as in sepsis)
- (ii) The release or exposure of procoagulant material into the bloodstream (such as in cancer or in obstetrical cases).

Systemic infections with bacteria or viruses can lead to DIC. Specific cell membrane components of microorganisms cause a generalized inflammatory response, characterized by the systemic occurrence of pro-inflammatory cytokines. There is solid evidence that cytokines also play a pivotal role in the occurrence of DIC in trauma patients (Roumen, Hendriks et al. 1993). Ebola virus surface glycoproteins induce the release of pro- and anti-inflammatory cytokines (Wahl-Jensen, Afanasieva et al. 2005, Hutchinson and Rollin 2007) thus potentially contributing to DIC syndrome in Ebola virus infected patients.

3.1.3.2. DIC pathogenesis

Two principal simultaneously mechanisms play a role in the pathogenesis of DIC (**Figure 17**), fibrin deposition and impairment of fibrin removal. Fibrin deposition is a result of tissue factor-mediated thrombin generation that is insufficiently balanced by dysfunctional physiologic anticoagulant mechanisms (such as the antithrombin system and the protein C system). In addition, impairment of fibrin removal is a result of the impairment of the fibrinolytic system, mainly caused by high circulating levels of PAI-1, a fibrinolytic inhibitor. During Ebola virus infection, the appearance in the tissues of fibrin deposits, coinciding with detectable viremia and infectious virus, strongly supports a direct link between DIC and viral infection (Geisbert, Young et al. 2003).

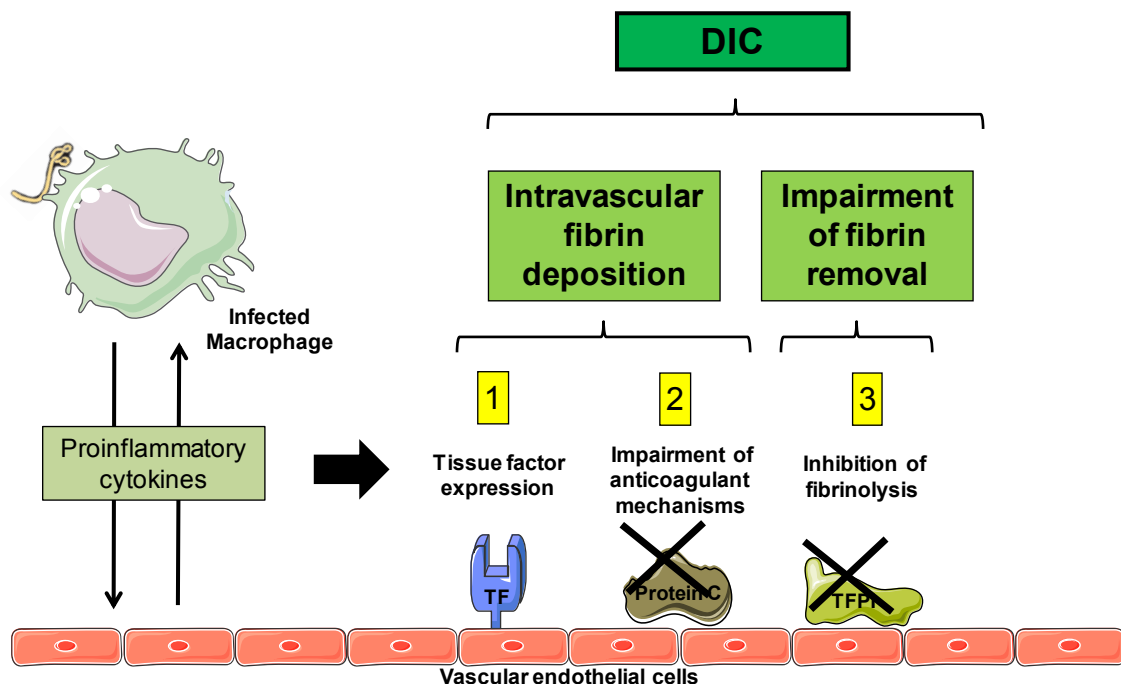


Figure 17.- Schematic representation of pathogenic pathways in disseminated intravascular coagulation. During systemic inflammatory response syndromes, both perturbed endothelial cell function and activated mononuclear cells may produce proinflammatory cytokines that mediate coagulation activation. Activation of coagulation is initiated by tissue factor expression on activated macrophages, monocytes and endothelial cells. In addition, downregulation of physiological anticoagulant mechanisms (like Protein C) and impairment of fibrinolysis (by inhibition of Tissue Factor Pathway

Inhibitor -TFPI-) will further promote Disseminated Intravascular Coagulation (DIC).

In somewhat more detail, the following processes are involved:

1. Tissue factor expression
2. Impairment of anticoagulant mechanisms
3. Impairment of fibrin removal

3.1.3.2.1. Tissue factor expression

TF is best known as the key initiator of coagulation in vivo, but it is also a member of the cytokine receptor superfamily and functions as a signaling receptor. Not only vascular injury, but also inflammatory cytokines and other factors can lead to TF expression, thus linking inflammation and coagulation. Monocytes are the predominant cell type to express TF after appropriate stimulation and are partially responsible for the activation of coagulation in endotoxemia. LPS and the subsequent host response also induce endothelial cells to express TF. Indeed, monocytes and ECs do not express TF constitutively but only after stimulation by endotoxin immune complexes, certain cytokines, and platelets. ECs have also been shown to release procoagulant TF in response to TNF α and IL-6. Recent studies have shown that activated platelets release TF from their alpha granules and provide another source of intravascular TF. Other leukocytes do not express TF but can acquire TF by binding with monocyte-derived microparticles (MPs).

The initiation of coagulation requires the exposure of TF to VIIa, which is circulating at low concentration in the blood. The TF/FVIIa complex activates both IX and X, leading to generation of fibrin clots (**Figure 14**). In line with this observation, abrogation of the tissue factor/VIIa pathway by monoclonal antibodies resulted in a complete inhibition of thrombin generation in endotoxin-challenged chimpanzees and prevented the occurrence of DIC and mortality in baboons that were infused with *E. coli* (ten Cate, Schenk et al. 1994, Biemond, Levi et al. 1995, Taylor 2001). Importantly, a study showed that samples obtained from Ebola virus infected macaques contained increased levels of TF

associated with lymphoid macrophages. Plasma from these macaques also contained increased numbers of TF-expressing membrane microparticles (Geisbert, Young et al. 2003). Interestingly, another study showed that administration of recombinant nematode anticoagulant protein c2 (rNAPc2), a potent inhibitor of tissue factor-initiated blood coagulation, to Ebola virus infected macaques prolonged survival time, with a 33% survival rate. Moreover, rNAPc2 also attenuated the coagulation in all treated animals (Geisbert, Hensley et al. 2003).

As seen before, tissue factor can also activate thrombin, which itself has several roles in coagulation, including:

- The conversion of fibrinogen to fibrin, activation of factors V, VIII, XIII, protein C, and thrombin-activatable fibrinolysis inhibitor (TAFI).
- The ability to elicit responses in leukocytes, platelets and endothelial cells
- Cleavage of proteins outside the coagulation pathway, such as complement components C3 and C5.
- Stimulate the production and secretion of inflammatory cytokines principally through NF- κ B signaling pathways. It activates a pro-inflammatory response through its interaction with specific receptors known as protease-activated receptors or PARs, which are expressed on ECs, mononuclear cells, platelets, fibroblasts, and smooth muscle cells. IL-6 production enhanced by thrombin leads to TF expression through a positive feedback loop.
- Enhancement of monocytes or monocyte-derived macrophages adhesion and increase of their production of pro-inflammatory cytokines with downregulation of anti-inflammatory cytokines.
- Stimulation of endothelial cell production of platelet- activating factor (PAF), whose role is to attract neutrophils and contribute to their adhesion.

Therefore, it should be noted that circulating thrombin itself has several potent pro-inflammatory properties (thus contributing to coagulation): from activation of proinflammatory cytokines to induction of clotting through the conversion of fibrinogen into fibrin.

3.1.3.2.2. Impairment of anticoagulant mechanisms

Activation of coagulation is regulated by four major anticoagulant factors: antithrombin (AT), thrombomodulin (TM), the activated protein C (PCA) and tissue factor pathway inhibitor (TFPI). An impaired function of various natural regulating pathways of coagulation activation may amplify thrombin generation and contribute to fibrin formation (Okajima 2001). The dysregulation of these anticoagulants are detailed below:

Antithrombin (AT)'s role in coagulation involves the inhibition of several coagulation modulators including factors Xa, IXa, XIa, and thrombin. AT's anti-inflammatory actions include:

- Inhibition of leukocyte activation (rolling and adhesion)
- Binding of AT to heparin sulfate proteoglycan (HSPG) on the endothelial surface and blockade of the expression of pro-inflammatory cytokines. Pro-inflammatory cytokines also contribute to decreased production of endothelial HSPGs, reducing the efficacy of AT.
- Mediation of inflammation, for example, by direct binding to leukocytes and thereby attenuation of cytokine receptor expression.

Thrombomodulin (TM) is a membrane protein synthesized predominantly by ECs and is ubiquitously expressed. Some of its functions include:

- TM binding to thrombin and generation of thrombin-TM complexes which activate protein C by a 100-fold. This complex also blocks the thrombin-mediated conversion of fibrogen into fibrin and inhibits the binding of thrombin to other cellular receptors on platelets and inflammatory cells.
- Inhibition of leukocyte adhesion to activated endothelium.

- Acceleration of the activation of the plasma carboxipeptidase thrombin-activatable fibrinolysis inhibitor (TAFI), an important inhibitor of fibrinolysis.
- TM is considered anti-inflammatory through its interference with complement activation and the neutralization of LPS.

Serum specimens from the Sudan Ebola virus outbreak in 2000 in Uganda were analysed for several biomarkers and elevated levels of thrombomodulin were associated with mortality and haemorrhaging (McElroy, Erickson et al. 2014).

Activated protein C (PCA) inhibits, through proteolytic cleavage, coagulation factors Va and VIIIa, providing critical negative feedback regulation of thrombin generation. In addition, PCA possesses cytoprotective properties including anti-apoptotic mechanisms (inhibits endothelial cells apoptosis), endothelial barrier stabilization, and the inhibition of inflammation. In addition, binding of activated protein C to the protein C receptor has been shown to inhibit endotoxin-induced tissue factor expression on mononuclear cells.

As with thrombin, most of PCA's actions are mediated through its interaction with PAR-1 and via the endothelial protein C receptor (EPCR). EPCR is expressed on ECs as well as on leukocytes. Endothelial NF- κ B activity, reduces thrombomodulin and EPCR expression and increases their shedding from the endothelial surface, thus rendering the endothelium less capable of activating Protein C (White, Schmidt et al. 2000).

Animal experiments based on the study of severe inflammation-induced coagulation activation showed that compromising the protein C system results in increased morbidity and mortality rates, whereas restoring an adequate function of activated protein C improves survival and organ failure (Levi, van der Poll et al. 2004). The analysis of samples obtained from 25 macaques infected with Zaire Ebola virus showed a rapid decrease in plasma levels of protein C concomitant with progression of disease (Geisbert, Young et al. 2003). In agreement with this study, treatment with recombinant activated Protein C (rPCA) of rhesus macaques that had been challenged with a lethal dose of

Zaire Ebola improved NHP survival in at an 18% rate (Hensley, Stevens et al. 2007).

TFPI, a protein produced by ECs, inhibits the TF-VIIa complex to prevent further generation of Xa. TAFI has also been shown to be the primary enzyme responsible for inactivation of complement factor C5a, thus providing protection against complement-mediated injury in the micro-vasculature. Studies evaluating recombinant TFPI found that it significantly blocks inflammation-induced thrombin generation, but no conclusions have been drawn from phase I or II clinical trials (O'Brien 2012). In addition, another study showed that TFPI is capable of preventing mortality during systemic infection and inflammation. Altogether data suggest that high concentrations of TFPI are capable of modulating tissue factor-mediated coagulation (de Jonge, Dekkers et al. 2000).

3.1.3.2.3. Impairment of fibrin removal

Dissolution of the fibrin clot by enzymes with fibrinolytic activity is required to restore integrity to intravascular and extravascular sites of thrombus formation. The amount of fibrin deposited in tissues is generally highest in the smallest vessels but may be influenced by the local rate of blood flow and the activity of local clearance mechanisms. The effect of fibrin deposition can lead to organ ischaemia and failure. Passage of red cells through fibrin strands can also cause red blood cell distortion and fragmentation and subsequent haemolysis and local platelet aggregation.

There are principally two systems to remove fibrin:

1. Plasmin is the primary fibrinolytic pathway of plasma, but platelets and neutrophils also contain fibrinolytic proteases. The fibrinolytic proteases of leukocytes, such as elastase, are found in secretory granules and are capable of degrading fibrin at neutral pH (Plow 1982).
2. A secondary fibrinolytic response to fibrin deposition results from the activation of the plasminogen activator (PA) from endothelium. There are two types of PA: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), found in storage sites in vascular endothelial cells. t-PA is principally synthesized by endothelial cells in

normal blood vessels and u-PA is produced and secreted by a variety of cell types including monocytes, macrophages, trophoblasts and epithelial cells. The plasmin formed after PA action can generate fibrin degradation products (FDP) which, as for fragments X and Y, have a direct antithrombin action. Other FDP fragments such as D (D-Dimers) and E interfere with fibrin formation by inhibiting its polymerisation.

Both fibrin deposition in tissues, and the rapid increase in D-dimer plasma levels are hallmark features of Ebola hemorrhagic fever in cynomolgus macaques and humans. Indeed, samples obtained from Ebola virus infected macaques showed the development of fibrin-degradation products (D-dimers) and evidence of fibrin in tissues (spleen, hepatic sinusoids and in blood vessels of the renal medulla) in association with viral antigen. In agreement with this, blood samples from patients infected with the Sudan species of Ebola virus (EBOV), obtained during an outbreak of the disease in Uganda in 2000, were analysed and d-Dimer levels in blood specimens were drastically increased in both patients with fatal and nonfatal infections but were 4 times higher in patients with fatal cases than in patients who survived. (Geisbert, Young et al. 2003, Rollin, Bausch et al. 2007). Importantly, in another study, when Ebola virus infected macaques were treated with rNAPc2, the coagulation response was attenuated, as evidenced by modulation of various important coagulation factors, including plasma D dimers, which were reduced in nearly all treated animals; less prominent fibrin deposits and intravascular thromboemboli were observed in tissues of some animals that succumbed to Ebola virus (Geisbert, Hensley et al. 2003).

3.1.3.3. Inflammatory response and DIC

After pathogen exposure there is increased production of pro-inflammatory cytokines. Cells of the immune system secrete most of these inciting mediators. Cytokines act on many cell types to modulate the host's immune response. Under normal circumstances, cytokines exert their actions locally and their effects are restricted to neighboring cells.

During DIC syndrome, the excessive release of pro-inflammatory cytokines overwhelms the host's defenses and contributes to the morbidity and mortality of sepsis. The most common cytokines include members of the tumor necrosis factor (TNF), interleukin (IL), and interferon (IFN) superfamilies. The primary function of these molecules is to promote inflammation; they do this via signaling pathways that induce expression of inflammatory products. The by-products of this stimulation form a very long list that includes nitric oxide, platelet-activating factor, and eicosanoids. These products have direct effects on the host vasculature and facilitate the transmigration of leukocytes to the site of infection. TNF α and IL-6 are both noted for inducing the expression of procoagulant proteins such as TF.

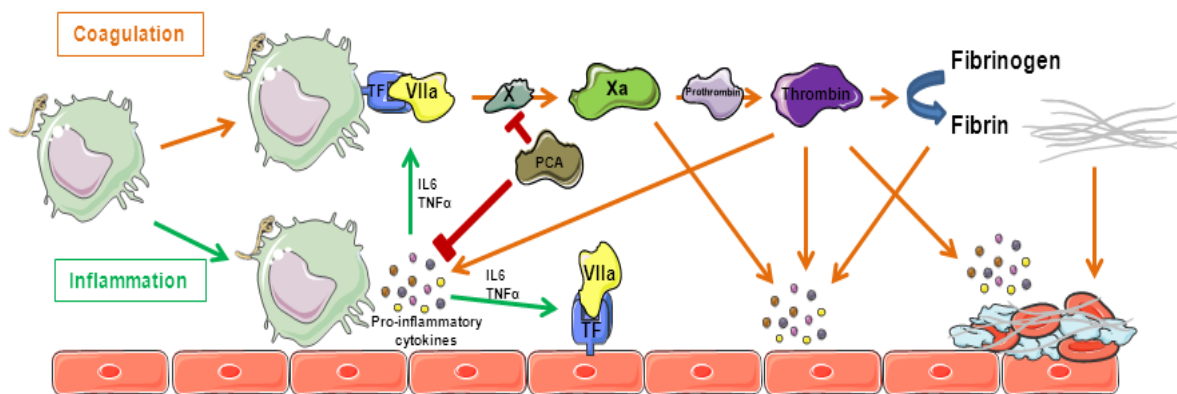


Figure 18.- Cross-talk between the inflammation and coagulation systems.

Infection of monocytes/macrophages will lead to the expression of tissue factor, directly due to infection or indirectly through cytokine release as consequence of Ebola virus infection. The inflammatory process would then induce the coagulation pathway. Several coagulation factors induce the release of proinflammatory cytokines, thus creating a cross-talk between coagulation and inflammation.

3.1.3.4. Cross-talk between inflammation and coagulation

Inflammation and coagulation play an important role during Ebola virus pathogenesis. Both constitute two host defense systems with complementary roles in eliminating invading pathogens, limiting tissue damage, and restoring homeostasis.

There is cross-talk between these two systems, whereby inflammation leads not only to activation of coagulation, but coagulation also considerably affects inflammation. Some of the physiological processes involved in inflammation and coagulation that are regulated by several coagulation factors and cytokines are represented in **Figure 19**.

Coagulation and fibrin deposition can be activated due to inflammation and are essential in containing both the invading entity and the consequent inflammatory response to a limited zone. On the other side, coagulation can also modulate the inflammatory response; coagulation factors including thrombin and anticoagulant proteins, such as activated protein C, activate mononuclear and endothelial cells, which in turn affect cytokine production and inflammatory cell apoptosis. Coagulation activation yields proteases that interact with the coagulation protein zymogens, but also with specific cell receptors to induce signalling pathways that mediate inflammatory responses. Coagulation proteins, such as Xa, thrombin and fibrin, can activate endothelial cells, eliciting the synthesis of pro-inflammatory cytokines and growth factors (van der Poll, de Jonge et al. 2001).

The most important mechanisms by which coagulation proteases influence inflammation is by binding to protease activated receptors (PARs), localized in the vasculature on endothelial cells, mononuclear cells, platelets, fibroblasts and smooth muscle cells (Coughlin 2000). In addition, physiological anticoagulant pathways can influence inflammatory activity. Indeed, activated protein C has been found to inhibit endotoxin-induced production of TNF- α , IL-1b, IL-6 and IL-8 by cultured monocytes/macrophages (Yuksel, Okajima et al. 2002). It has also been shown that activated protein C abrogates inflammatory

activity and improves organ function and survival during sepsis in baboons (Taylor 1994).

It has also been shown that activated platelets play an important role in inflammation. First, platelet adhesion to the subendothelial matrix supports leukocyte rolling, adhesion, and transmigration through interaction of platelet P-selectin with leukocyte P-selectin glycoprotein ligand-1. Platelets can also release various proinflammatory cytokines (such as CD40 ligand and IL-1 β) and chemokines (such as RANTES and platelet factor-4), which may result in further activation of monocyte integrins and thus lead to further monocyte recruitment. These findings indicate that natural anticoagulants can regulate inflammatory responses as well as the coagulation system by inhibiting the production of pro-inflammatory cytokines monocytes/macrophages.

Infection with Ebola virus leads to the production of proinflammatory cytokines that, in turn, stimulate the production of tissue factor. Activation of the coagulation system and ensuing thrombin generation are dependent on the expression of tissue factor; acute inflammation results in DIC, a systemic activation of coagulation. Conversely, activated coagulation affects inflammatory cells and endothelial cells and thereby modulates the inflammatory response.

The basic elements of clotting and inflammation include platelets, leukocytes, monocytes/macrophages, and an array of coagulation enzymes, but the essential cooperation and interactions between clotting and inflammation are essential to preserve both pathways. This reciprocal relationship is well-regulated and tightly controlled under normal health conditions but can lead to significant morbidity and mortality in disease.

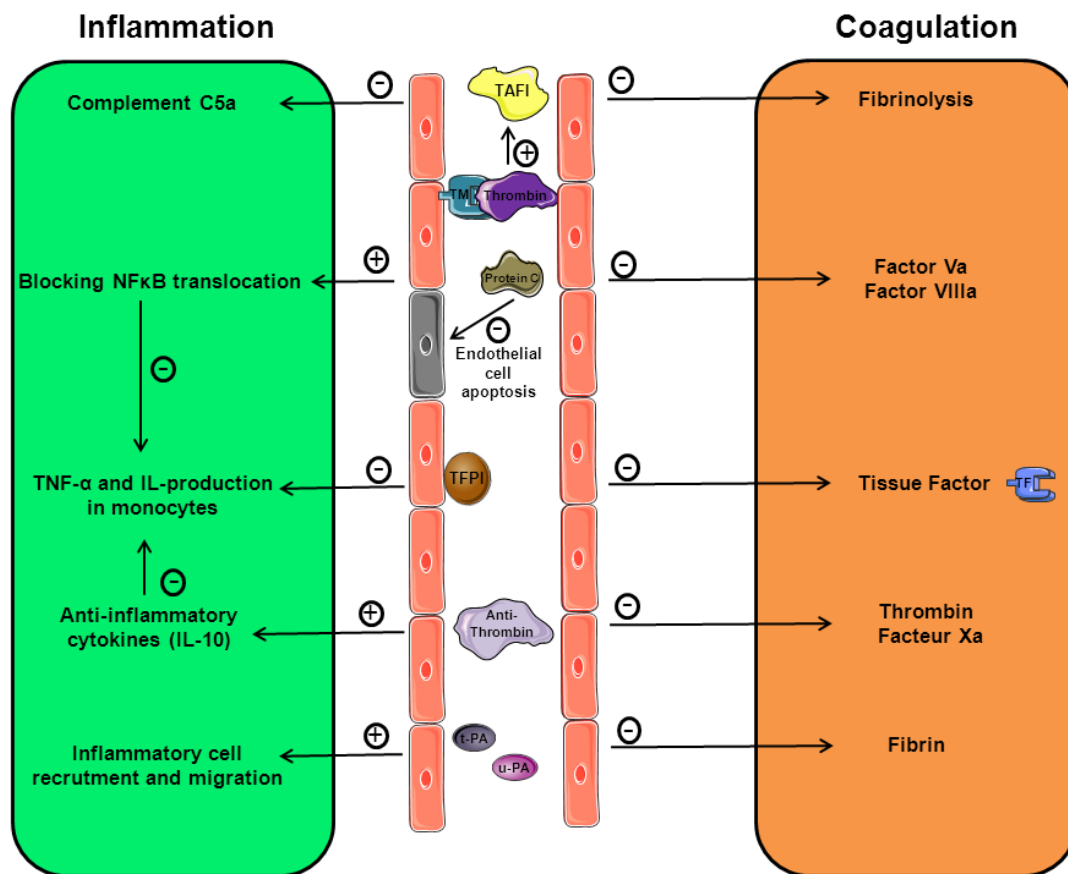


Figure 19.- Effects of physiological anticoagulant systems and fibrinolysis on coagulation and inflammation. Thrombomodulin plays a central role as an endothelial receptor and activator of protein C and TAFI respectively and has direct effects on endothelium. Activated protein C and TAFI have effects on coagulation/fibrinolysis as well as in inflammation. TFPI is the endothelial cell associated inhibitor of tissue factor but also has direct effects on cytokine production by mononuclear cells. Antithrombin binds to glycosaminoglycans, which causes a more efficient inhibition of activated coagulation proteases and also affects inflammation. Release of plasminogen activators (PA) from endothelial cells will induce local fibrinolysis; the complex of u-PA and u-PAR plays an important role in inflammatory cell recruitment and migration. Plus (+) and minus (-) signs indicate stimulatory and inhibitory effects respectively. Adapted from (Williams 2007).

3.1.3.5. DIC diagnosis

The detection of molecular markers for activation of coagulation or fibrin formation may be the most sensitive assays with which to establish a diagnosis of DIC, but unfortunately, the specificity of such tests is low (Horan & Francis, 2001).

Another problem is that a reliable test for quantifying soluble fibrin in plasma is not available and a wide discordance been shown among various assays (McCarron et al, 1999). The dynamics of DIC can also be judged by measuring activation markers that are released upon the conversion of the coagulation factor zymogen to an active protease, such as prothrombin activation fragment (Bauer & Rosenberg, 1987; Boisclair et al, 1990). Indeed, these markers are notably elevated in patients with DIC but again specificity is a problem. Other tests that can be used are: plasma thrombin clotting time, fibrinogen levels, assessment of antithrombin III - thrombin complex, fibrin degradation products (FDP) in serum, etc...(Levi 2004).

3.1.4. Another player in the coagulation pathway: MBL

MBL, also known as mannan/mannose binding protein, is a glycoprotein and belongs to a subfamily of proteins called the collectins because family members possess both collagenous regions and also lectin domains. MBL is synthesized in the liver and secreted into the blood where it has an important role in the innate immune system as an acute-phase reactant (Eisen and Minchinton 2003).

MBL is composed of subunits comprising three identical peptide chains of 32kDa. Each chain is characterised by a lectin or carbohydrate-recognition domain (CRD), a hydrophobic neck region, a collagenous region and a cysteine-rich-N-terminal region. The collagenous regions of three such chains interact to give a classical triple helix. This can then generate disulphide-bridge links at the N-terminal extremity thus forming higher order structures. MBL circulates in the serum mainly as a hexameric molecule (6 trimeric subunits with 18 CRD) (**figure 20**).

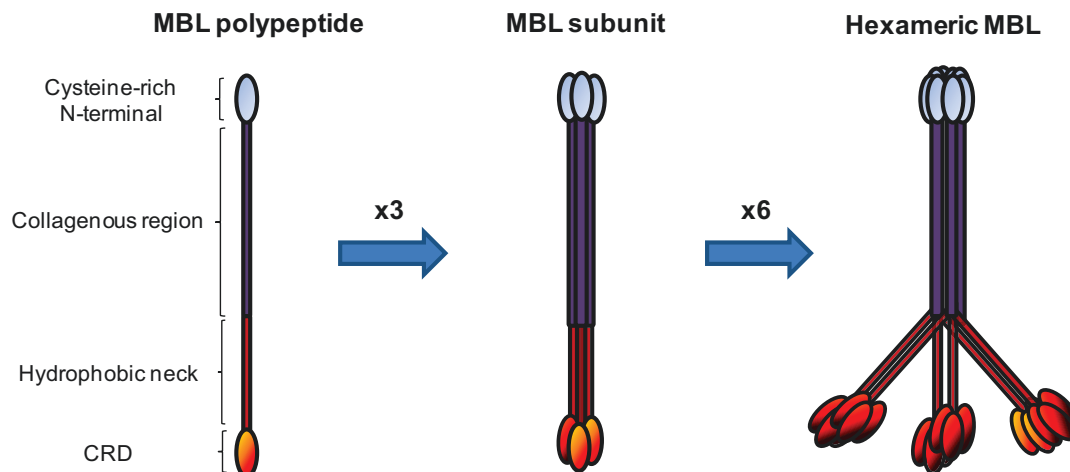


Figure 20.- MBL structure.- MBL is a three polypeptide protein constituted by a carbohydrate-recognition domain (CRD), a hydrophobic neck region, a collagenous region and a cysteine-rich-N-terminal region. MBL can associate several polypeptides to form functional multimeric units.

In the serum, MBL binds to terminal sugar residues via the carbohydrate recognition domain (CRD) (lectin) heads. The binding is calcium-dependent to sugars that have hydroxyl groups on carbon-3 and carbon-4; this gives MBL affinity for mannose, fucose, glucose, N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-mannosamine. This specificity allows MBL to bind to these sugar arrays on the surfaces of microorganisms, including bacteria, viruses and fungi, but not to most human glycoprotein glycans which generally terminate in galactose or sialic acid. The affinity of a single CRD for carbohydrate is very weak (10^{-3} M), however, the simultaneous engagement of multiple CRDs of an MBL molecule with a cluster of glycans can generate a much higher avidity of binding, which is physiologically significant. MBL circulates in the serum at an average concentration of ~ 1.2 - 1.6 $\mu\text{g/ml}$, however, the concentration varies greatly between individuals, from below 50 ng/ml to above 10 $\mu\text{g/ml}$. This variation between individuals is caused by several identified polymorphisms in the coding sequence and promoter regions of the MBL gene. The coding sequence polymorphisms disrupt the collagenous region destabilizing the collagen triple helix formation and preventing oligomerisation and consequently heterozygotes have low levels of MBL in the blood. Low levels of MBL have been linked to severe and recurrent infections (Arnold, Dwek et al. 2006).

MBL has at least four distinct functions: (i) the promotion of (complement-independent) opsonophagocytosis; (ii) the activation of complement; (iii) the modulation of inflammation; and (iv) the promotion of apoptosis (**figure 22**). Firstly, it acts directly as an opsonin, promoting phagocytosis of foreign material to which it has bound (**figure 22(i)**). The second mechanism through which MBL functions is by triggering the lectin pathway of complement activation via MBL associated serine proteases (MASPs). Complement activation leads to opsonisation of the material and cell lysis. MASPs are homologues of the complement C1 serine proteases C1r and C1s. Three MASPs have been identified to date; MASP-1, MASP-2 and MASP-3 and also a small 19 kDa protein lacking a serine protease domain named MASP-4. Upon MBL binding to targets, MASP-2 cleaves the complement proteins C4 and C2, resulting in the formation of a C3 convertase, C4b2a. C3 is cleaved by C4b2a, generating the fragments C3a and C3b (**figure 21**). C3b is further cleaved to form the iC3b opsonin. Activation of C3 also leads to cell lysis through the formation of the membrane attack complex (MAC) (**figure 22(ii)**).

There is evidence from studies of MBL–disease associations that point to the protein having a major role in the modulation of inflammation. Some studies suggest that the MBL protein exerts a complex effect on cytokine release by monocytes (Jack, Read et al. 2001) (**figure 22(iii)**). When *N. meningitidis* organisms are incubated with increasing concentrations of MBL and then added to MBL-deficient whole blood, the release of the cytokines TNF- α , IL-1 β and IL-6 from monocytes was enhanced at MBL concentrations below 4 $\mu\text{g/ml}$ but reduced at higher concentrations. This suggests that both the *MBL* genotype and the presence or otherwise of an on-going acute phase response will help to determine the nature of such cytokine responses in any given individual.

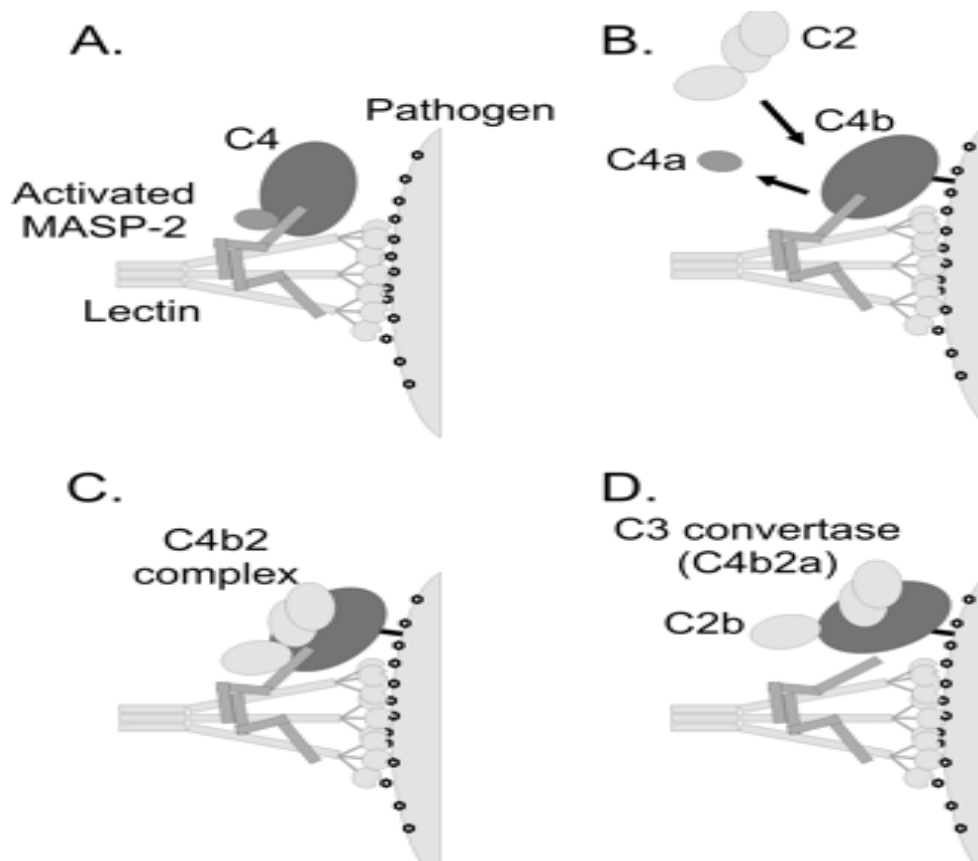


Figure 21.- MBL binding to certain patterns activates MASPs and subsequently activates the lectin complement pathway. (A) MASP-2 fixes and cleaves C4, leading to the formation of C4a and C4b. Following cleavage of C4, C4b still binds to MASP-2. **(B)** Free C2 then binds to C4b and is also cleaved by MASP-2 to produce C2a and C2b. **(C)** C4b and C2b combine on the surface of the pathogen to form C3-convertase **(D)**, while C4a and C2a act as chemoattractants. Adapted from (Wallis, Dodds et al. 2007)

It has also been shown that MBL (like C1q) can bind to apoptotic cells and initiate their uptake by macrophages (Ogden, deCathelineau et al. 2001). This process appears to require cell surface calreticulin (cC1qR), which binds to the collagenous region of the MBL. Although cC1qR has no transmembrane domain, it appears to associate with CD91 (the α_2 -macroglobulin receptor) on the macrophage surface and thereby initiate engulfment of apoptotic cells through macropinocytosis (**figure 22(iv)**).

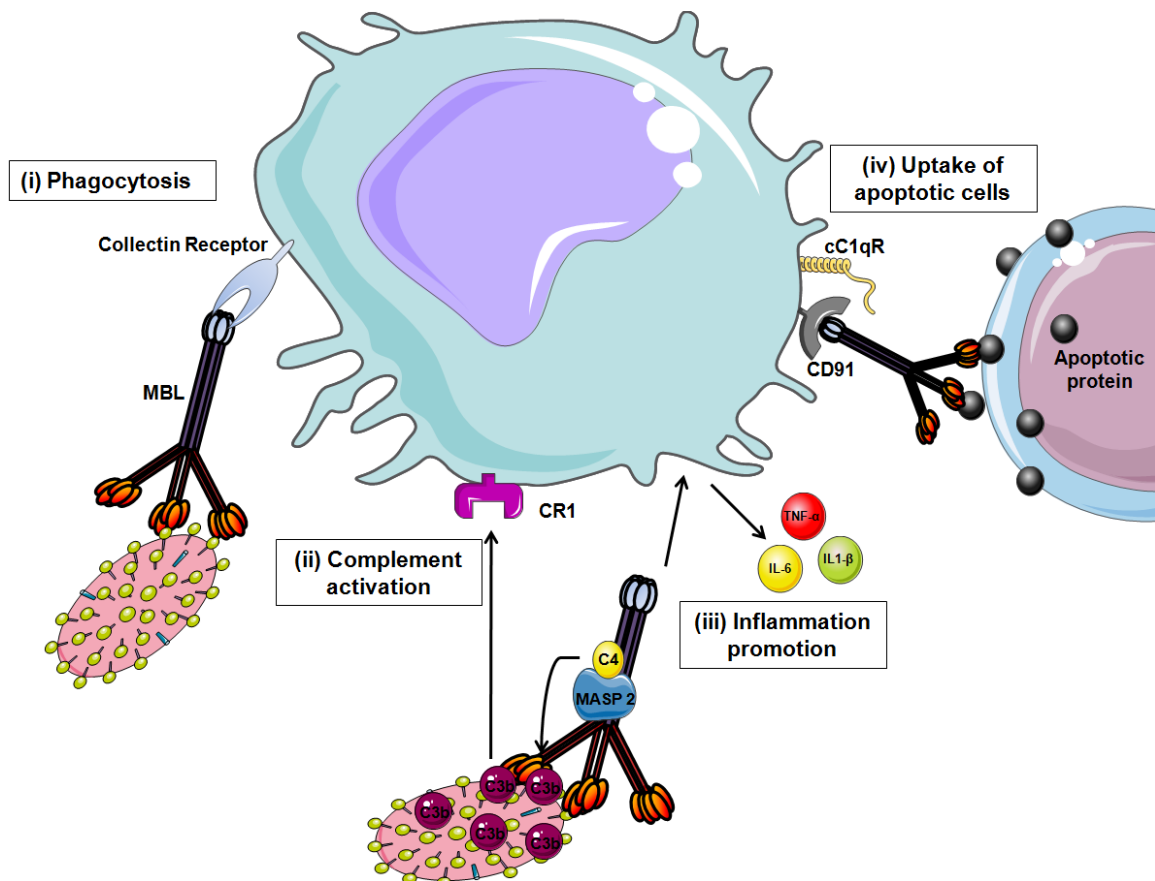


Figure 22.- Role of MBL during infection. MBL, which is found in a soluble form in blood, has several functions during infection: **(i)** promotion of opsonisation/phagocytosis, **(ii)** activation of complement, **(iii)** induction of inflammatory cytokines release and **(iv)** initiation of uptake of apoptotic cells.

The Ebola glycoprotein is heavily glycosylated and contains both N-linked and O-linked carbohydrate structures. N-linked carbohydrates are normally terminated by mannose residues and it has been shown that DC-SIGN, which has a high affinity for high-mannose sites on N-linked glycans, binds to Ebola glycoproteins and that this interaction is inhibited by the high-mannose carbohydrate mannan (Ji, Olinger et al. 2005).

It has previously been shown that recombinant human MBL (rhMBL) binds EBOV GP1,2 lentiviral virion-like particles and wild-type-like EBOV in a specific and dose-dependent manner (Ji, Olinger et al. 2005, Michelow, Dong et al. 2010). Treatment of EBOV infected mice with supraphysiological rhMBL was also shown to have a protective effect (Michelow, Lear et al. 2011). It has also been shown that the CRD domain of MBL is capable of binding Ebola virus via

highly specific interaction to N-linked glycans; cyanovirin-N, a lectin that targets N-linked high mannose, was shown to compete with MBL binding to viruses. Moreover, deglycosylation of N-linked glycans with PNGase F inhibits Ebola virus infection (Brudner, Karpel et al. 2013).

MBL has a complex role in infections caused by Ebola virus. MBL effectively inhibits infection of human cells by Ebola virus GP-pseudotyped lentiviral virions and wild type-like Ebola virus in the presence of active complement in serum (Michelow, Dong et al. 2010, Michelow, Lear et al. 2011). In contrast to this, MBL seems to have a paradoxical function depending on the levels of complement. It has been shown that rhMBL and native MBL enhanced pseudotyped and wild type-like Ebola virus infection of human cells when complement components were reduced or removed. It has been speculated that MBL would concentrate virus at the surface of the target cell (Brudner, Karpel et al. 2013)

According to the currently accepted paradigm, MBL deficiency predisposes children and immunocompromised individuals to diseases whereas normal or high levels of MBL are protective. Therefore, due to MBL single nucleotide polymorphisms (SNPs), different individuals containing different levels of MBL may respond differently to EBOV infection. Taken together, these data suggest that MBL and the lectin complement pathway can influence the course of EBOV infection.

3.2.Direct cytotoxicity caused by the virus itself and GP protein

Humans with fatal infections have up to 10^{10} copies of viral RNA per milliliter, whereas much fewer copies (10^7 copies/ml) are found in the serum of those who survive Ebola virus infection (Zampieri, Sullivan et al. 2007). High rates of viral replication lead to lysis and necrosis in cells of many organs, including the liver, spleen, kidneys and gonads. Much of the observed necrosis is virally induced, as there is little immune infiltration within infected tissues, and extraordinary numbers of viral particles are present in the necrotic debris. In addition, microscopic examination of infected human tissues shows a

correlation between tissue damage and the presence of viral antigens, nucleic acid and sites of viral replication. This observation indicates that direct viral damage of tissues and organs may lead to organ failure and shock. However, despite widespread viral replication, the observed damage to endothelia, liver and other organs is not sufficiently severe to cause terminal shock and death and, as mentioned before, a pathogenic role of the host response contributes to the high pathogenicity of Ebola virus (Zampieri, Sullivan et al. 2007).

Part of the cytotoxicity of Ebola virus can be explained by viral glycoprotein of surface GP. In fact, several studies *in vitro* have shown that Ebola virus surface glycoprotein GP, through its highly glycosylated mucin domain, can induce the rounding and detachment of infected cells (**Figure 23**) (Yang, Duckers et al. 2000).

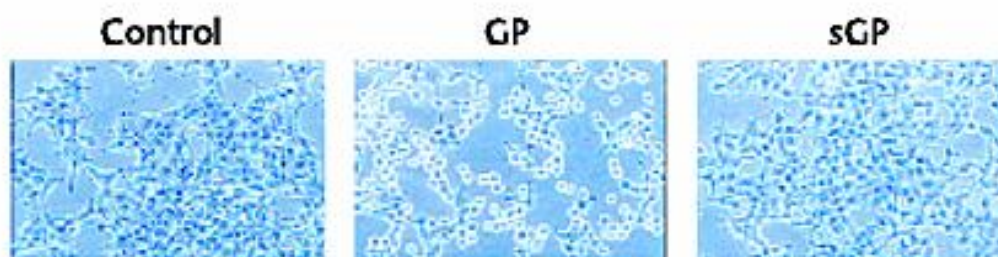


Figure 23.- Induction of cytopathic effects by Ebola virus glycoproteins
Morphology of 293T cells 24 hours after transfection with plasmids expressing Ebola (subtype Zaire) GP, sGP or a 'no-insert' control. (Yang, Duckers et al. 2000)

It was thought that direct damage to endothelial cells by virus replication was the first determinant of vascular injury and permeability. However, as mentioned above, Ebola virus histological analysis of autopsy tissues from several of the early outbreaks did not identify vascular lesions (Zampieri, Sullivan et al. 2007). Similarly, no evidence of substantial vascular lesions in non-human primates infected with Ebola virus exists (Jaax, Davis et al. 1996).

However, several studies have shown that Ebola virus GP down-regulates a range of cell surface markers including several involved in cell adhesion (such as integrin molecules, especially $\beta 1$ - and α_v -integrin subunits, and PECAM-1

and VCAM) and immune surveillance (like MHC-I). In fact, it has been shown that overexpression of mature GP on the plasma membrane results in the masking of cellular surface epitopes (Reynard, Borowiak et al. 2009). The high degree of GP glycosylation (including N- and O-linked sugars) and the fact that surface GP is a trimer, explains this masking phenomena by Ebola virus GP (Figure 24).

The loss of cell adhesion as well as the specific loss of MHC-I and adhesion molecules involved in stabilizing cell-to-cell interactions is likely to negatively impact both the stimulation of an anti-Ebola virus CD8⁺ T-cell response and the ability of T cells to kill infected cells.

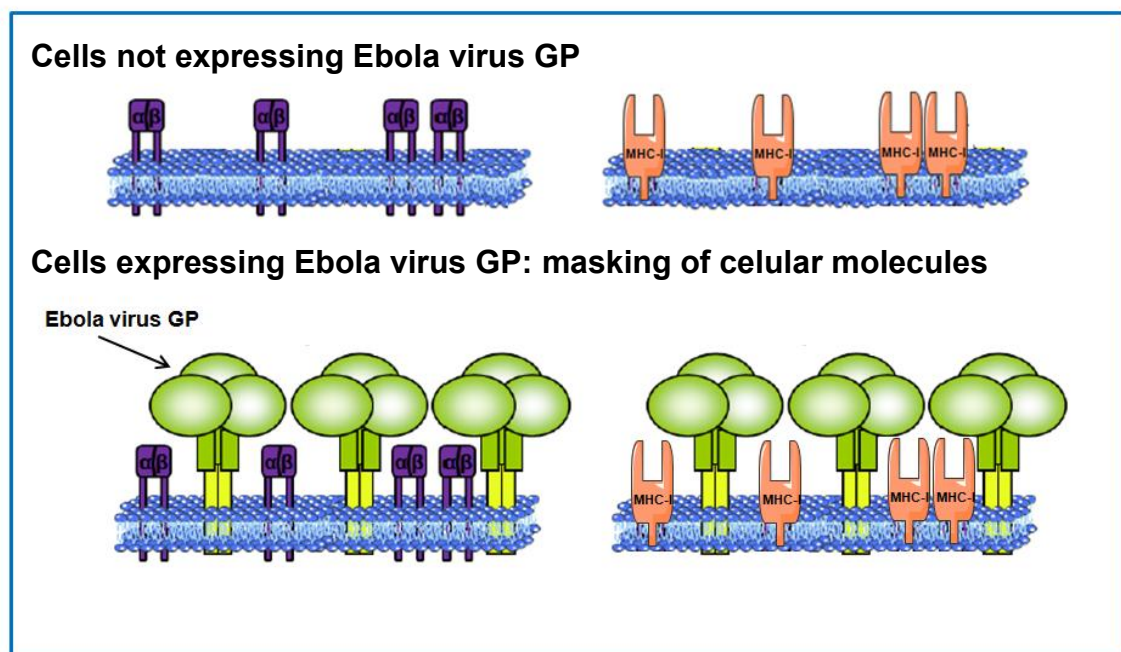


Figure 24.- EBOV GP masks cellular surface proteins. Surface markers such as MHC-I and $\beta 1$ -integrins are masked by Ebola GP expression in these cells due to the highly level of glycosylation of Ebola virus GP.

Integrins also play a critical role in the trafficking of leukocytes through the endothelium to sites of infection. Specifically, the initial tethering and rolling of leukocytes along the endothelium, followed by the arrest and final diapedesis into sites of infection, all require regulated integrin function. Infection of endothelial by cells Ebola virus, however, inhibits this antiviral state from being induced. This inhibition is at least partially due to the Ebola virus protein VP35, which acts as a potent alpha interferon antagonist. However, the down-modulation/masking of PECAM-1 and MHC-I in endothelial cells by GP alone indicates that GP also plays a major role in this process (Simmons, Wool-Lewis et al. 2002). Indeed, the more specific down-modulation of MHC-I in HUVEC, macrophages, several cell lines and endothelial cells may suggest that such host defense molecules are the intended target of the down-regulation.

Shielding of MHC-I and integrins suggests that Ebola virus GP may play an important role in the evasion of antiviral immunity. Masking of surface proteins may result in the inhibition of receptor-ligand interactions and signaling and thus contribute to a functional destabilization of virus-infected cells.

3.3.Overview of Ebola virus pathogenesis

In striking contrast with fatal outcome, effective control of EBOV infection is associated with balanced immune responses. A transient and well-regulated inflammatory response is observed early in the disease not only in patients that survive, but also in asymptomatic subjects. This response may help to control the infection and to induce specific immunity. An early and robust humoral response is strongly correlated with survival in symptomatic patients. In asymptomatic EBOV-infected subjects, antibodies are detected about 3 weeks after infection and in moderate amounts. In survivors and asymptomatic subjects, high antibody titres can persist for years.

The factors underlying the radically different outcomes of EBOV infection are unclear, but may include the route of infection, the size of the inoculum, the cell types that are initially infected, previously acquired immunity, heterologous immunity, and MHC status. However no difference was found in the coding

sequences of viruses isolated from survivors and fatalities and different outcomes can be observed in patients infected with the same viral source (Leroy, Gonzalez et al. 2011).

Ebola virus induced pathophysiology is explained by a combination of factors, including uncontrolled and non-specific inflammatory responses, viral-induced immunosuppression and direct viral destruction of several cell types; collectively contributing to the collapse of the vascular system, multiorgan failure and a septic shock-like syndrome in lethal infection.

OBJECTIVES

Ebola virus, a member of the Filoviridae family, causes lethal hemorrhagic fever in man and non-human primates, displaying up to 90% mortality rates. A unique feature of EBOV is that, following infection, virus glycoproteins are released from cells in soluble forms. High amounts of truncated surface GP, named shed GP, are detected in the blood of patients and experimentally infected animals. Even if shed GP has been discovered more than 10 years ago, besides its role as a decoy factor, no other roles have been yet determined for this soluble glycoprotein. This topic is increasingly relevant in the face of the reoccurring disease threat posed by this continually remerging and highly pathogenic virus.

In this context, the objective of the work done in the scope of this thesis was to produce and to study the role of shed GP during Ebola virus pathogenesis. Overall, we provide new insights into the biological role of shed GP during infection and its contribution to the high pathogenicity of Ebola virus.

Since surface GP is almost not cleaved by TACE when it is transiently transfected, we first generated a system where we were able to refine and optimize production of shed GP in high enough amounts to be able to study it in BSL-2 conditions. We have identified its primary targets to include DCs and macrophages. We have also determined that shed GP has a direct, by itself, and indirect, through cytokine release, effect on cells of the immune system and we have characterised that this activation is, principally but not exclusively, achieved by binding of glycosylated shed GP to TLR4.

We were also interested in determining if monocytes, which remain a paradoxal site of replication for Ebola virus, could be numbered amongst shed GP's early targets. To do this, we have studied if shed GP could bind and allow Ebola virus infection of monocytes.

Moreover, since impairment of coagulation and vascular disruption are both typical phenomena caused by Ebola virus infection, we sought to study the possible role of shed GP in their disruption. Particularly, we demonstrate that EBOV soluble protein shed GP binds to Mannose Binding Lectin (MBL) and

activates the MBL-associated proteases MASP-1 and MASP-2, implicated in the coagulation pathway.

Importantly, we also show that shed GP causes DCs, monocytes, macrophages and HUVECs to increase the expression of tissue factor (TF). Importantly, TF has been shown to be the primary endothelial cell response that results in alterations in both permeability and procoagulant activity. We have also revealed that shed GP activates permeability of HUVECs, both directly and indirectly through cytokine release.

Finally, we have also studied the role of shed GP in affecting infection of cells by the virus itself. We have performed competition experiments during infection to characterise the impact of the presence of shed GP in Ebola virus binding and internalisation.

Taken together the objective of this work was to delimit a new role for EBOV shed GP. Overall data provide a potential explanation as to a major contributing factor of the excessive host responses observed during EBOV infection.

RESULTS

RESULTS I: ROLE OF SHED GP IN INNATE IMMUNE RESPONSE

Production and characterisation of EBOV soluble glycoproteins

As discussed above, EBOV shed GP is structurally identical to virion surface GP except for the lack of its carboxy-terminal part consisting of 13 amino acids upstream of the membrane anchor, the anchor itself and a short cytoplasmic tail. Consequently, it was of interest to investigate whether shed GP and

potentially sGP, that shares 365 amino-terminal amino acids with surface GP, can interact with and affect the function of DCs and macrophages, that are the major primary targets for viral infection in vivo. While transient expression of sGP from plasmid provides sufficient amounts of the protein secreted into the medium, the low efficiency of GP shedding seen with transient expression systems has hampered production of large quantities of shed GP protein equivalent to those observed during EBOV infection. To surmount these difficulties we modified the TACE cleavage site by substituting amino acids at positions D637A and Q638V and thus increased efficiency of GP shedding. This newly generated sequence resembles that previously shown to be recognized by TACE for efficient release of TNF α (Caescu, Jeschke et al. 2009). The plasmid expressing this mutant was designated pHCMVGP-HS and GP expressed from this plasmid as GP-HS (high shedding).

As already mentioned, during EBOV infection significant amounts of soluble glycoproteins, including shed GP and secreted sGP, are released from virus-infected cells. As demonstrated in **Figure R1** the amounts of shed GP present in the blood of animals significantly exceed those used in our study. Quantification of shed GP in the sera of infected animals (500 TCID₅₀/animal of guinea pig-adapted EBOV), based on amounts of truncated GP₂, reveals approximately 85 times higher concentrations of shed GP (34 μ g/ml) than those used in this study.

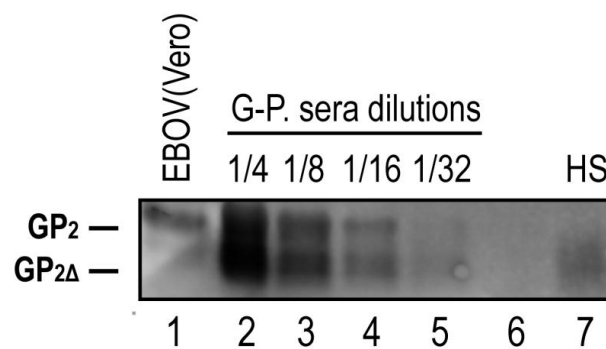


Figure R1. Production of recombinant EBOV shed GP and analysis of shed GP release in the blood of infected guinea pigs. (A) Guinea pigs (strain Hartley) were infected intraperitoneally with 500 TCID₅₀/animal of guinea pig-adapted EBOV (Mateo, Carbone et al. 2011). Serial dilutions of sera collected at day 6 post-infection (5μl, dilutions 1/4, 1/8, 1/16 & 1/32, lanes 2-5), supernatant from EBOV-infected Vero E6 cells (20μl, EBOV Vero, lane 1) and a sample of concentrated recombinant shed GP (5μl, HS, lane 6) were analyzed by Western blot using anti-GP2 antibodies. Estimation of shed GP amounts in the sera of animals in comparison with shed GP from GP-HS expressing cells was performed using ImageQuantTL software (GE Lifesciences). Sera of animals contained approximately 85 times higher amounts of shed GP than those used in experiments with immune cells.

Another difficulty faced in the production of recombinant EBOV glycoproteins results from their strong cytotoxic effects causing cell rounding, loss of cell attachment, and possibly the release of cellular factors caused by eventual cell death. In order to address solely the potential functions of shed GP and to allow for the possible effects caused by cytotoxicity, an EBOV GP mutant lacking shedding properties was used in this study as a negative control. This mutant contains a substitution L635V (GP-LS, low shedding) that was previously shown to prevent TACE cleavage and result in increased surface GP expression (Dolnik, Volchkova et al. 2004). Of note, expression of this mutant results in similar, if not higher, cytotoxicity than that caused by wild-type GP (GP-WT) and provides culture supernatants containing the same potential extracellular factors

released from GP expressing cells whilst displaying undetectable amounts of shed GP.

It is important to stress that the expression and production of shed GP, sGP and also of all control proteins used in this study was performed in cells cultured in FCS free medium (VP-SFM). This prevents putative interaction between soluble EBOV glycoproteins and sera lectins including mannose-binding lectin (MBL), which as described above has previously had been shown to bind surface GP of EBOV (Ji, Olinger et al. 2005). In this manner, 293T cells were transfected with the plasmids expressing mutated GPs, GP-WT, sGP as well as GFP as a non-relevant control. Western blot analysis of cell lysates and culture supernatants revealed a dramatic difference in efficacy of GP shedding between GP-WT and mutated GPs as expected (**Figure R2 A**). Of note, due to the removal of the region spanning the transmembrane anchor, after cleavage by TACE the GP₂ within shed GP shows a lower molecular mass and faster migration on SDS-polyacrylamide gels compared to GP₂ in intracellular GP_{1,2}. While similar amounts of intracellular GP (represented by endoplasmic reticulum GP precursor - preGP_{per}- and mature GP_{1,2}) were seen in cells expressing either GP-WT or GP-LS (compare lines 1 and 3), GP-LS did not show any detectable levels of shed GP in the medium when compared with GP-WT. Notably, relatively low amounts of intracellular GP were detected with GP-HS whilst increased levels of shed GP were detected in the medium. As expected EBOV sGP is efficiently secreted from 293T cells transfected with corresponding plasmid (**Figure R2 B, lane 4**). Using concentrators the volume of culture supernatants was reduced by 10 fold and no differences were detected in total amount of extracellular protein between samples, as also illustrated in **Figure R2 B** by Coomassie staining. The presence of shed GP in concentrated samples from GP-HS expressing cells and its absence in samples from GP-LS expressing cells was confirmed by Western Blot analysis (**Figure R2 B, right panel**).

Using serial dilutions of a commercially available soluble, recombinant baculovirus expressed EBOV GP lacking the transmembrane domain (recGP), we were able to quantify the amount of GP-HS contained in concentrated

supernatants by analysis with western blot and ImageQuantTL software. The standard curve was linear with an R^2 value of 0.99 over the range used for quantification and the GP-HS sample was found to contain 1 $\mu\text{g/ml}$ (**Figure R2 C**) and was used at 0,4 $\mu\text{g/ml}$ in subsequent experiments.

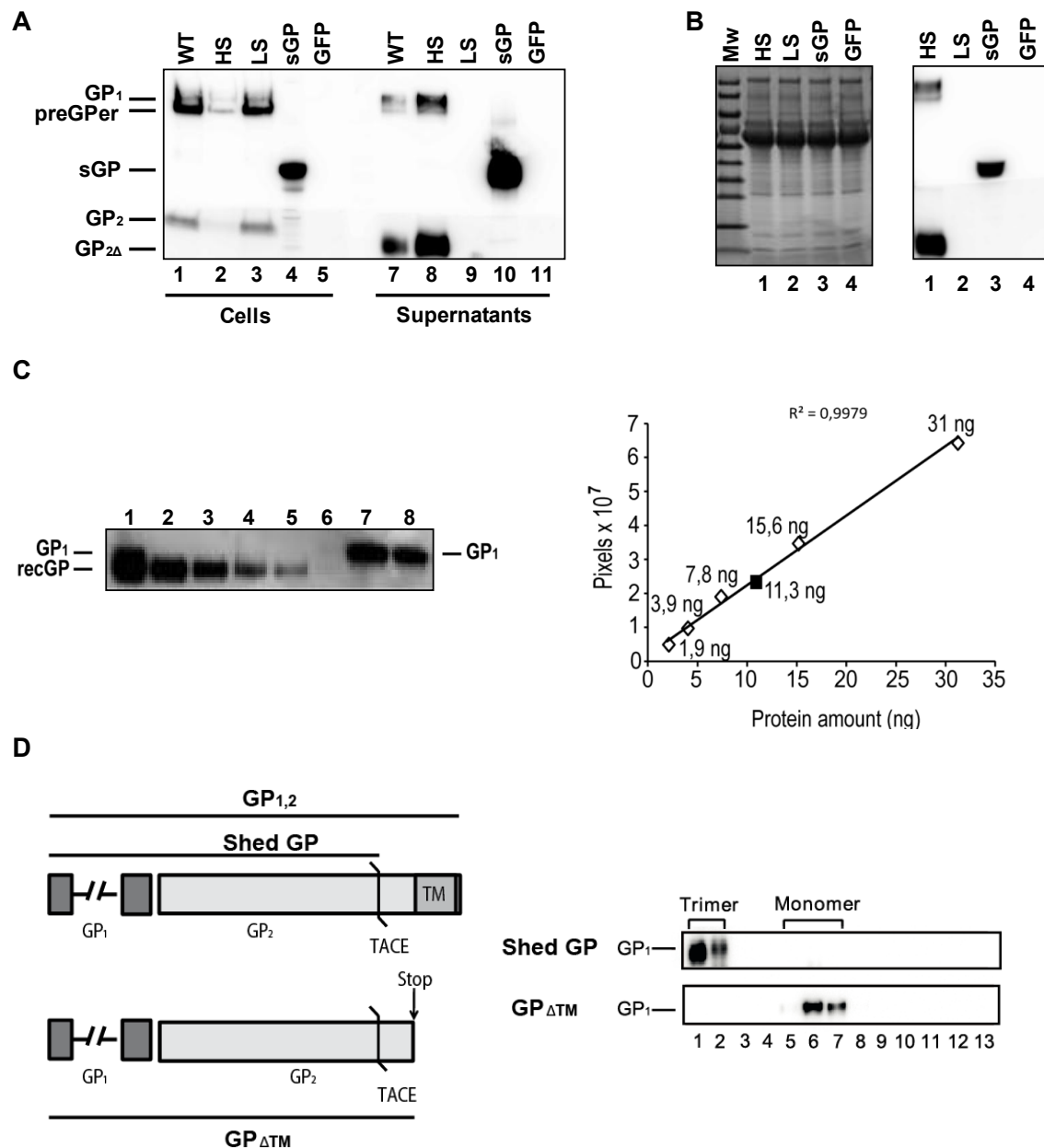


Figure R2. Shed GP production and characterisation. (A) Western Blot analysis of culture medium from 293T cells expressing different recombinant EBOV GP constructs. 293T cells were transfected with wild type GP (WT), shed GP (HS), low shedding GP (LS), sGP and GFP constructs and 36h p.t. cells

and supernatants were collected and analyzed by Western Blot using α -GP₁ and α -GP₂ antibodies. GP₁, endoplasmic reticulum precursor GP (preGPer), sGP, GP₂, and truncated GP₂ Δ are indicated. **(B)** Culture medium supernatants were concentrated and total protein Coomassie staining was performed in order to ensure an equal amount of proteins for all samples (left panel). Samples were also analysed by Western blot with appropriate antibodies (right panel). **(C)** Quantification of shed GP produced in GP-HS- and sGP-expressing 293T cells. Serial dilutions of recombinant EBOV GP as indicated, produced in insect cells (recGP, IBT Bioservices) were used to estimate amounts of HS (dilutions lane 7 and 8) in concentrated culture supernatants (lane 1 with 31 ng; lane 2 with 15,6 ng; lane 3 with 7,8 ng; lane 4 with 3,9 ng and lane 5 with 1,9 ng). Samples were analyzed by Western blot using anti-GP1 antibodies and ImageQuantTL software (GE Lifesciences). As illustrated the standard curve was linear with an R² value > 0.99 over the range used for quantification (open diamonds). Note: the molecular mass of shed GP is ~160kDa in comparison to recGP of ~ 110 kDa. Shed GP sample (closed square) corresponds to ~11.3 ng of recGP and thus contains ~1 μ g/ml in the concentrated supernatants. **(D)** Schematic representation of EBOV glycoproteins (left). Sucrose gradients of 5-25% in Co-IP buffer were performed with supernatants containing shed GP and GP1,2 Δ in order to determine their oligomerisation state. Fractions from 1 to 13 are shown after Western blot in reducing conditions (right).

Of note, our GP construction and production approach differed from that used in an earlier study investigating soluble EBOV glycoproteins. In the previous study the authors had used purified HA-tagged soluble glycoproteins produced through the addition of a stop codon immediately upstream of the transmembrane domain that allows efficient secretion of GP into the extracellular media (Wahl-Jensen, Kurz et al. 2005). However, we have observed that when GP is produced in this way the truncated GP is clearly present in a monomeric form, as evidenced by sedimentation through a sucrose gradient (**Figure R2 D**). In contrast, shed GP that is released through TACE cleavage is present in a trimeric form, as observed during infection (Dolnik, Volchkova et al. 2004) and as seen with our GP-HS construction.

EBOV soluble glycoproteins bind to DC and macrophages

Human monocyte-derived DCs and macrophages (MØ) as well as peripheral blood lymphocytes (PBL) containing T lymphocytes, B lymphocytes and NK cells, were incubated with the samples of culture medium containing the soluble EBOV GPs described above. To assess the ability of shed GP to bind to DCs and macrophages (MØ) flow cytometry was performed using anti-GP specific antibodies that recognize both sGP and shed GP. DCs were shown to bind both shed GP and sGP while macrophages could only bind shed GP (**Figure R3 A**). As expected, incubation of the cells with GP-LS failed to evidence any GP specific binding in agreement with the undetectable levels of shed GP in samples. It appears that neither B (**Figure R3 A**) nor T lymphocytes or NK cells (data not shown) bind shed GP or sGP; this is in agreement with earlier data revealing an absence of surface GP binding to these cells (Marzi, Moller et al. 2007, Dube, Schornberg et al. 2010).

In light of data revealing an interaction between surface GP and MBL, we also investigated whether human sera containing MBL is capable of affecting the binding of shed GP to cells. As shown in Figure 2A, pre-incubation of shed GP with such sera completely blocked shed GP binding to both DCs and MØ. Of note, shed GP produced in 293T cells cultured with 5% FCS also failed to show binding to immune cells (data not shown) supporting the importance of using sera-free medium for shed GP production.

Next, it was of interest to investigate if shed GP could interact with TLR4, which is known to recognize highly glycosylated molecules containing O-linked mannosyl residues and has previously been demonstrated to interact with surface GP of EBOV (Okumura, Pitha et al. 2010). To address this question DCs and MØ were pre-treated with mouse anti-TLR4 antibodies (HTA125) and then incubated with shed GP. As a control, cells were first incubated with mouse isotype control antibodies and then with shed GP. Binding of shed GP to both DCs and MØ was dramatically reduced upon treatment of the cells with anti-TLR4 antibodies whereas no effect on GP binding was observed when control antibodies were used (**Figure R3 B**). Of note, it is known that MBL can also bind TLR4 (Shimizu, Nishitani et al. 2009, Wang, Chen et al. 2011, Ma,

Kang et al. 2013). In this regard the MBL-mediated reduction in shed GP binding to the cells could be explained either by the interference of MBL in shed GP binding to TLR4 and/or by sequestering shed GP via direct interaction with MBL. In an attempt to clarify this question we performed experiments where cells were pre-treated with sera containing MBL with subsequent washing and incubation with shed GP. As demonstrated in **Figure R3 C**, shed GP binding to the cells was also blocked under these conditions, in this case most likely via interaction of TLR4 with MBL. In agreement, pre-incubation of cells with MBL deficient sera did not affect shed GP binding.

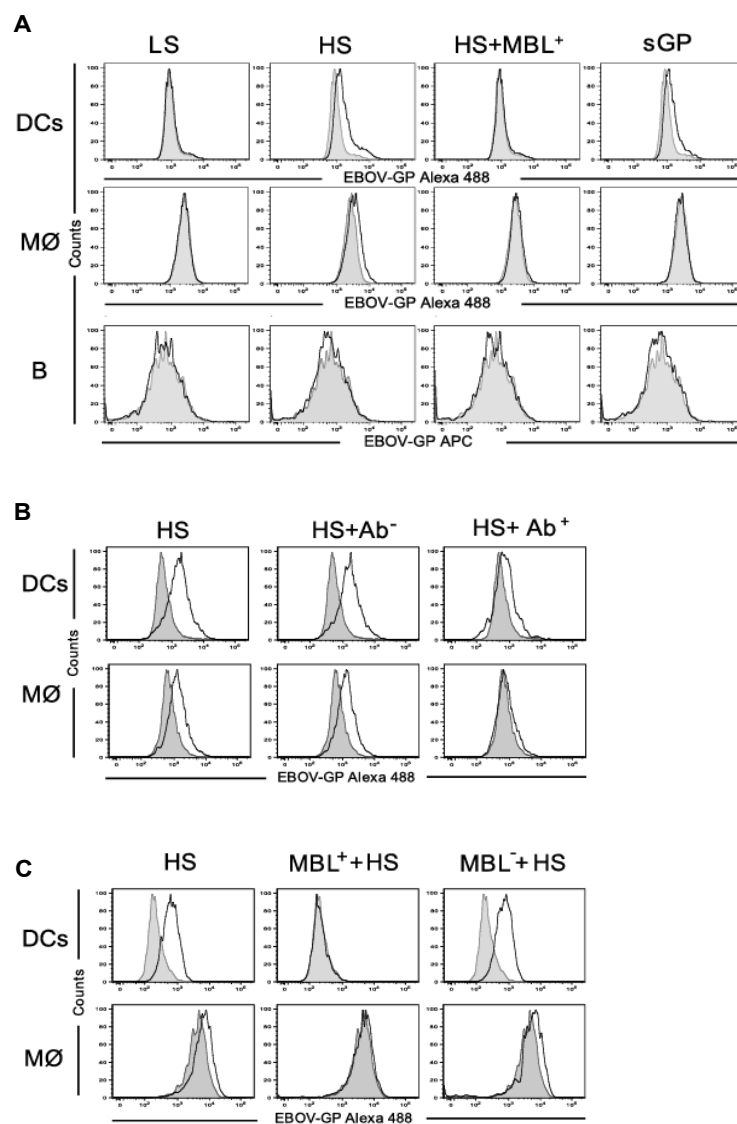


Figure R3. DCs and MØ are primary targets of shed GP. (A) Human monocyte-derived dendritic cells (DCs), monocyte-derived macrophages (MØ)

and PBL containing T lymphocytes (T), B lymphocytes (B) and NK cells were incubated with supernatants containing soluble proteins (HS-GP and sGP) or supernatants containing low shedding (LS) or HS-GP+MBL as negative controls. Bound protein was detected by subsequent incubation with mouse α -GP1 antibody and α -mouse Alexa 488 in DCs and macrophages and α -mouse APC in lymphocytes. The fraction of B lymphocytes was stained using CD20-FITC antibodies. Fluorescence was analyzed by flow cytometry. **(B)** DCs and macrophages (MØ) were pre-treated with anti-TLR4 antibody (Ab⁺), isotypic control antibodies (Ab⁻) or MBL as control, prior to shed GP treatment and binding to cells was analyzed by flow cytometry. **(C)** DC and macrophages were pre-incubated with serum containing MBL (MBL⁺+HS), MBL deficient sera (MBL⁻+HS) or culture media alone (HS) before washing and incubation with HS-GP. Binding was again detected by flow cytometry as above. All flow cytometry data are representative of three independent experiments.

EBOV shed GP induces upregulation of cytokine transcription in both DCs and MØ

To address whether shed GP binding to immune cells results in the activation of these cells, monocyte-derived DCs and MØ were incubated with samples of culture supernatants containing the soluble EBOV glycoproteins detailed above and expression of TNF α , IL6, IL10 and IL12p40 mRNA was measured by real-time PCR at 4, 8, 12 and 24h post-treatment. In controls, cells were treated either with culture supernatants from cells expressing GFP (Mock) or LPS. Culture supernatant from cells expressing GP-LS was used as an additional negative control. Prior to experiments, all culture supernatants were tested for the absence of endotoxin and showed endotoxin concentrations below 0,25 units/ml and were thus considered to be endotoxin-negative.

In a sharp contrast to all negative controls used in this experiment, treatment of DCs and MØ with either shed GP or LPS results in transcriptional activation of a number of analysed genes (**Figure R4 A and B**). Upregulation of TNF α and IL6 occurred as early as 4h post treatment whereas IL10 and IL12p40 were

activated only 8h and 12h post treatment, respectively. The increase in mRNA levels appears to be higher in MØ than in DCs and when compared to LPS, cell activation caused by shed GP appears to be more durable. Treatment of the cells with sGP did not reveal any significant effects on cytokine mRNA synthesis. In agreement with the above experiments concerning shed GP binding, pre-treatment of both DCs and MØ with anti-TLR4 antibody significantly reduced shed GP-induced activation of TNF α mRNA synthesis (**Figure R4 C**). A similar but even stronger neutralizing effect of anti-TLR4 antibody was observed in the case of LPS treatment, in agreement with previous publications demonstrating that LPS acts on cells via TLR4 (Beutler 2002, Beutler 2002, Salomao, Martins et al. 2008, Wang, Chen et al. 2011). Furthermore as shown in **Figure R4 D**, pre-treatment of shed GP with human sera containing MBL results in the abrogation of shed GP-dependent TNF α activation in a dose dependent manner. Accordingly, no such blocking effect was observed after pre-treatment of shed GP with MBL-deficient sera. As expected, both human sera used did not themselves activate DCs or MØ.

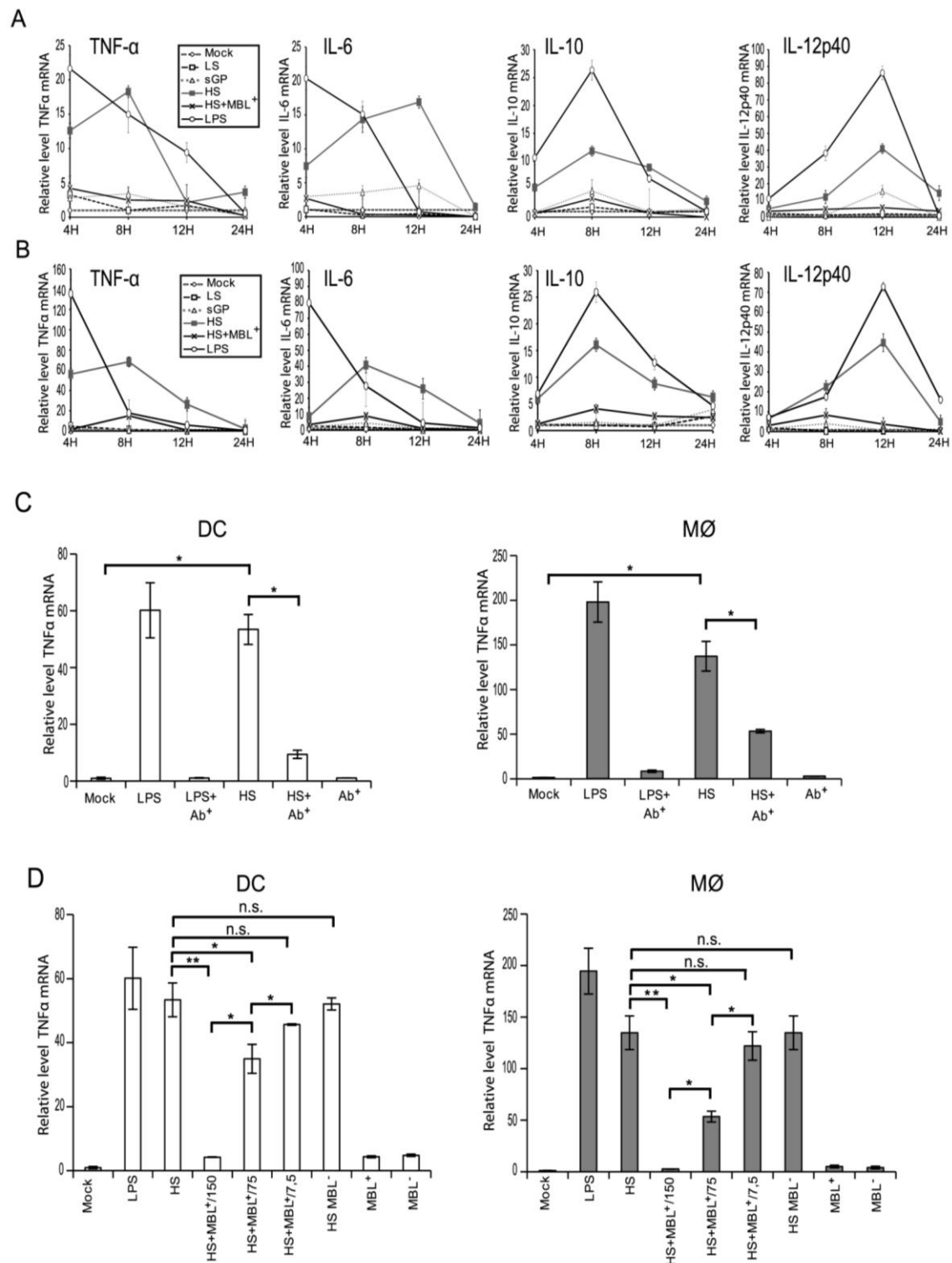


Figure R4. Shed GP induces transcriptional activation of cytokines in DCs and MØ. DCs and MØ (5×10^5 cells) were incubated with concentrated culture supernatants as above (figure1R C,D) and with LPS. Cells were collected at 4, 8, 12 and 24h post-incubation as indicated. Expression levels of mRNA encoding TNF- α , IL-6, IL-10 IL-12p40, and GAPDH were measured by RT-Q-

PCR in DCs **(A)** and MØ **(B)**. Relative levels of the cytokines were obtained by normalization to GAPDH and Mock and shown as : open circles, broken lines (Mock); closed squares, broken lines (LS); closed triangles, broken lines (sGP); closed squares, solid lines (HS); crossed squares, closed lines (HS+MBL⁺ for shed GP pre-incubated with MBL-containing sera) and closed circles, dark lines (LPS). Data are representative of three independent experiments. **(C)** As above, DCs and MØ were treated with concentrated culture supernatants for 8h as follows : culture medium alone (Mock), LPS, shed GP (HS) or the cells were pre-incubated with anti-TLR4 antibody (Ab⁺) and then treated with shed GP or LPS. Expression levels of mRNAs encoding TNF α and GAPDH were analysed by RT-Q-PCR as above. Relative levels of TNF α mRNA were obtained by normalization to GAPDH and Mock. The data are representative of three independent experiments and presented as mean \pm sd of triplicates. **(D)** DCs and MØ were treated with concentrated culture supernatants as above. In addition HS culture supernatants were pre-incubated with different concentrations of MBL-containing sera as indicated (150 ng/ml, 75 ng/ml and 7,5 ng/ml) before addition to DCs or MØ. An MBL-deficient serum (MBL-) was used as a negative control. Cells were collected 8 hours post treatment and analysed by RT-Q-PCR as above. Human sera did not itself activate DCs or MØ. The data are representative of three independent experiments and presented as mean \pm sd of triplicates. Statistical significance (paired-sample *t* test) compared to GP-HS is shown as follows: * - $p < 0.05$ and ** - $p < 0.01$; n.s. – not significant.

EBOV shed GP induces release of pro- and anti-inflammatory cytokines from DCs and MØ

Given the transcriptional activation observed above it follows that upon stimulation DCs and MØ would release a range of cytokines into the medium. Accordingly, culture supernatants of DCs and MØ collected 24h after addition of shed GP (as detailed above) were assayed using a Multiplex cytometric bead array against a panel of cytokines previously shown to be upregulated during EBOV infection (Stroher, West et al. 2001, Baize, Leroy et al. 2002). As

expected, shed GP induced the secretion of TNF α , IL6, IL10, IL12, IL8, IL1 β and IL1RA in both DCs and M \emptyset (**Figure R5 A**). Comparable levels of these cytokines were also observed in the medium when cells were treated with LPS. All negative controls used in this study, including samples of medium from GP-LS-expressing cells did not show any significant release of the cytokines tested. Incubation of the cells with the sGP-containing sample also did not result in detectable cytokine secretion. Expectedly, pre-treatment of shed GP-containing samples with MBL before their use resulted in a complete block in cytokine release that correlates well with the absence of activation of cells seen in Figure 3A and B. In accordance with the results presented above, pre-treatment of cells with an anti-TLR4 antibody considerably reduced the release of TNF α , IL6, IL10, IL12, IL8, IL1 β and IL1RA (**Figure R5 B**). The neutralizing effect of anti-TLR4 antibody was again stronger where cells were treated by LPS.

As activation of DCs and M \emptyset is usually associated with an increase in expression of phenotypic activation markers such as the co-stimulatory molecules CD86, CD80, CD83 and CD40, we investigated whether the binding of shed GP or sGP to these immune cells could induce the expression of these surface molecules. As demonstrated in **figure R5 C and D**, exposure to shed GP but not to sGP induced the expression of co-stimulatory molecules on both DCs and M \emptyset . Similar expression of these surface markers was observed upon treatment of the cells with LPS. Samples of culture medium from GP-LS expressing cells used here as a negative control did not reveal any effect on immune cells. Pre-treatment of cells with MBL prevents cell activation as demonstrated by the absence of any increase in co-stimulatory molecule expression.

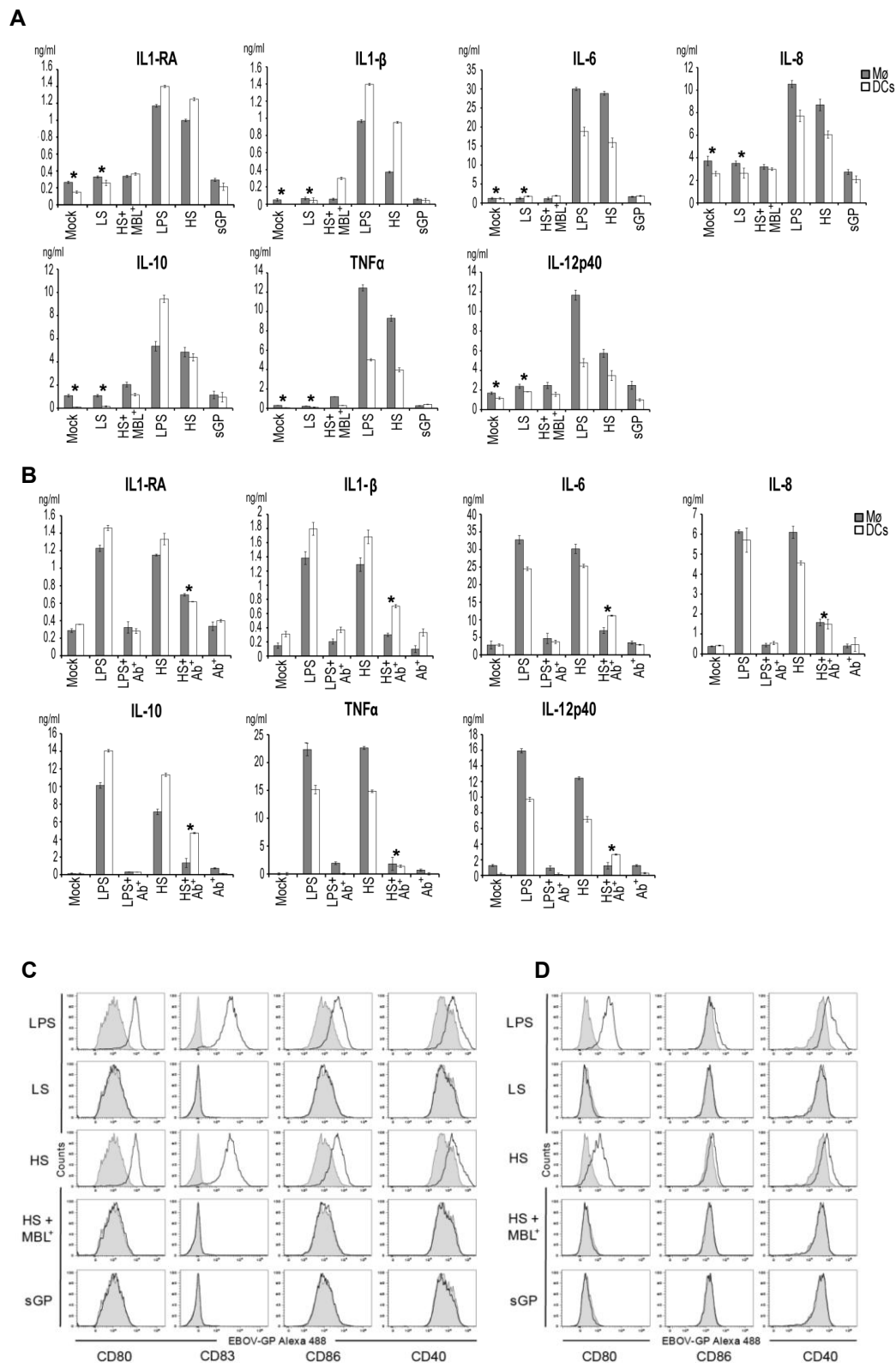


Figure R5. Shed GP induces secretion of pro- and anti-inflammatory cytokines from DCs and MØ and induces their phenotypic maturation. (A)

MØ (in grey) and DC (in white) were incubated for 24h with concentrated culture supernatants as in figure 3. The concentration of each cytokine in duplicate was measured using Multiplex cytometric bead array by Luminex MAGPIX. The data are representative of three independent experiments and presented as mean \pm sd of triplicates. Statistically significant differences (paired-sample *t* test) compared to GP-HS are shown as follows: * - $p < 0.05$. **(B)** MØ (in grey) and DCs (in white) were incubated for 24h with concentrated culture supernatants as above and, in addition, the cells were pre-incubated with an anti-TLR4 antibody (Ab+) followed by treatment with HS or LPS. The concentration of each cytokine in duplicate was again measured using Multiplex cytometric bead array by Luminex MAGPIX. The data are representative of three independent experiments and presented as mean \pm sd of triplicates. Statistically significant differences (paired-sample *t* test) compared to GP-HS are shown as follows: * - $p < 0.05$. **(C and D)** DCs **(C)** and MØ **(D)** were incubated with concentrated culture supernatants as above. The cells were harvested at 48h post-incubation and expression of CD80, CD86, CD40 and CD83 was analyzed by flow cytometry. Filled histograms represent staining with an appropriate isotype-matched control antibody. Data are representative of three independent experiments.

Glycosylation of shed GP is vital to activate DCs and MØ

Since anti-TLR4 antibodies were capable of blocking shed GP binding and also to prevent immune cell activation we speculated that the glycosylation pattern seen with GP might serve as a pathogen associated molecular pattern (PAMP) recognized by TLR4. In order to verify this idea, shed GP was treated under non-denaturing conditions with a mix of N- and O- glycosidases and then used for incubation with immune cells, as detailed above. As shown in Figure 5A treatment with deglycosylation enzymes reduced the molecular weight of shed GP as expected, in comparison to shed GP treated with the same mix of deglycosidases that were previously inactivated by heating for 30min at 80°C **(Figure R6 A, compare lanes 1 and 2)**. DCs and MØ were incubated with shed GP samples or LPS alone for 24h and then culture supernatants were analysed

by ELISA for amounts of secreted TNF α . As an additional control cells were subjected to incubation with LPS following incubation with the deglycosylated shed GP/deglycosydases mixture. Strikingly, as shown in **Figure R6 B**, deglycosylation of shed GP considerably decreases release of TNF α in both DCs and M ϕ , confirming the importance of the glycosylation pattern of shed GP for its activity. Cells incubated with deglycosylated shed GP remained viable and capable of responding to LPS stimuli ruling out a possible negative effect of deglycosydases on cells.

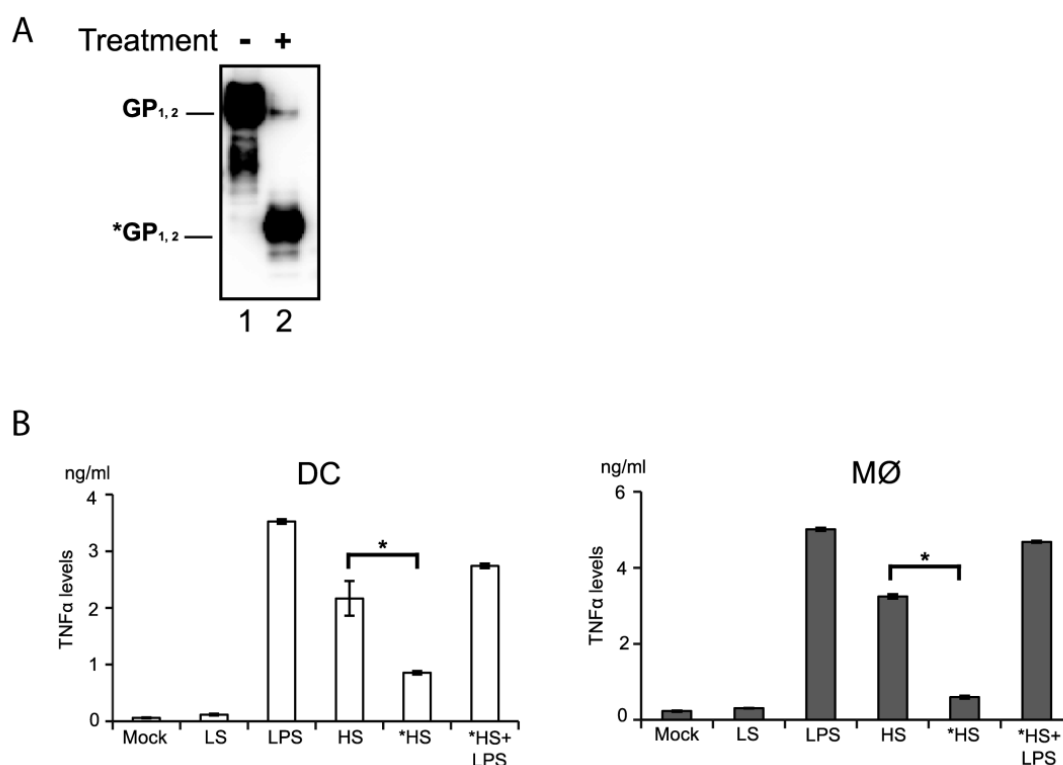


Figure R6. Deglycosylation of shed GP affects activation of DCs and macrophages. **(A)** Concentrated culture supernatants of 293T cells expressing GP-HS (HS) were treated either with inactivated deglycosylation enzymes or a mix of deglycosylases (*HS). Samples were analyzed by Western blot under non-reducing conditions, line 1 and 2, respectively and shown. Deglycosylated EBOV GP is indicated as *GP_{1,2} Δ . **(B)** DCs (in white) and M ϕ (in grey) were incubated for 24h with concentrated culture supernatants of 293T cells expressing GP-HS (HS), GP-LS (LS) after treatment with inactivated deglycosylation enzymes, LPS as a positive control and HS after treatment with

a mix of deglycosylation enzymes (*HS). In addition the cells were incubated with *HS following LPS treatment (*HS+LPS). The concentration of TNF α in duplicate was measured using Multiplex cytometric bead array in Luminex MAGPIX. The data are representative of three independent experiments and presented as mean \pm sd of triplicates. Statistically significant differences (paired-sample *t* test) compared to GP-HS are shown as follows: * - $p < 0.05$.

In summary, in this section we have identified cell targets for shed GP, namely DCs and macrophages and we have seen that upon binding of shed GP these cells can become activated leading to the release of a panel of pro-inflammatory cytokines and phenotypic maturation of these immune cells. We also show the principal receptor involved is TLR4 and that it is the glycosylation pattern of shed GP that is important for its activity. This activity can also be blocked partially by the use of anti-TLR4 antibodies or completely by treatment with MBL-containing sera.

**RESULTS II:
EBOLA VIRUS SHED
GP ALLOWS
MONOCYTE
INFECTION BY
EBOV**

Shed GP binds to monocytes

Monocytes are the most abundant APCs in the blood; however, data concerning EBOV infection of these cells is quite controversial. Interestingly, it has recently been shown that EBOV can bind monocytes and that this, in fact, can induce cellular differentiation that will promote Ebola virus entry into monocytes (Martinez, Johnson et al. 2013). We sought to determine if shed GP could induce monocyte differentiation and permissiveness to EBOV infection.

Human monocytes were incubated with culture medium containing shed GP (HS), low shedding GP (LS), sGP and shed GP with MBL containing serum (HS+MBL⁺) and flow cytometry detection was performed in order to determine if shed GP and sGP were capable to bind human monocytes. Shed GP was shown to bind monocytes whereas sGP did not show any significant binding (**Figure R7**). As expected, supernatant containing LS did not show any specific binding. Incubation of shed GP with MBL prior to binding assay completely blocked shed GP interaction with monocytes as had been observed previously for DC and MØ.

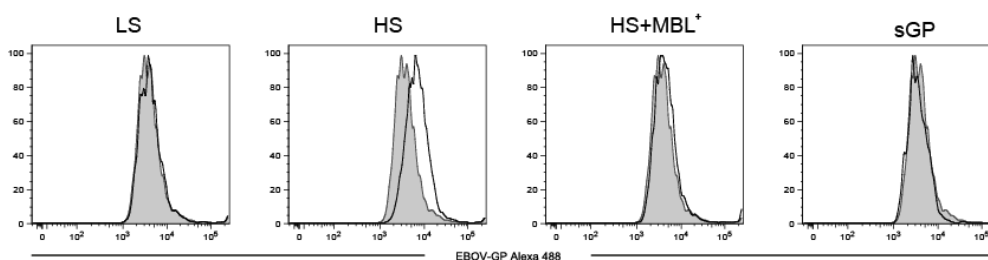


Figure R7.- Ebola virus shed GP binds to human monocytes. Human monocytes were incubated with isotype control antibodies (in grey) and supernatants containing soluble glycoproteins (HS and sGP) and supernatants containing low shedding (LS) or shed GP with sera containing MBL (HS+MBL⁺)

as negative controls (in white). Bound protein was detected by α -GP antibody and α -mouse Alexa 488. Fluorescence was analyzed by flow cytometry.

Shed GP does not activate human monocytes

Since we observed that shed GP was capable to bind human monocytes we wanted then to determine whether shed GP would induce upregulation of cytokine mRNA transcription and release. Monocytes were stimulated for 24 hours with LPS, supernatants containing soluble glycoproteins shed GP (HS), sGP and negative controls containing GFP supernatant (mock), low shedding (LS) and shed GP previously incubated with MBL (shed GP+MBL⁺) (**Figure R8**) and release of cytokines was analyzed by Multiplex ELISA. Surprisingly, no release of TNF- α , IL-6, IL-10 or IL12p40 was observed. Positive control LPS did induce TNF- α , IL-6, IL-10 and IL12p40 release into the medium by human monocytes.

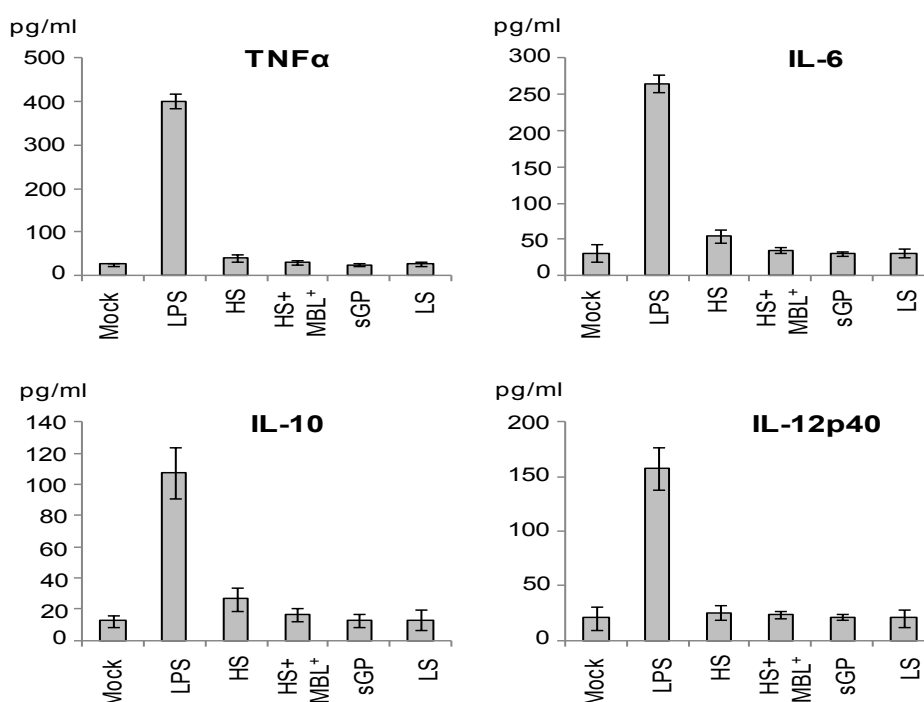


Figure R8.- Ebola virus shed GP does not induce mRNA synthesis and release of cytokines from human monocytes. Monocytes were treated for 24 hours with LPS (as positive control), soluble glycoproteins (HS and sGP) and low shedding (LS) and shed GP with MBL (HS+MBL⁺). Concentration of cytokines in the medium was measured by multiplex ELISA.

Shed GP turns monocytes into permissive cells

Human peripheral blood monocytes were incubated for 48 hours with mock supernatant (medium from Vero cells) and ultracentrifuged supernatants from recombinant EBOV with mutated TACE cleavage site: EBOV-HS, EBOV-LS and EBOV-WT which express respectively high, low and wild-type amounts of shed GP (**Figure R9 A**). As shown, comparison between supernatants from wild-type virus (WT) and the high shedding mutant 637 (HS) clearly reveals a difference in the ratios of GP observed as: (i) virions (upper band, GP2) which is higher in WT and (ii) shed GP (lower band, GP2Δ), higher in HS. After ultracentrifugation to remove virions, only shed GP is visible with Western blot.

Following incubation, cells were infected with recombinant Ebola virus expressing GFP (rEBOV-GFP) at an MOI of 0,5. After 48 hours, an obvious difference in infection of monocytes was observed. Cells treated with HS supernatant (EBOV-HS) did become permissive, whereas supernatants containing a negligible (and undetectable) quantity of shed GP (EBOV-LS) did not render monocytes permissive to infection (**Figure R9 B**). Interestingly, EBOV-WT supernatant made cells more permissive to EBOV infection when compared to LS-EBOV. Cells treated with mock supernatant presented an overlapping profile showing that rEBOV-GFP by itself did not render monocytes permissive to infection.

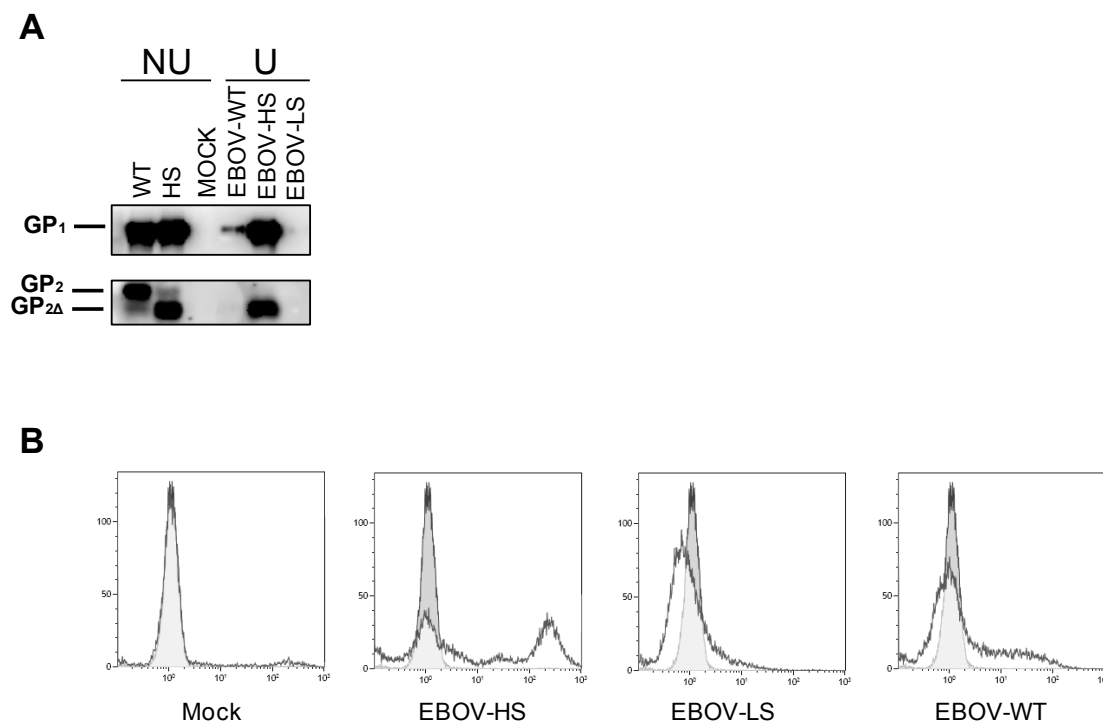


Figure R9.- Ebola virus replication in human monocytes after shed GP treatment. (A) Vero cells were infected with nothing (Mock), EBOV-HS, EBOV-LS and EBOV-WT and supernatants were collected. Non-ultracentrifuged supernatants (NU) are shown as wild-type (WT) and high shedding (HS). Ultracentrifuged (U) supernatants (EBOV-HS, EBOV-LS and EBOV-WT) contain majoritarily shed GP (GP_{2Δ}). **(B)** Monocytes were pre-treated for 48 hours with supernatants (in white) containing a high (EBOV-HS), low (EBOV-LS) or wild type (EBOV-WT) quantity of shed GP or mock (medium). After 48 hours, monocytes were infected with recombinant Ebola virus expressing GFP (rEBOV-GFP) at an MOI of 0,5. A mock infection served as a control (in grey). Infection was determined by flow cytometry.

Overall, we demonstrate that shed GP can bind to monocytes but that this binding does not lead to cytokine release over the first 24 hours of incubation. However we show that shed GP can render these cells permissive to EBOV infection with longer incubation time and posterior infection.

**RESULTS III:
ROLE OF SHED GP
IN COAGULATION
AND VASCULAR
PERMEABILITY**

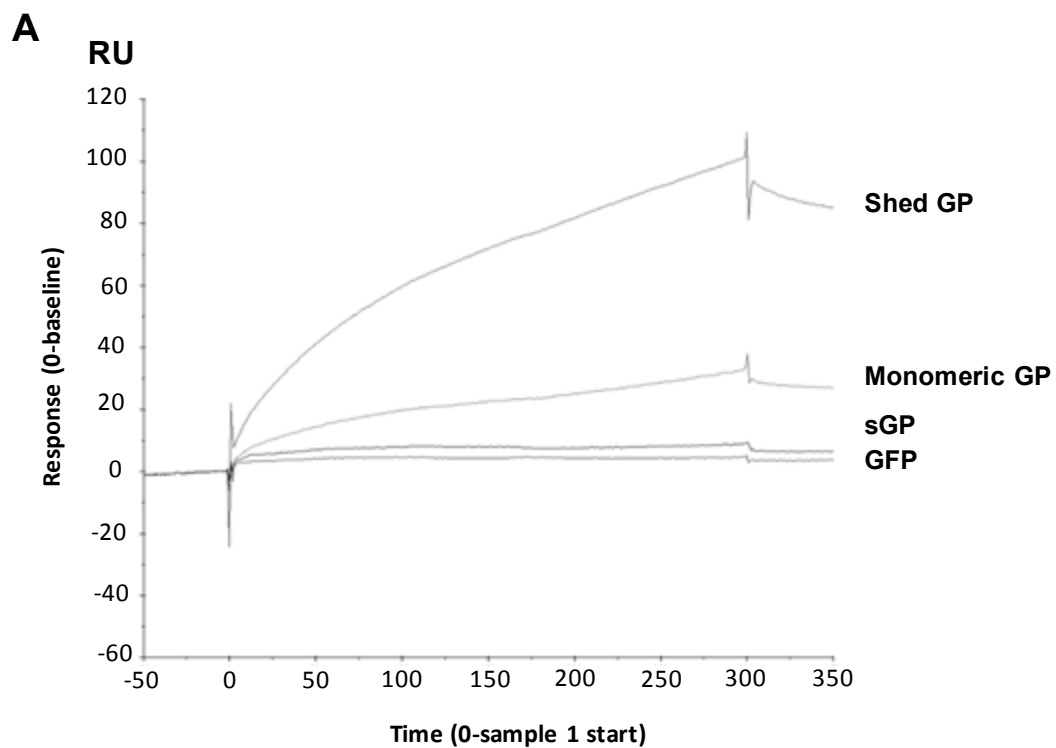
Soluble EBOV glycoproteins bind to rMBL

C-type lectins bind high mannose residues of N-linked glycans in a calcium-dependent manner (Michelow, Dong et al. 2010). Previous studies have shown that particles pseudotyped with Ebola and Marburg glycoproteins bound significantly to MBL (Ji, Olinger et al. 2005).

To determine whether soluble glycoproteins interact with MBL, we performed surface plasmon resonance (SPR) using the Biacore T100 system. We immobilized recombinant MBL (rMBL) on a CM5 sensorchip and tested binding of soluble glycoproteins in the presence of CaCl_2 . The data obtained showed that soluble glycoprotein shed GP specifically interacts with MBL. Only trimeric shed GP bound strongly to immobilized rMBL. Since the monomeric form is similar to trimeric shed GP it may have conserved interaction sites to MBL but differences in oligomerisation showed lower affinity to MBL (**Figure R10 A**). Analytes containing sGP, considerably less glycosylated than shed GP, and mock supernatant (from GFP expressing cells) did not show any binding to MBL ligand.

In order to confirm this data, we performed an ELISA (**Figure R10 B**). Maxisorp plates previously coated with MBL were incubated with soluble glycoproteins from transiently transfected cells (HS, $\text{GP}_{\Delta\text{TM}}$, LS, sGP and mock -from GFP expressing cells-) and UV inactivated VSV-GP and VSV-G as positive and negative control respectively. As expected, VSV-GP was shown to strongly bind to rMBL. Shed GP also bound to MBL in a dose-dependent manner and, as observed with surface plasmon resonance and dot blot, $\text{GP}_{\Delta\text{TM}}$ binding levels to rMBL were lower when compared to shed GP. Negative controls LS, sGP, mock, inactivated VSV-G and MBL with no supernatant were not shown to unspecifically bind to MBL.

Figure 6



B

Protein fixation
to MBL (OD)

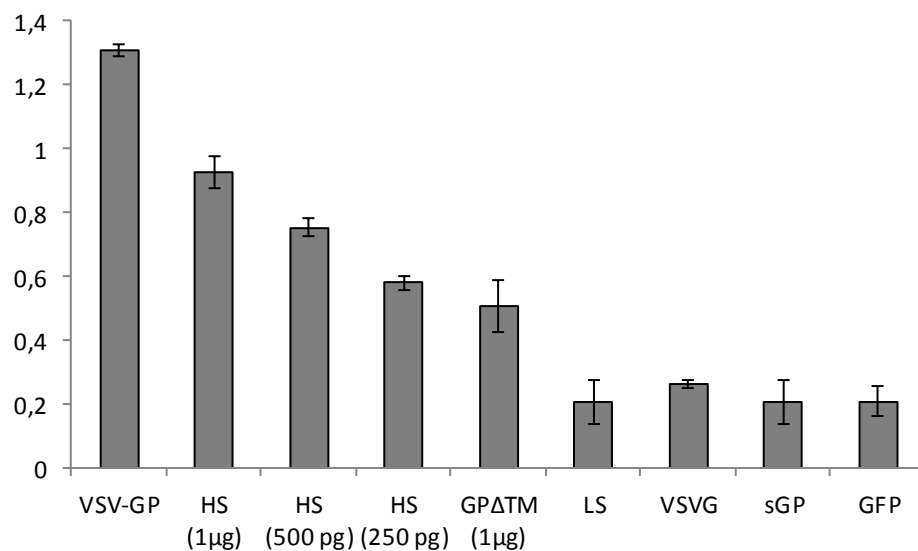


Figure R10.- EBOV glycoproteins interact with rMBL. (A) SPR sensorgram overlays was performed for the binding of different soluble glycoproteins to surface-immobilized rMBL. Shed GP curve indicates strong binding to rMBL.

whereas the monomeric form of shed GP showed a weaker interaction. Analytes containing sGP and mock supernatant didn't show specific binding to MBL. **(B)** Purified rMBL protein was coated on maxisorp plates before incubation with soluble glycoproteins and supernatants containing VSV-GP and VSV-G as controls. Decreasing amounts of shed GP were added (1 µg, 500 pg and 250 pg per well). After blocking with milk and washing, plates were revealed with antibodies against EBOV GP1 as in a classical ELISA.

Shed GP Activates MASP1

After confirming MBL binding to shed GP, we wanted to determine if this binding was capable of activating MBL associated protein MASP-1. To explore the activity of MASP-1, which is known to have a thrombin-like substrate specificity, we performed a fluorescence-based assay by using the thrombin substrate VPR-AMC, a short peptide conjugated to 7-amino-4-methyl coumarin (AMC). This probe becomes fluorescent when MASP-1 associates to MBL and becomes active, thereby cleaving the substrate to release the probe in a fluorescent form.

Mannan and VLPs expressing GP (VLP-GP), as positive controls, and soluble HS, LS and sGP glycoproteins were bound to α-EBOV GP specific antibody-coated maxisorp plates. Next, MBL containing serum was added and VPR-AMC cleavage was monitored at 60 (white), 80 (grey) and 100 (black) minutes. We could observe that MBL fixation and activation of MASP-1 in wells containing HS reached the same levels of fluorescence as that observed with the positive control VLP-GP (**Figure R11 A**). Mannan, which was also used as a positive control for MBL activation, showed the highest activation of AMC cleavage. Furthermore, thrombin-like activity demonstrated time dependency, confirming that the activity is not only MBL-dependent but also depends on enzymatic activation of MASP-1. Wells containing sGP and low shedding (LS) did not show significant activation of AMC cleavage and gave a basal level of fluorescence that did not increase over time.

Similar results were observed when the assay was repeated using supernatants from recombinant EBOV, as described in figure R9. Briefly, maxisorp plates coated with α -EBOV GP specific antibody were incubated with supernatants from recombinant Ebola virus containing mutations affecting release of shed GP in such a way that culture medium contains high (EBOV-HS), low (EBOV-LS) or medium (EBOV-WT) levels of shed GP. As in the previous experiment, HS-EBOV supernatant activates the cleavage of AMC probe to a higher extent than seen with EBOV-LS or EBOV-WT (**Figure R11 B**). MBL alone did not show significant AMC cleavage.

The interactions of soluble glycoproteins with MBL were also investigated in competition experiments (**Figure R11 C**). Maxisorp plates were coated with either EBOV-GP VLPs or mannan. VLP-GP and supernatants from transiently transfected cells containing HS, LS and sGP were incubated with MBL and then added to coated plates. In order to determine if MBL binding to VLPs and mannan coated in plates was affected in the presence of glycoproteins, AMC cleavage was measured as previously described. In the case where MBL can bind to soluble glycoprotein present in the supernatant it will no longer be able to bind to VLPs or mannan coated in the plate, thus reducing any fluorescent signal as a measure of MASP activation. As expected, in EBOV-GP VLP-coated plates, MBL pre-incubated with supernatant containing HS and EBOV-GP VLPs, showed weaker cleavage of AMC probe compared to MBL with no supernatant (as control), low shedding (LS) or sGP containing supernatants (**Figure R11 C, left**). It is thus likely that, as predicted, MBL pre-incubated with shed GP (HS) and VLPs binds to GP and is thus unable to bind to VLPs coated in the plate. MBL in non-coated wells showed a basal level of AMC cleavage (Mock). The same result was obtained upon analysis of mannan-coated plates. Again MBL binding was influenced by its prior incubation with shed GP (HS) or EBOV-GP VLPs, ratifying interactions of shed GP with MBL (**Figure R11 C, right**).

As mentioned before, MBL deficiency results from genetic defects caused by single nucleotide polymorphisms (SNPs) that occur in 5–30% of the human population. Combinations of these SNPs result in reduced blood concentrations

of and/or dysfunctional MBL proteins (Takahashi 2011). As a result, human MBL blood levels vary significantly in individuals. We first wanted to quantify individual MBL variability in sera. Standards (represented by black circles from s1-s6) were made with serial dilutions of MBL containing serum with the following MBL concentrations s1: 2µg/well; s2: 1.3 µg/well; s3: 0.88 µg/well; s4: 0.59 µg/well; s5: 0.395 µg/well and s6: 0.26 µg/well. Sera from 20 healthy adult individuals were collected and assayed for the levels of MBL by ELISA (**Figure R11 D**). As expected, different individuals showed a wide variation of MBL concentrations with a mean value of 1.2 µg/ml (ranging from 400 ng/ml to 2 µg/ml), which corresponds well with the median values of MBL generally observed in humans (median of 1.43 µg/ml) (Babovic-Vuksanovic, Snow et al. 1999).

We next tested whether there was a correlation between MBL content in sera and MASP-1 activity when it binds to shed GP (**Figure R11 E**). Briefly, plates were coated with shed GP (HS) and incubated with sera from 20 donors. In this manner MBL in sera can then bind to immobilized shed GP. Notably, the degree of binding to shed GP reflects the serum concentration and a positive correlation was observed for the activation of MBL in different serum samples ($R^2=0.8401$, grey diamonds). As expected, MBL concentrations modulated the efficiency of MASP-1 activation; sera from individuals with higher blood MBL levels activated MASP-1 more effectively than those with lower MBL levels. Thus, individual sensibility to MASP-1 activation is MBL-dose dependent. Sera containing less than 5ng of MBL showed the lowest MASP-1 activation (white circle).

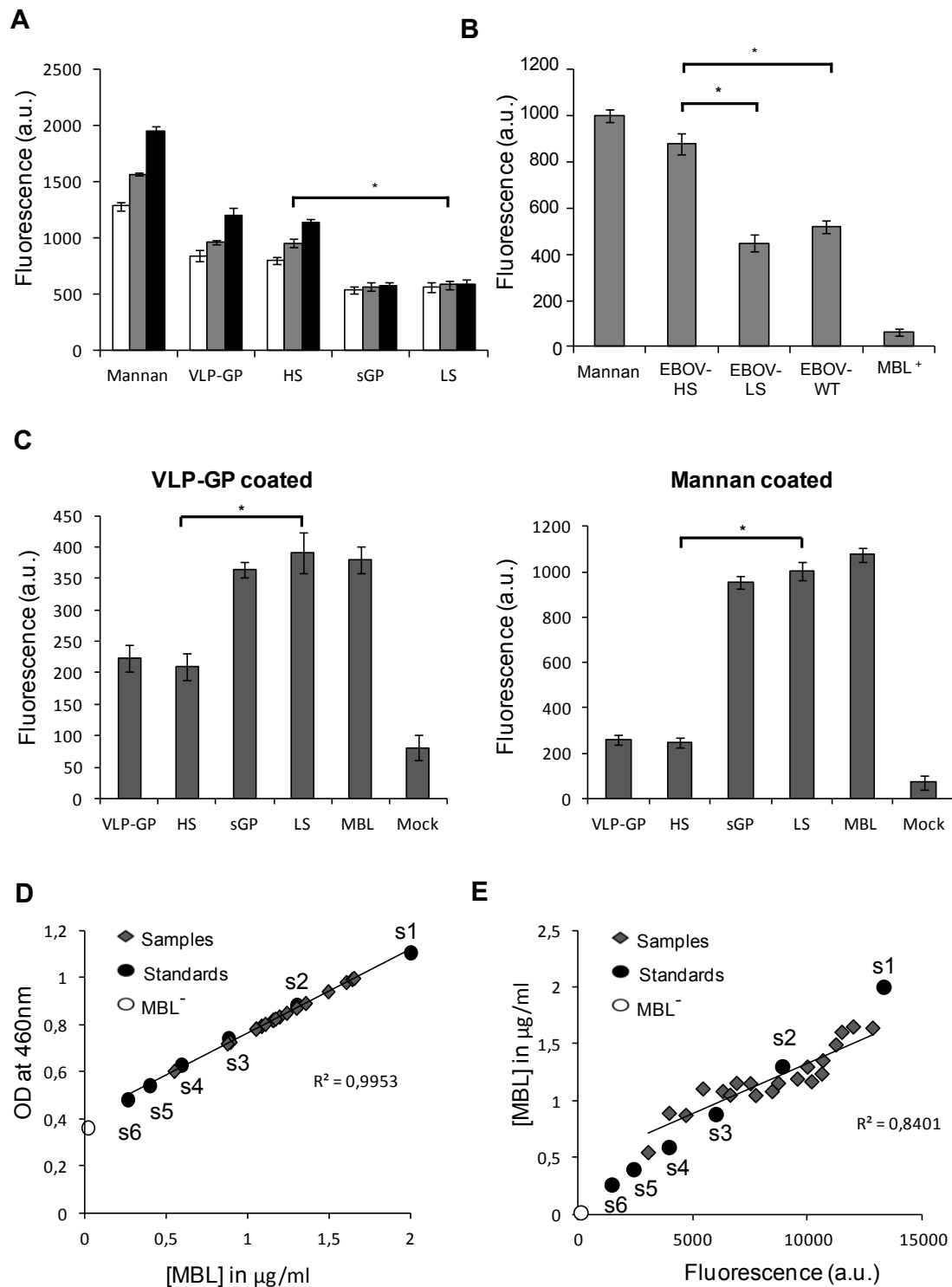


Figure R11. Shed GP induces VPR-AMC cleavage through MASP-1. A) VPR-AMC cleavage was quantified after addition of mannan, VLPs pseudotyped with EBOV surface GP and supernatants containing HS, LS and sGP. VPR-AMC cleavage was measured after 60 (white), 80 (grey) and 100 (black) minutes. The mean of triplicates from one representative experiment

from three independent experiments is shown. Statistically significant differences (paired-sample *t* test) compared to GP-HS are shown as follows: * - $p < 0.05$. **B)** VPR-AMC cleavage induced by supernatants from recombinant Ebola virus was measured. Supernatant from EBOV-HS (supernatant with high shed GP in medium) infected cells activated probe cleavage to higher levels than that seen with EBOV-LS (supernatant with low shed GP in medium) and EBOV-WT (supernatant with wild type shed GP in medium). The mean \pm sd of triplicates from one representative experiment from three independent experiments is shown. Statistically significant differences (paired-sample *t* test) compared to EBOV-HS are shown as follows: * - $p < 0.05$. **C)** MBL competition assay was performed in VLP-GP or mannan coated plates. Supernatants from cells transiently transfected with HS, LS and sGP plasmids or VLPs-GP were incubated with MBL prior to incubation in pre-coated plates and VP-AMC cleavage was measured. In both experiments VPR-AMC cleavage was monitored by emission at 460 nm and is shown as arbitrary units. The mean of triplicates from one representative experiment from three independent experiments is shown. Statistically significant differences (paired-sample *t* test) compared to GP-HS are shown as follows: * - $p < 0.05$. **(D)** MBL content of 20 healthy donors (grey diamonds) was quantified by ELISA using with serial dilutions of MBL containing serum (black circles) with the following MBL concentrations s1: 2 $\mu\text{g}/\text{well}$; s2: 1.3 $\mu\text{g}/\text{well}$; s3: 0.88 $\mu\text{g}/\text{well}$; s4: 0.59 $\mu\text{g}/\text{well}$; s5: 0.395 $\mu\text{g}/\text{well}$ and s6: 0.26 $\mu\text{g}/\text{well}$ and control sera with no MBL (less than 5ng, white circle) **(E)** Correlation between MBL content in human sera, as shown in figure 10D, and MASP-1 activation through shed GP was quantified by ELISA and fluorescence respectively. Standards were the same as above. A positive correlation was present between serum MBL levels and activation of MASP-1 through binding to shed GP ($R^2=0,84$). In all experiments fluorescence is shown as arbitrary units (a.u.). Data are means of three duplicates measurements from three independent experiments.

Shed GP activates C4 fixation and cleavage through MASP-2

Since the above experiments indicated that soluble glycoprotein shed GP can bind to MBL and activate MASP-1, we wanted to determine if this binding could also activate MASP-2, which is directly implicated in C4 cleavage during the lectin complement pathway.

Supernatants from the recombinant viruses described above (EBOV-HS, EBOV-LS and EBOV-WT) were coated in maxisorp plates and C4 fixation was quantified by ELISA after addition of MBL (**Figure R12 A**). EBOV-HS supernatant, which contains high amount of shed GP compared to LS and WT variants, fixed significantly more C4 than EBOV-LS and EBOV-WT. Accordingly EBOV-WT, that contains more shed GP than EBOV-LS fixed more C4 compared to EBOV-LS. Negative control containing no supernatant but only MBL showed the lowest C4 fixation. All samples were normalised to mock controls (supernatant from non-infected cells).

In order to confirm these results, we performed a competition assay as in **Figure R11 B**. Briefly, mannan, VLPs-GP and supernatants from cells transiently transfected with HS, LS and sGP were incubated with MBL and the mixture was subsequently added to plates previously coated with VLP-GP. Finally, C4 was added and its fixation and cleavage was measured by ELISA (**Figure R12 B**). As expected, MBL previously incubated with HS, EBOV-GP VLPs and mannan showed weaker fixation of C4 compared to incubation with LS, sGP or nothing (MBL alone, as positive control). These data show that MBL which is already bound to shed GP, EBOV-GP or mannan, does not allow MBL fixation to EBOV-GP VLPs coated in the plate and thus prevents fixation and cleavage of C4. Thus, lectin complement pathway activation could be achieved through activation of MASP-2 by shed GP.

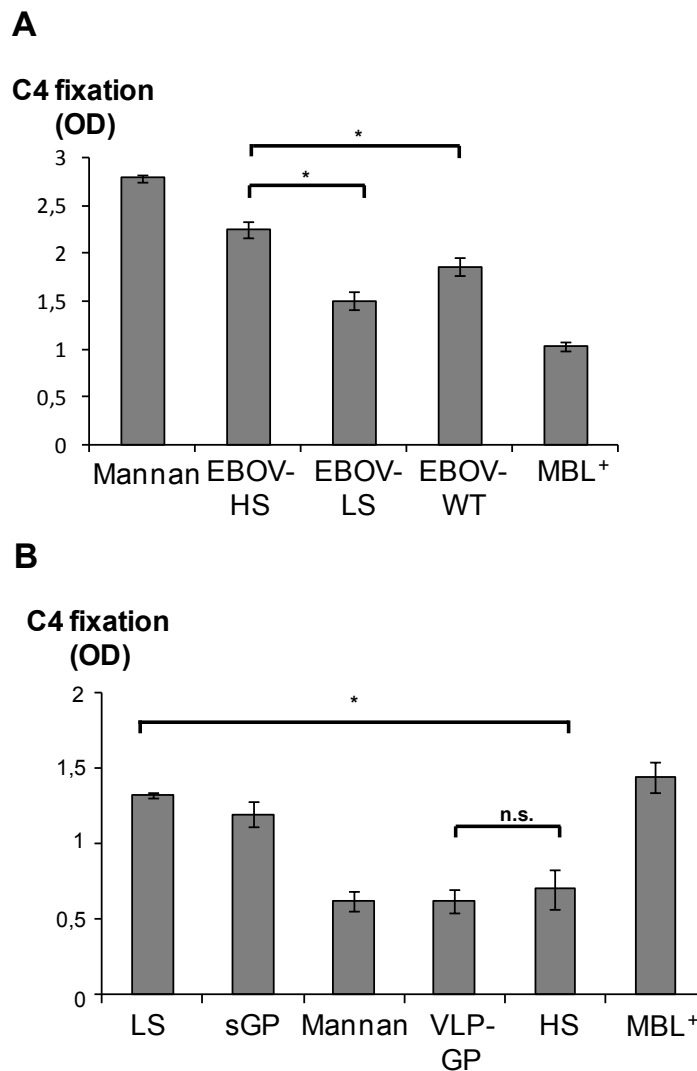


Figure R12.- C4 fixation and cleavage by shed GP through MBL. Human serum containing MBL was incubated with **(A)** supernatants from recombinant Ebola virus and C4 fixation was measured by ELISA. The mean \pm sd of triplicates from one representative experiment from three independent experiments is shown. Statistically significant differences (paired-sample *t* test) compared to EBOV-HS are shown as follows: * - $p < 0.05$. **(B)** Competition assay between MBL and EBOV soluble glycoproteins. MBL was incubated with mannan, VLP-GP and supernatants from cells expressing HS, LS and sGP; this was then added to VLP-GP coated plate and C4 fixation and MBL cleavage by MASP-2 was detected by ELISA and is shown as arbitrary units. The mean \pm sd of triplicates from one representative experiment from three independent experiments is shown. Statistically significant differences (paired-sample *t* test) compared to GP-HS are shown as follows: * - $p < 0.05$.

Shed GP induces expression of tissue factor in monocytes, DCs, macrophages and HUVEC

TF is the primary initiator of blood coagulation. Modulation of TF expression plays an important role in regulation of endothelial thrombogenicity and vascular tone. Expression of tissue factor (TF) is known to be induced during EBOV infection (Geisbert, Young et al. 2003).

In order to test if soluble glycoproteins are able to induce the expression of tissue factor in EBOV primary targets (monocytes, DCs and macrophages) and also in human endothelial cells (HUVECs), these cells were exposed for 24 hours to LPS and supernatants from transiently transfected cells containing: Mock (in grey), sGP, LS, HS and, as detailed above, HS previously incubated with MBL (HS+MBL) (all in white). Cells were then collected and TF expression was analysed by flow cytometry (**Figure R13 A**). Expression of tissue factor was strongly induced in monocytes by HS and LPS but not by LS or sGP. TF activation by HS was completely abrogated when this supernatant was previously incubated with MBL (HS+MBL). Expression of TF was also induced by LPS and HS in macrophages and HUVEC but not in DCs. LS, sGP and HS+MBL did not induce TF expression. We then wanted to determine if soluble glycoproteins could activate coagulation by increasing soluble levels of tissue factor (sTF) and decreasing soluble thrombomodulin (sThM), which has anticoagulant properties. Supernatants from HUVEC, treated as in the previous experiment, were collected and their content of sTF and sThM was analysed by Multiplex ELISA. In the case of sTF both TNF- α and HS incubation led to an increase in soluble levels of TF (**Figure R13 B**). Controls containing Mock, sGP, LS and HS+MBL did not affect sTF release. Surprisingly, in the case of sThM, supernatants from transiently transfected cells containing shed GP (HS), LS, or HS+MBL did not induce the release of anticoagulant biomarker thrombomodulin (ThM) when compared to positive control, TNF- α . Supernatant containing sGP seemed to slightly decrease sThM in medium when compared with negative controls.

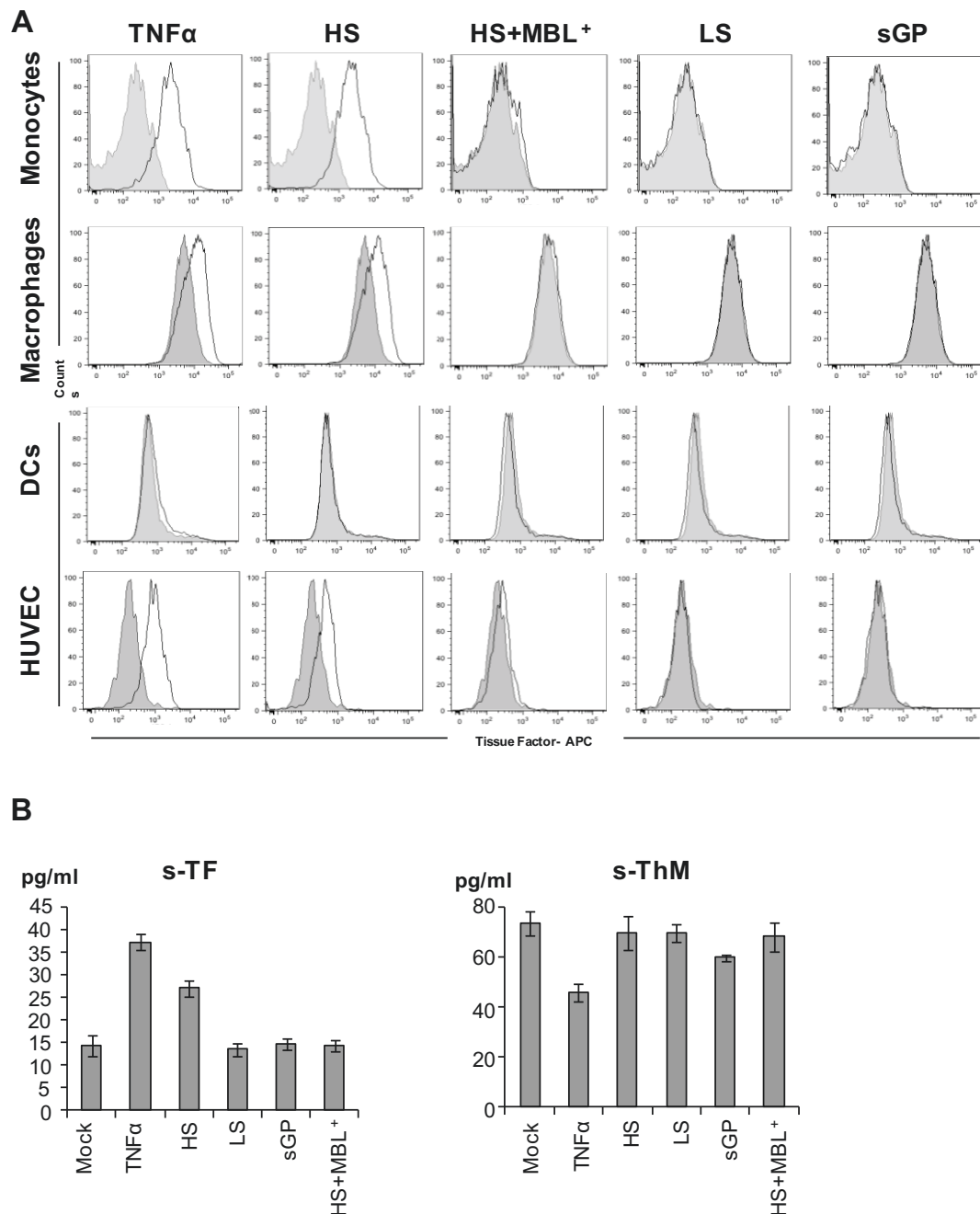


Figure R13. Expression of tissue factor in monocytes, DC, macrophages and HUVEC cells. (A) Monocytes, macrophages, DCs and HUVEC were treated with LPS, and supernatants from transfected cells containing: Mock (in grey) and sGP, LS, HS and HS+MBL (in white). Expression of tissue factor was determined by flow cytometry. The data are representative of three independent experiments. **(B)** Supernatants from HUVEC cells were analysed by Multiplex cytometric bead array to determine the presence of soluble tissue factor (sTF)

and thrombomodulin (sThM). The mean \pm sd of triplicates from one representative experiment from three independent experiments is shown.

ICAM-1 and VCAM expression on HUVEC cells

It is well known that upon stimulation, endothelial cells express cell adhesion molecules such as ICAM-1 and VCAM-1, which are known to be important mediators in cellular migration and infiltration and in inducing TF expression in monocytes and macrophages (Celi, Pellegrini et al. 1994, McGilvray, Lu et al. 1997). Therefore, we evaluated the expression of ICAM-1 and VCAM-1 on the vascular endothelium after treatment with EBOV soluble glycoproteins.

HUVECs were cultured in the presence of EBOV soluble glycoproteins and expression of ICAM-1 and VCAM-1 on the surface of human endothelial cells was studied by flow cytometry analysis. Release of soluble forms of tissue factor, thrombomodulin, ICAM-1, VCAM-1 and E-Selectin was assayed using a Multiplex cytometric bead array (Millipore). As shown in **Figure R14 A**, ICAM-1 and VCAM-1 were expressed on HUVECs after 24hours of incubation with supernatant containing shed GP (HS) and its level of expression was considerably increased. Supernatants containing controls Mock (in grey), LS and sGP supernatant were not shown to increase ICAM-1 and VCAM-1 levels. In agreement with previous results, when supernatant containing shed GP was incubated with MBL containing serum, no increase in expression of ICAM-1 and VCAM-1 was observed.

Soluble levels of ICAM-1, VCAM-1 and E-Selectin correspond with data observed with surface expression of these molecules (**Figure R14 B**); whereas supernatant containing shed GP (HS) and TNF- α did induce the release of these molecules into HUVEC culture medium, supernatants containing LS, and sGP did not induce ICAM-1, VCAM-1 and E-Selectin release. Again, supernatant containing shed GP together with MBL containing serum (HS + MBL⁺) lost the capacity to induce the release of these coagulation and permeability biomarkers (**Figure R14 B**).

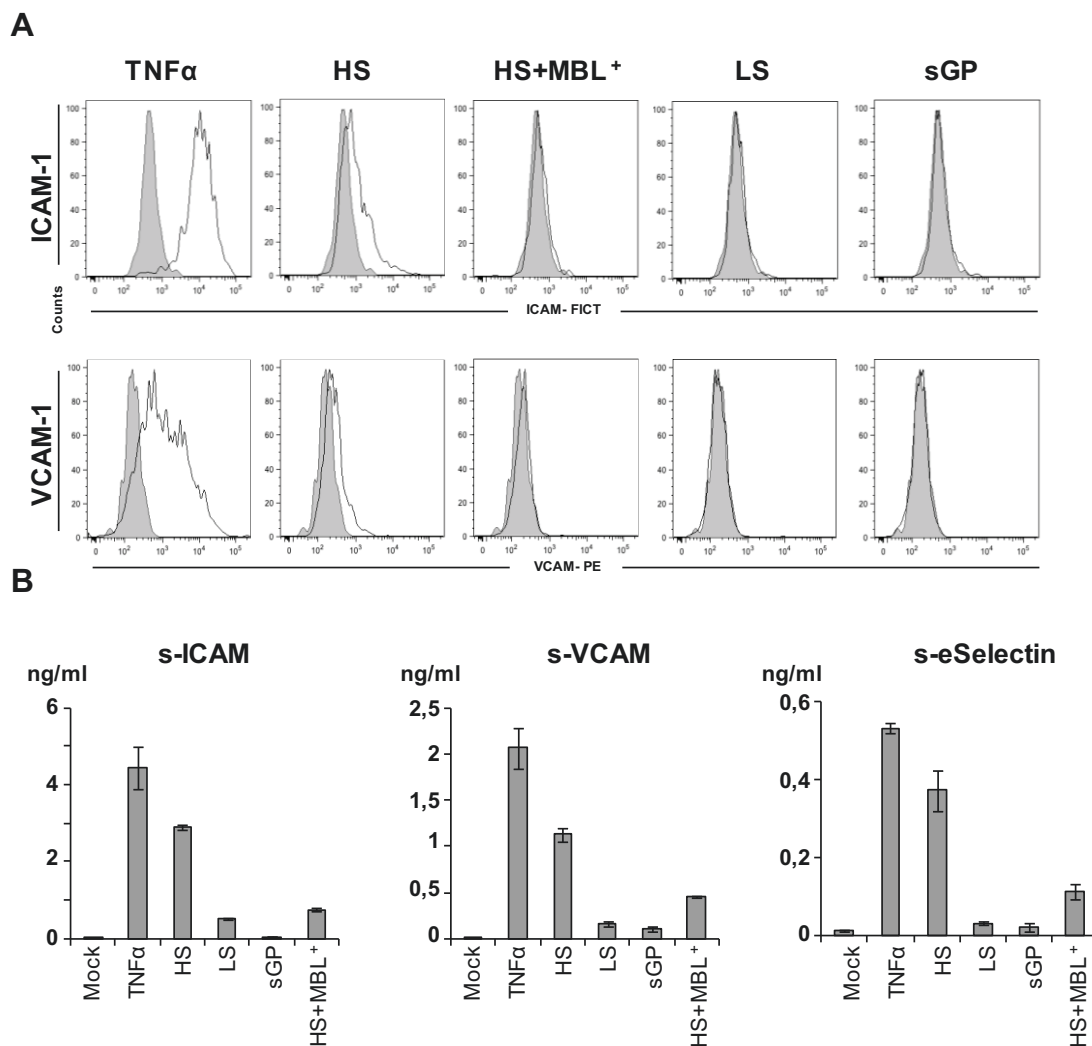


Figure R14.- Surface expression and release of coagulation and permeability markers by HUVEC. **(A)** HUVEC were treated with supernatants from transfected cells containing Mock (in grey), HS, LS, sGP and HS+MBL⁺ (all in white) and surface cell expression of ICAM-1 and VCAM-1 was determined by flow cytometry. The data is representative of three independent experiments. **(B)** Supernatants from HUVEC cells were recollected 24 hours after stimulation with TNFα, and supernatants containing soluble glycoproteins (HS, sGP and HS+MBL⁺) or controls (LS and Mock), and were analysed using a Multiplex cytometric bead array in MAGPIX. The mean± sd of triplicates from one representative experiment from three independent experiments is shown.

Modulation of endothelial monolayer permeability by EBOV shed GP

Vascular dysregulation and instability are thought to be crucial symptoms during EBOV infection (Ryabchikova, Kolesnikova et al. 1999, Ryabchikova, Kolesnikova et al. 1999, Zaki and Goldsmith 1999, Feldmann and Geisbert 2011). While endothelial cells are susceptible EBOV targets in vitro (Yang, Duckers et al. 2000, Simmons, Reeves et al. 2003, Wahl-Jensen, Afanasieva et al. 2005) they are considered to be rather late virus targets in vivo (Baskerville, Fisher-Hoch et al. 1985, Geisbert, Young et al. 2003, Hensley and Geisbert 2005) suggesting an indirect effect of EBOV infection on these cells. A dysregulated inflammatory host response to EBOV infection is believed to facilitate increased permeability of endothelial barriers that would eventually cause haemorrhaging (Royall, Berkow et al. 1989, Geisbert, Young et al. 2003, Geisbert, Young et al. 2003, Wahl-Jensen, Afanasieva et al. 2005). In this regard proinflammatory cytokines, in particular TNF α , released from immune cells activated by shed GP could affect endothelial barrier integrity.

To answer this question, an *in vitro* monolayer cell permeability assay was performed using HUVECs. HUVEC monolayers grown in transwell plates on semi-permeable membranes were treated for 22h with samples containing soluble glycoproteins or culture supernatants from macrophages previously stimulated for 24h with either HS or sGP and respective controls. Following treatment, FITC-dextran was added to the apical part of semi-permeable membranes in cell culture plate and the permeability of the monolayer induced directly by soluble glycoproteins (**Figure R15 A**) or indirectly through supernatants from treated macrophages (**Figure R15 B**) was assessed by measuring fluorescence in the basal compartment.

Cells were treated with samples of culture medium containing Mock, HS, LS, sGP, LPS, MBL containing sera (MBL⁺), MBL-deficient sera (MBL⁻) and TNF α (10ng/ml in Figure 11A and 1ng/ml in figure 11B). Integrity of HUVEC monolayers was verified with a fluorometer both before and after 22h of incubation with supernatants containing soluble glycoproteins. HUVEC monolayers treated with supernatants containing soluble glycoproteins (**Figure**

R15 C) or supernatants from macrophages previously stimulated with soluble glycoproteins (**Figure R15 D**) were also photographed under a light microscope.

As seen in **Figure R15 A and C**, supernatants containing HS, LPS and TNF α significantly increased the permeability of HUVEC monolayer when compared with controls Mock, LS, MBL⁺, MBL⁻ and sGP. Moreover, culture supernatants containing HS in the presence of MBL (HS+MBL⁺) were significantly less capable of inducing an increase in permeability of the HUVEC monolayer compared supernatants containing HS in the presence of MBL-deficient sera.

As we confirmed that HS is capable of activating immune cells for release of different cytokines including TNF α , we sought to determine if the levels of such soluble modulators were sufficient to contribute to the decreased barrier function of the endothelium (**Figure R15 B and D**). As expected, supernatants from macrophages incubated with the relevant soluble factors had a greater effect in disrupting the integrity of a HUVEC monolayer when compared to direct treatment with supernatants containing soluble glycoproteins, reaching comparable effect as that seen with LPS/TNF α -treated macrophages or LPS/TNF α alone used as a controls (compare Figure R15A with 15B). Somewhat surprisingly given the absence of detectable activation in previous assays, a moderate increase in permeability was observed when culture medium from GP-LS-expressing cells was used. Nevertheless, the effect observed by HS is significantly superior when compared to LS.

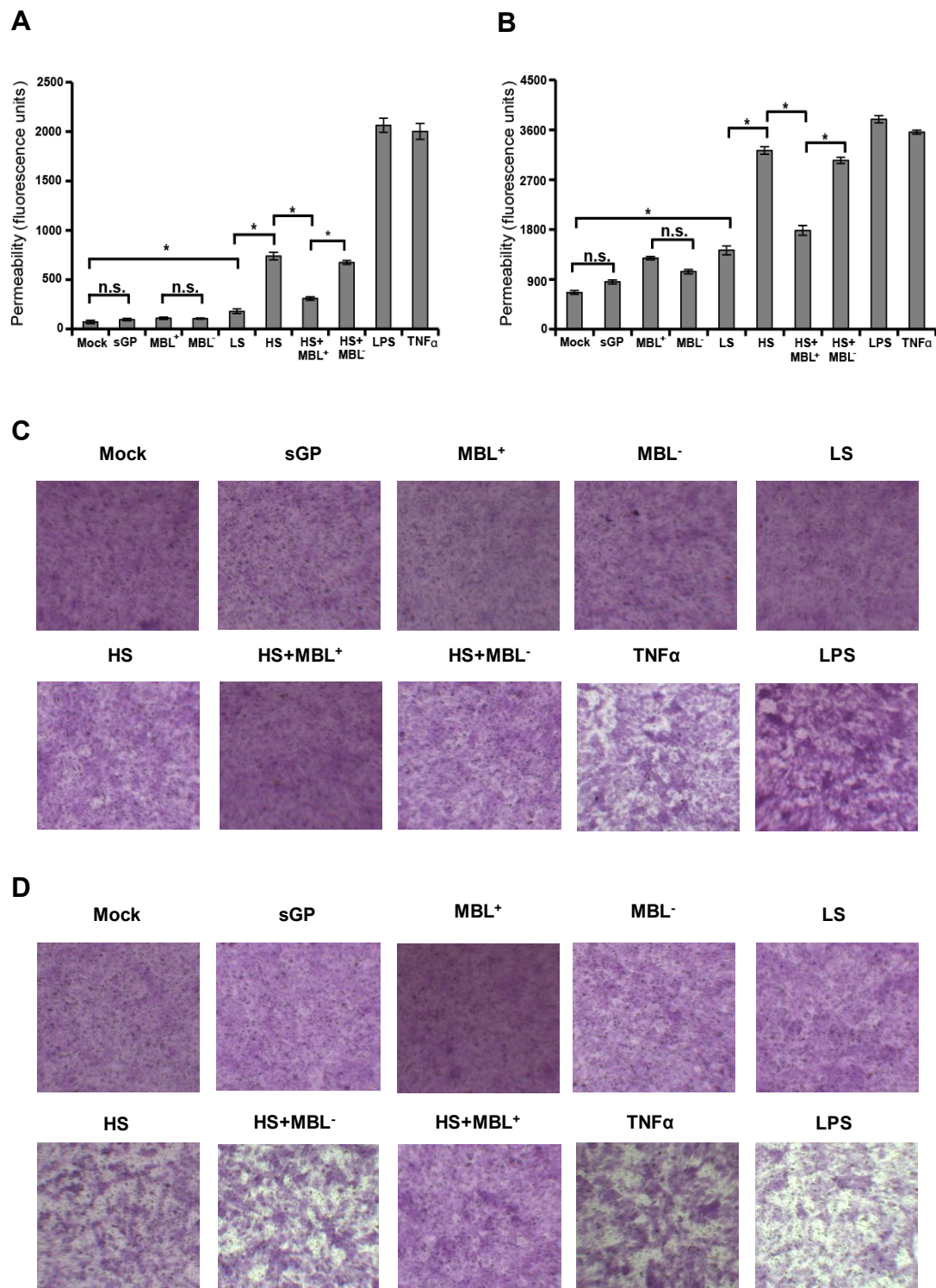


Figure R15. EBOV shed GP increases cell permeability of HUVEC *in vitro*.

HUVEC cells were seeded onto the culture inserts of permeability assay chambers and incubated for 22h with concentrated culture supernatants from 293T cells expressing GFP (Mock), sGP, GP-LS (LS), GP-HS (HS) and LPS (10ng/ml). Cells were also incubated with human sera containing MBL (MBL⁺),

MBL-deficient sera (MBL⁻), a mixture of HS/MBL-containing sera (HS+MBL⁺) and HS/MBL-deficient sera (HS+MBL⁻). As a permeability control the cells were treated with TNF α (20 ng/ml) **(A and C)**. In parallel, HUVEC cells were also incubated for 22h with culture supernatants from macrophages previously stimulated with samples of concentrated culture supernatants and controls as above. As a permeability control the cells were treated with TNF α (100 ng/ml) **(B and D)**. In all cases following incubation FITC-Dextran was added to each insert and FITC-Dextran leaking out into the bottom chamber was assayed using a fluorometer and presented as absorbance fluorescent units. Bars represent \pm SD of triplicates. The data are representative of three independent experiments. * - indicates statistically significant differences ($P < 0.05$) or n.s. - statistically not significant **(A and B)**. The integrity of HUVEC monolayers assayed in A and B was also observed with a MacroFluo microscope after fixation and coloration **(C and D)**.

In summary, we have demonstrated that shed GP binds to MBL. This binding leads to activation of MASP-1 and MASP-2. We also show that there is a clear correlation between the amount of MBL in human sera and MASP-1 activation upon shed GP binding to MBL. In addition, shed GP increases expression of TF on the surface of monocytes, macrophages and HUVEC cultures. Incubation of HUVECs with shed GP also leads to an increase in levels of secreted TF and ThM and induces both increased expression and release of adhesion molecules VCAM-1, ICAM-1 and E-Selectin. Finally, shed GP and supernatants from macrophages previously incubated with shed GP induce permeability in endothelial cell monolayers.

RESULTS IV: IMPACT OF SHED GP ON EBOV ENTRY

Shed GP decreases rVSV-GP binding and replication and rEBOV-GFP binding to Vero cells

Since EBOV surface GP and shed GP have a very similar structure and glycosylation pattern and according to our data they also share cellular targets and types of receptor, we thought that shed GP might also affect infection of cells by competing with surface GP present on viral particles with cellular target proteins or by modulating the surface expression of potential cellular targets after binding. In order to study this, and as viral attachment can be experimentally separated from entry by temperature, Vero cells were precooled

to 4°C and exposed to supernatants containing HS, LS or mock prior to incubation with R18 labeled VSV-GP (**Figure R16 A**). A marked diminution in rVSV-GP binding was observed when cells had been pre-incubated with HS (white) supernatant compared to mock (grey with grey line) and LS (grey with black line) controls supernatants. As expected, time course studies of rVSV-GP replication in cells and release into the supernatant after HS, LS or mock incubation (**Figure R16 B**) confirmed binding results showing that at 4, 8 and 12 hours post rVSV-GP infection, replication and therefore, the amount of virus in the supernatant, was lower when cells were pretreated with HS supernatant than with LS and mock controls.

Infection of Vero cells with recombinant Ebola virus expressing GFP during infection (rEBOV-GFP) showed similar results as rVSV-GP experiments. Vero cells were incubated with mock supernatant (in grey) or supernatants (in white) from recombinant viruses releasing high, low or normal amounts of shed GP into the media (named EBOV-HS, EBOV-LS and EBOV-WT respectively). We could observe that infection by rEBOV-GFP virus was clearly reduced when cells had previously been incubated with HS supernatant (**Figure R16 C**). Cells incubated with LS and WT did not show any reduction when compared to cells that were not treated with any supernatant (in grey).

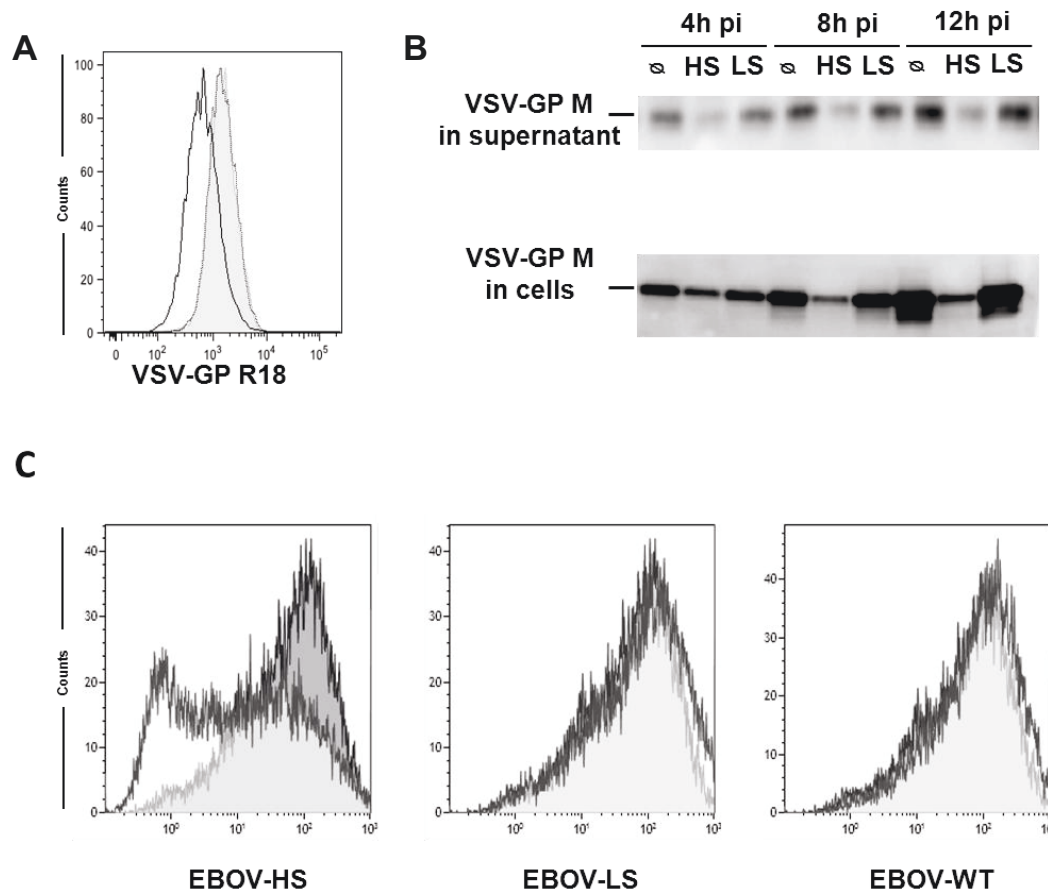


Figure R16. Pre-incubation of cells with Shed GP reduces virus binding and replication. (A) Vero E6 cells were treated with supernatants containing HS, LS and mock as previously described. Cells were then infected with R18-labeled-rVSV-GP and binding was determined by flow cytometry. (B) VSV-GP replication in Vero E6 after treatment was analyzed through VSV-M detection by Western blot 4, 6 and 8 hours post-infection. (C) Replication of rEBOV-GFP in Vero E6 cells was studied after treatment with mock supernatant (in gray) and supernatants (in white) from recombinant EBOV expressing high shedding (rEBOV-HS), low shedding (rEBOV-LS) or wild-type (EBOV-WT) levels of shed GP, respectively. rEBOV-GFP replication was determined by flow cytometry 48 hours post infection. Data are representative of three independent experiments.

Overall, in this section we demonstrate that shed GP prevents virus binding to target cells by competing with viral surface glycoprotein for cellular attachment factors. This competition may favor EBOV systemic dissemination in a host organism.

DISCUSSION

Shed GP targets the immune system

The molecular mechanisms behind the pathogenesis of Ebola virus are yet not completely understood, however it is known that the pathogenic potential of EBOV is enhanced through several factors and both viral gene products and dysregulation of host proteins seem to be detrimental to the host's chances of survival.

Ebola virus infection is characterized by immune suppression and a systemic inflammatory response that causes impairment of the vascular, coagulation and immune systems in addition to lymphopenia. These are all hallmarks of pathogens that subvert both innate and adaptive immune responses (Feldmann and Geisbert 2011). An impaired and ineffective host response leads to high concentrations of virus and proinflammatory mediators in the late stages of disease, which play important detrimental roles in the pathogenesis of haemorrhaging and shock. It has been shown that Ebola virus-infected cells produce and release high amounts of viral soluble glycoproteins into the blood vessels in both humans and animals (Feldmann, Volchkov et al. 1999, Volchkova, Klenk et al. 1999); once released, these proteins are free to interact with host factors and cells. However, the precise role of these soluble glycoproteins during viral pathogenesis still remains unknown.

Studying the interaction of these soluble proteins with host cells and their role in pathogenesis is complicated due to the difficulty in producing these soluble proteins in large quantities equivalent to those observed during Ebola virus infection. In the case of shed GP, the efficiency of shedding in cells transiently expressing GP from transfected plasmids is much lower compared to virus infection, probably due to the low activity of TACE or another potential unidentified cellular process in uninfected cells. Moreover, the high glycosylation and postranslational modifications of these soluble proteins hinders the use of other commonly used systems such as bacteria or yeast, which are more efficient. For these reasons we sought to generate several constructs with different shedding properties that allowed us to study this protein: High shedding (HS) and Low shedding (LS) constructs, which respectively induce more or less secretion of shed GP into the media compared

to GP wild-type. The fact that we have produced our proteins in serum-free medium means, on one side that different serum lectins didn't interact with our soluble glycoproteins and on the other side, we could generate a negative control for the heavily glycosylated shed GP through the subsequent addition of sera lectin MBL.

One earlier study addressed some of the same issues that we did, in that they used purified, secreted HA-tagged glycoproteins in order to study activation of macrophages (Wahl-Jensen, Kurz et al. 2005). That study differed however, in that (i) the insertion of a HA tag and stop codon before the transmembrane domain allows efficient secretion into the extracellular media. However, we have observed that when GP is produced in this way, the truncated GP is clearly present in a monomeric form, as evidenced by sedimentation through a sucrose gradient (Figure 1D); in contrast, in our construct shed GP is released through TACE cleavage and is present in a trimeric form, as also observed during infection. In addition, (ii) in the previous study, macrophage culture and experiments were done in the presence of human sera which is known to contain lectins, including MBL amongst others, that can interact with glycoproteins. These differences could explain that, in their case, no role was found for secreted glycoproteins until now.

Depending on the cytokine profile observed its effect can be either protective or damaging. Filovirus-induced expression of such mediators results in an immunological imbalance that partly contributes to the progression of disease. The balance in cytokine release is influenced by the route, the titer and the strain of the incoming virus as well as factors specific to the individual host immune response. In this respect, it should be expected that cytokine profiles could differ between individuals and EBOV strains. It has previously been shown that there is a link between clinical status and inflammatory responses (Baize, Leroy et al. 2002); in fact, human blood samples from survivors and nonsurvivors obtained during the five outbreaks that occurred between 1996 and 2003 in Gabon and Republic of Congo were studied and it was observed that fatal outcome is associated with exacerbated innate immune responses and with global suppression of adaptive immunity (Wauquier, Becquart et al.

2010). This exaggerated innate immune reaction is characterized by a “cytokine storm,” with aberrant secretion of numerous proinflammatory cytokines, chemokines and growth factors that seem to be indicative of fatal outcome. At even moderately elevated concentrations these soluble mediators act at various points in the first line of defence by recruiting circulating mononuclear cells to the site of infection, increasing endothelial permeability, activating macrophage and DC cytotoxic functions, and inducing adaptive immune responses by providing co-stimulatory signals for naïve T cells. Therefore, compared to the exacerbated release of cytokines observed during Ebola virus infection, a proper and controlled inflammatory response against the virus during the early steps of infection would help to counteract Ebola virus pathogenesis.

Several studies on infected human and nonhuman primates have demonstrated the interaction of Ebola virus with different cells of the immune system [19,20]. This corresponds well with the data obtained during our study where we observed that cultured primary DCs and macrophages were activated upon treatment with Ebola virus shed GP soluble glycoprotein, resulting in an increase of a number of mediators including pro- and anti-inflammatory cytokines, namely TNF α , IL6, IL10, IL12, IL8, IL1 β and IL1RA. This has also been observed in previous studies using VLPs on DCs (Bosio, Moore et al. 2004) and macrophages (Wahl-Jensen, Kurz et al. 2011). This observation leads to the conclusion that neither the whole virus nor viral replication are prerequisites for activation of primary target cells during Ebola virus infection. As observed during our study the secreted form of the viral glycoprotein spike, soluble glycoprotein shed GP, is sufficient to activate DCs and macrophages and thus, probably together with other viral factors, be partially responsible for the high pathogenicity of Ebola virus. Here we compare for the first time activation of primary targets DCs and macrophages by soluble glycoproteins. Notably, we also observe that macrophage activation and release of cytokines is considerably higher when compared to DCs.

Since susceptibility of monocytes to infection is controversial (Feldmann, Bugany et al. 1996, Stroher, West et al. 2001, Dube, Schornberg et al. 2008, Dube, Schornberg et al. 2010, Martinez, Johnson et al. 2013), human-

monocyte-derived cells were also tested as possible targets for EBOV soluble glycoproteins. Even if shed GP appears to bind to monocytes, it doesn't appear capable of activating the release of significant amounts of pro- and anti-inflammatory cytokines 24 hours post-treatment with supernatants containing soluble glycoproteins. Interestingly, monocytes treated with shed GP for 48 hours became permissive to EBOV infection. These data suggest that monocytes are not incompetent for EBOV entry, rather, entry and replication is delayed or requires monocyte maturation/differentiation. Similar data has recently been shown wherein EBOV treatment of monocytes renders them increasingly permissive with time, downregulating IFITM expression (IFN-inducible proteins that can restrict entry of viruses) and conversely, cathepsin B and NPC1 (which are essential entry factors for EBOV) are considerably upregulated during monocyte differentiation (Martinez, Johnson et al. 2013). In agreement, we show that delay in permissiveness may be explained by the fact that treatment of monocytes with shed GP differentiates them and thus, they become permissive to infection. Therefore monocyte differentiation appears to be a requirement for EBOV entry and this differentiation could be due to shed GP. This would suggest that soluble shed GP could target non-infected monocytes and render them permissive for infection, thus considerably increasing EBOV cellular targets in vivo. Further experiments should be done in order to determine expression of cell surface markers and compare them with macrophages and DCs to fully characterise the maturation phenotype associated with permissive EBOV infection.

Since lymphocyte apoptosis is observed during EBOV infection, we thought that shed GP could be the cause of this cell death; however, shed GP was shown not to bind to lymphocyte. The simple release of proinflammatory cytokines could indirectly induce lymphocyte apoptosis but further research should be done in this field to test this hypothesis. Notwithstanding, the fact that shed GP can clearly bind to DCs and macrophages raises an interesting hypothesis; it is known that TIM-4 is restrictively expressed on the surface of DCs and macrophages and that it is the ligand of TIM-1, which is principally expressed on activated T lymphocytes, and that this delivers a costimulatory signal necessary for T cell proliferation (Meyers, Chakravarti et al. 2005).. This data

also suggested that at low concentrations, TIM-4 inhibits T-cell responses, but, at high concentrations, TIM-4 mediates positive costimulation. Interestingly, TIM-1 and shed GP share some features: (i) both can be soluble (Bailly, Zhang et al. 2002), (ii) TIM-1 is more expressed and released into a soluble form during kidney injury (KIM-1) (Ichimura, Bonventre et al. 1998) and (iii) both have a highly glycosylated mucine domain important in binding to their receptors (Rodriguez-Manzanet, DeKruyff et al. 2009). Therefore, we believe that during EBOV infection, the binding of shed GP or viral particles to TIM-4 in DCs and macrophages could prevent the interaction between TIM-1 and TIM-4 and that this could induce apoptosis of T cells due to the fact that they are receiving stimulation via CD3 and CD28 receptors but not from TIM-1. However, this hypothesis should be tested.

Further investigation should be also done in order to determine if soluble protein shed GP may induce changes in primary or secondary cell targets thus indirectly inducing lymphopenia.

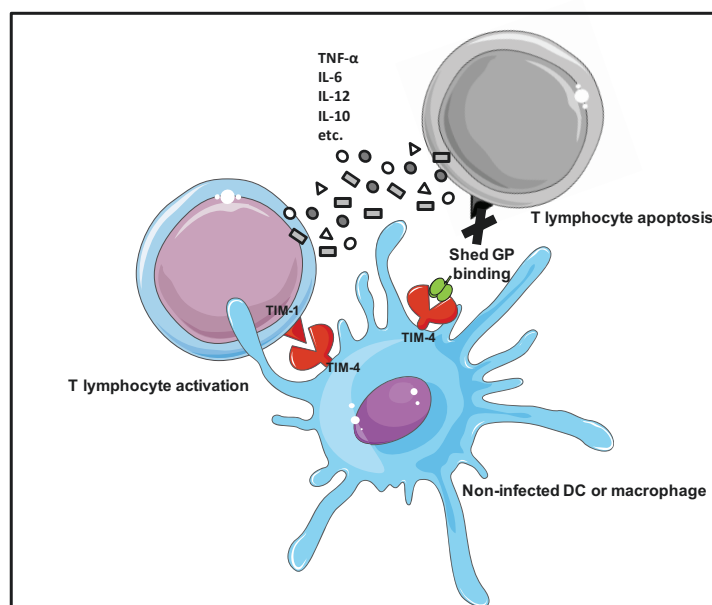


Figure 25. Possible role of shed GP in inducing lymphocyte apoptosis.- Shed GP is able to bind DCs and macrophages and activate the release of pro- and anti-inflammatory cytokines that will recruit and partially activate T lymphocytes. Potential binding of shed GP to TIM-4 could mean that

lymphocytes are not able to receive co-stimulatory signals through TIM-4 and could induce their apoptosis.

A series of studies have shown that EBOV stimulates antigen-presenting cells to release large quantities of proinflammatory cytokines, chemokines and other mediators (Geisbert, Hensley et al. 2003, Hutchinson and Rollin 2007, Mohamadzadeh, Chen et al. 2007). In fact, virus spread rapidly causes an intense systemic inflammatory syndrome resembling septic shock (Feldmann, Jones et al. 2007). Ebola virus infection and septic shock present fairly similar innate responses. The major clinical features of septic shock are brought about through the action of cytokines, chemokines, and other mediators produced by monocytes, macrophages, and related cells as a result of the binding of lipopolysaccharide (LPS) and other bacterial components to pattern-recognition receptors, including Toll-like receptors. This produces the typical traits of an inflammatory response (Bray and Mahanty 2003). As with septic shock caused by bacterial pathogens, fatal cases of Ebola virus infection are thought to involve inappropriate or maladapted host responses including an exacerbated release of proinflammatory cytokines. The triggering mechanism by which Ebola virus stimulates induction of proinflammatory cytokines remains unknown. The apparent similarity in the response of immune cells to shed GP and LPS highlights a likely common triggering of TLR4 and shared downstream pathway for activation and cytokine release. Given this common pathology, we wondered whether EBOV soluble glycoproteins might affect TLR4 as LPS does during septic shock. Indeed, when presented in VLPs EBOV GP has been shown to interact with TLR4, leading to the induction of proinflammatory cytokines as well as SOCS1 in both a human monocytic cell line (THP-1) and 293T cells expressing a functional TLR4/MD2 receptor (Okumura, Pitha et al. 2010). Thus, in the case of EBOV both the virus particles themselves and importantly shed GP as a soluble mediator would be able to activate TLR4 on target cells, making it distinct from a number of other viruses whose surface glycoproteins have been shown to act as ligands for TLR4 recognition, including respiratory syncytial virus, mouse mammary tumor virus and human CMV (Haynes, Moore

et al. 2001, Burzyn, Rassa et al. 2004, Tal, Mandelberg et al. 2004, Xie, Liu et al. 2008). In this way activation of non-infected immune cells through soluble shed GP would contribute to the systemic inflammatory syndrome and high pathogenicity seen with EBOV infection.

In agreement with this idea, we show that activation of DCs and macrophages by Ebola virus soluble glycoproteins is principally triggered through TLR4 receptor. These results indicate that the stimulation of TLR4 by soluble glycoprotein shed GP leads to an innate host response that may enhance virus egress through enhanced recruitment of new potential targets for viral infection and potentially by providing an activated immune state favorable for viral replication. This observation with shed GP corresponds very well with those cited above for EBOV surface glycoproteins (Okumura, Pitha et al. 2010) and with another study where it was shown that Toll-like receptor agonist augments virus-like particle protection towards EBOV with transient immune activation (Ayithan, Bradfute et al. 2014). Importantly, our study suggests that induction of innate immune signaling by shed GP via TLR4 is not a by-product of the disease but that it may strongly influence disease severity by being a key event during the early steps of EBOV infection. Modulation of this exacerbated inflammatory pathway, through counteracting the effects of shed GP could, therefore, potentially be used to protect EBOV infected patients.

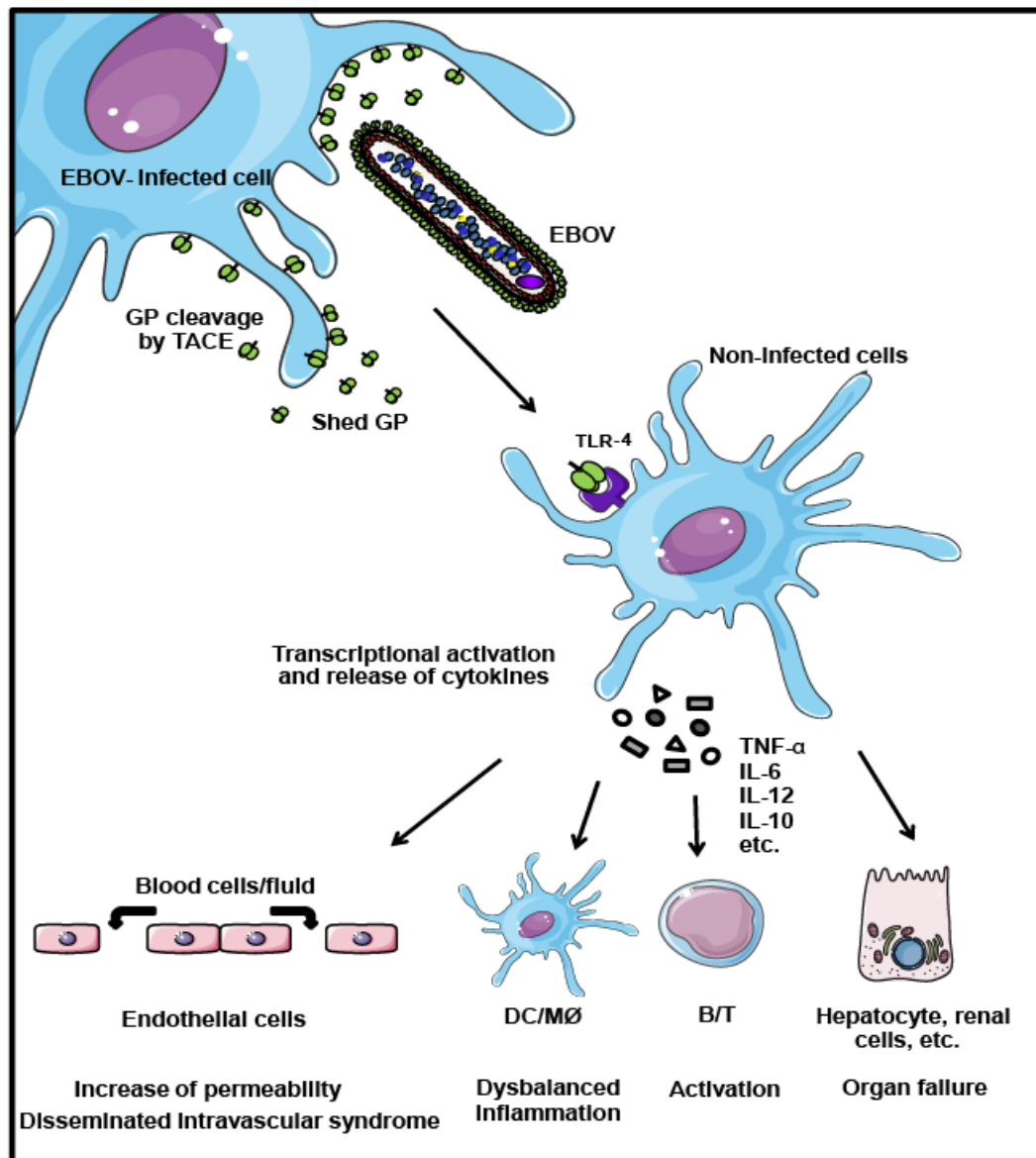


Figure 26. Potential effects of shed GP on the immune system during EBOV infection.- In our model, EBOV infected cells release both virions and shed GP into the bloodstream. Shed GP is able to bind TLR4 on the surface of macrophages and DCs thus inducing the release of pro- and anti-inflammatory cytokines. The soluble nature of both shed GP and cytokines enables them to trigger new, remote targets such as non-infected APCs, which will then amplify inflammation by recruiting and/or activating other cell types including; lymphocytes, that could explain the lymphopenia observed during EBOV infection; endothelial cells, which display increased permeability due to cytokines released from DCs and macrophages; and other cell types including hepatocytes, leading to the impairment of other functions and organ failure.

Indeed, in the case of EBOV both the virus particles themselves and importantly shed GP, as a soluble mediator, would be able to activate TLR4 on target cells, making it distinct from other viruses. In this way activation of non-infected immune cells through soluble shed GP would be a major cause of the systemic inflammatory syndrome seen with EBOV infection and would considerably amplify the host's immune response, thus contributing to the high pathogenicity of Ebola virus.

Likewise, it has also been shown that MBL is able to bind to TLR4 and suppress lipopolysaccharide-induced inflammatory cytokine secretion (Wang, Chen et al. 2011, Wang, Zhang et al. 2011) and microbial (Wang, Wang et al. 2013) and virus infectivity (Reading, Hartley et al. 1995, Saifuddin, Hart et al. 2000) by different mechanisms. Moreover MBL has also been reported to bind the membrane-anchored receptor CD14, which facilitates the interaction between LPS and TLR4 (Sano, Chiba et al. 2000, Chiba, Sano et al. 2001). As we can see in our study, blocking of TLR4 receptor with MBL or anti-TLR4 antibodies considerably reduces binding and activation of proinflammatory cytokines by shed GP in DCs and macrophages, a fact that is probably explained by the fact that MBL can block shed GP binding to TLR4 by both directly blocking TLR4 receptor and by binding to shed GP and thus preventing shed GP binding to TLR4.

It has been shown that O-glycosylations are specifically recognized by TLR-4 receptor and that this recognition induces release of proinflammatory cytokines (Netea, Gow et al. 2006). Indeed, as discussed above, surface glycoproteins from a number of other viruses have been shown to act as ligands for TLR4 recognition (Haynes, Moore et al. 2001, Burzyn, Rassa et al. 2004, Tal, Mandelberg et al. 2004, Xie, Liu et al. 2008). In agreement with this, we have observed that deglycosylation of shed GP resulted in the loss of its capacity to activate TNF α release in DCs and macrophages. This means that removal of N- and O-glycosylation of shed GP probably prevents the attachment of shed GP through TLR4 in DCs and macrophages. We speculate that shed GP activation of TLR4 could have a positive effect on viral replication by directly activating non-infected macrophages and dendritic cells, promoting subsequent infection

and allowing an initial round of viral replication or feedback of TLR4-induced cytokines, which may promote virus replication.

Further studies are required however in order to determine if shed GP receptors correspond to some of the attachment factors already described for EBOV i.e. C-type lectin membrane associated receptors, such as DC-SIGN and L-SIGN, or other attachment factors on the cell surface as β integrins (Takada, Watanabe et al. 2000, Schornberg, Shoemaker et al. 2009), Axl (Shimojima, Takada et al. 2006, Shimojima, Ikeda et al. 2007, Brindley, Hunt et al. 2011) or T-cell immunoglobulin mucin domain-1 (TIM-1) (Kondratowicz, Lennemann et al. 2011). A previous study has shown that carbohydrate-specific signalling through DC-SIGN involves cooperation with TLR4 (Gringhuis, den Dunnen et al. 2009). Even if our data suggests that TLR4 is the principal attachment receptor for shed GP, it does not exclude the fact that other attachment factors could participate in shed GP binding and that it is possible that a cross-talk exists between different signalling pathways or attachment factors (involving NF κ B pathway and TLR4 receptor among others) that could be crucial in the induction of pathogen-specific immune responses in EBOV targets. In fact, our data shows that treatment of DCs and macrophages with antibodies against TLR4 did not completely block shed GP binding nor the release of cytokines. Shed GP is also N-glycosylated and therefore it may also bind lectins such as Mannose receptor (MR) or DC-SIGN. The fact that we can still observe shed GP binding after blocking with TLR4 antibodies may be explained by the fact that immune sensing of Ebola virus probably requires other attachment factors on the surface of the cell. Importantly, our data shows that release of pro-inflammatory cytokines is downregulated but not completely abrogated, a fact that could indicate that TLR4 signalling could also be modulated by cooperative recognition of glycosylation by lectins and Toll-like receptors as it has previously been shown with HIV concerning TLR-4 and DC-SIGN. It should also be considered that, blocking TLR-4 with antibodies could prevent this modulation-cooperation (and even shed GP binding), suggesting the possibility that TLR4 acts in concert with other plasma membrane-associated proteins to mediate productive uptake of filoviruses.

Moreover, since GP distribution on the surface of Ebola virus is not the same as soluble shed GP even if their trimeric structure and glycosylation are almost identical, this may affect the type of receptors that they engage and could explain the wide range of receptors that surface GP on the virion can bind. It is also possible that shed GP binds similar receptors but, contrarily to VLPs and virus, does not activate cells response due to differences and indeed lack of viral spike conformation. More experiments including blocking different cell receptors with antibodies, silencing them or even using KO animal models, should be done in order to clarify this.

It is likely that C-type lectins increase filovirus attachment to cells rather than serving as cellular receptors that mediate internalization of the virus into endosomes (Matsuno, Nakayama et al. 2010). MBL, as a soluble C-type lectin receptor of Ebola virus, could target the virion and facilitate its binding to the surface of a cell. Although cellular MBL receptors have not been completely identified, potential receptors include C1qRp, CD35 and CD91 (McGreal and Gasque 2002, Takahashi and Ezekowitz 2005). However, we hypothesize that the high amounts of shed GP produced during Ebola virus infection may trigger MBL thus preventing virus binding to MBL and therefore, promoting viral attachment to cell surfaces.

Data concerning the role of MBL during virus infection is controversial. In the presence of complement, MBL has been shown to be able to neutralize VSV pseudotyped with Ebola virus glycoprotein (Ji, Olinger et al. 2005). Likewise, MBL has also been shown to have a protective effect during Ebola virus infection in vivo where mice whose recombinant MBL serum concentration was increased survived otherwise fatal Ebola virus infection and became immune to virus rechallenge (Michelow, Lear et al. 2011). Paradoxically, another study has recently shown that, in the absence of complement, MBL enhances infection of Ebola virus and other N-glycosylated viruses such as West-Nile virion-like particles and to a lesser degree, Nipah and Hendra viruses (Brudner, Karpel et al. 2013). However, it should be noted that this last study was done using considerably supraphysiological concentrations of recombinant MBL (at least 5 times higher than physiological levels). Another limitation was that lentiviral

pseudotyped viruses were used for mechanistic experiments. Ebola virus length ranges from 600 to 1400 nm, and peak infectivity is associated with 805-nm particles (Aleksandrowicz, Marzi et al. 2011); Therefore, pseudotyped systems have limitations because the morphology of the virions differs significantly from that of real Ebola virions (spherical and smaller for retrovirus or VSV-pseudotyped virions versus filamentous for authentic Ebola virus (Bhattacharyya, Warfield et al. 2010)). Hence, for all of these reasons, the observed interactions between HIV-EBOV-GP and host attachment factors during this study may be to some extent artificial.

In summary, the first part of this study has produced several novel findings. First we have identified primary targets of EBOV soluble glycoproteins; second, we have observed that shed GP is able to induce pro- and anti-inflammatory cytokine synthesis and secretion in DCs and macrophages, principally through TLR4 receptor, besides inducing their phenotypic maturation. Comparison of these two primary targets simultaneously under the same experimental conditions was never done before. Second, we have demonstrated the importance of shed GP glycosylation on the immunogenicity of the protein and that its activity can be blocked completely by the presence of the sera lectin MBL. Third, we have shown that both shed GP but also cytokines released from shed GP treated DCs and macrophages induce the disruption of endothelial permeability. Altogether we provide information to suggest that soluble glycoprotein shed GP plays an important role during EBOV pathogenicity by contributing to the exacerbated activation of inflammation which besides the dysregulation of the immune response, increases endothelial permeability.

Shed GP impairs coagulation and permeability pathways

Mannose-binding lectin (MBL) is known to have intrinsic antimicrobial activity and to be also implicated in the initialisation of coagulation and complement systems (Matsushita, Endo et al. 2013). Mannose-Binding lectin is a C-type lectin that recognizes hexose sugars including mannose, glucose, fucose, and *N*-acetylglucosamine on the surface of glycosylated pathogens. Several viruses, such as influenza virus, human immunodeficiency virus, herpes simplex virus type 2, severe acute respiratory syndrome coronavirus (SARS-CoV), Ebola virus, and Marburg virus have been shown to be recognized by MBL through *N*-linked high-mannose carbohydrate structures on their respective glycoproteins (Hartshorn, Sastry et al. 1993, Anders, Hartley et al. 1994, Reading, Hartley et al. 1995, Saifuddin, Hart et al. 2000, Gadjeva, Paludan et al. 2004, Michelow, Lear et al. 2011). This process involves attaching a high-mannose core to the amide nitrogen of asparagines followed by the adornment and the reorganisation of viral oligosaccharides in the endoplasmic reticulum and Golgi apparatus (Breitling and Aebi 2013). Importantly, surface glycoprotein structures are not only critical for virus stability, cell tropism, immune evasion and host cell invasion but they are characteristic of a broad group of viruses in which *N*-linked glycosylation contributes to viral virulence (Vigerust and Shepherd 2007). Ebola virus surface glycoprotein is heavily glycosylated and, like Marburg virus, contains both *N*-linked and *O*-linked carbohydrate structures (Feldmann, Will et al. 1991, Geyer, Will et al. 1992, Feldmann, Nichol et al. 1994). Since MBL has been shown to bind to both Ebola virus and Marburg virus glycoproteins (Ji, Olinger et al. 2005), due to the structural similarity between EBOV surface glycoprotein GP and soluble glycoprotein shed GP released during EBOV infection, it is easy to imagine that surface GP and shed GP might share some functions. Our results show that, as has been demonstrated with EBOV surface glycoprotein (Ji, Olinger et al. 2005), shed GP is able to specifically bind MBL and, importantly, the trimer structure of the protein is essential for this interaction. Moreover, we have shown that binding of shed GP to MBL activates at least two MBL-associated serine proteases (MASPs): MASP-1 and MASP-2, which have previously been shown to be implicated in coagulation and complement processes respectively (Matsushita, Endo et al. 2013). Our data

also show that there is a competition between shed GP and mannose to bind MBL, thus demonstrating specificity of binding between shed GP and MBL.

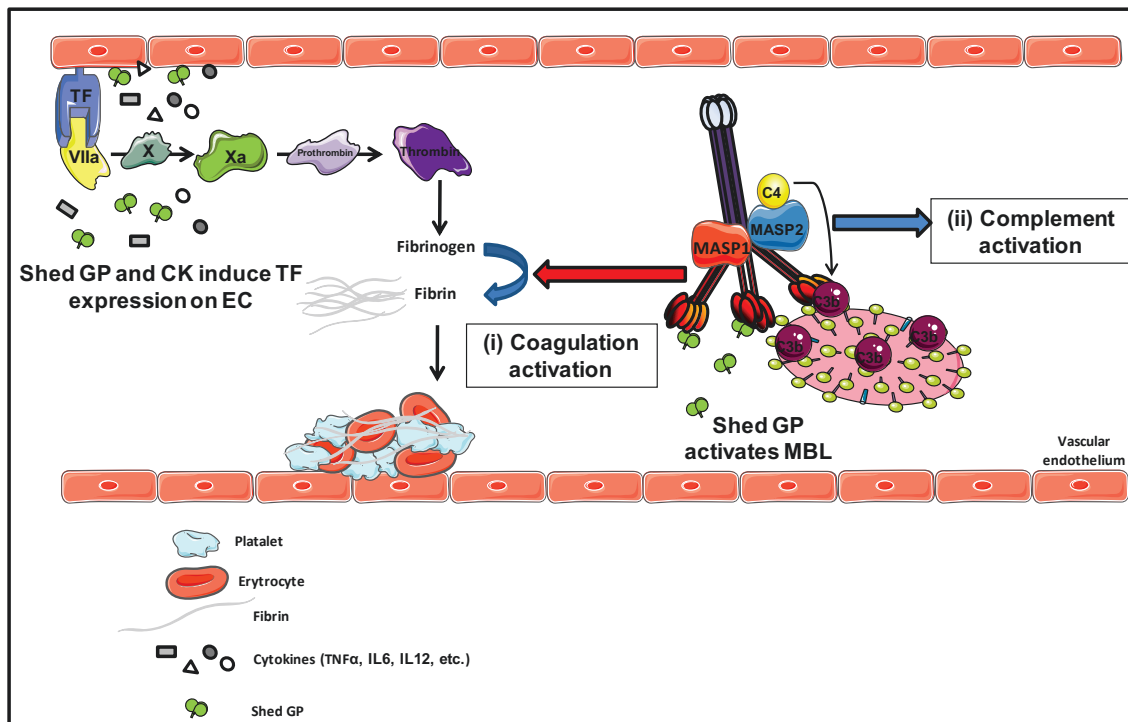


Figure 27. Role of shed GP in the impairment of coagulation.- EBOV shed GP is capable to directly and indirectly - through cytokines (CK) previously released by its primary targets DCs and macrophages - induce the expression of tissue factor (TF) in endothelial cells (EC), which will start the coagulation pathway that will produce blood clots that can lead to disseminated intravascular syndrome. At the same time, shed GP is able to bind Mannose binding lectin (MBL) and activate MASP-1, which has a thrombin-like activity that will contribute to impairment of the coagulation pathway. Likewise, shed GP is also able to induce MASP-2 activity, which fixes and cleaves serum C4 to form C4a (anaphylatoxin) and C4b (binds to proteins on a pathogens' surface). C4a will allow the formation and fixation of C3 convertase (represented in the picture as C3b) that at the end of the complement pathway will allow phagocytic cells to target pathogens and facilitate opsonitation.

Naturally occurring variation in MBL blood levels in the population has been shown to play a role in several diseases (Van der Ende, Van Baardewijk et al.

2013). MBL deficiency results from genetic defects caused by single nucleotide polymorphisms (SNPs) that occur in 5–30% of the population, depending on ethnicity. Combinations of these SNPs result in reduced blood concentration and/or dysfunctional MBL proteins. As a result, human MBL blood levels vary significantly (Takahashi 2011, Takahashi, Chang et al. 2011). Several clinical studies have associated MBL deficiency with increased susceptibility to infection (Sumiya, Super et al. 1991, Gadjeva, Paludan et al. 2004, Shi, Takahashi et al. 2004, Moller-Kristensen, Ip et al. 2006, Takahashi, Ip et al. 2006). Importantly, we have also shown that the degree of activation of MASP-1 by shed GP is dependent on the amount of MBL in sera of patients and therefore, that a correlation exists between MBL content in blood and its activation, meaning coagulation activation, by EBOV shed GP. It is interesting to speculate that these differences in MBL levels in blood could explain the wide range of individual susceptibility and severity in the symptoms observed in EBOV infected patients.

Interestingly, it has been observed that during DIC and organ failure, both phenomena observed during EBOV infection, there is a deficiency in MBL (Takahashi, Chang et al. 2011). Since the liver is the primary organ that synthesizes MBL (Uemura, Saka et al. 2002), and it is damaged during EBOV infection, this may have repercussions on MBL production. Damage to the liver, combined with massive viremia, leads to disseminated intravascular coagulopathy (DIC), further organ failure and systemic inflammation. We hypothesize that MBL ultimately does not neutralize Ebola virus during infection because MBL is depleted when it binds to shed GP; and also, that liver injury and impairment observed during EBOV infection may reduce MBL synthesis at later stages of infection. Moreover, the fact that MBL participates in coagulation processes, may, on one side, contribute to generate DIC syndrome and therefore thrombi and organ failure and, on the other side, this implication of MBL may reduce its availability for other processes, resulting in decrease of its opsonophagocytic activity and thus contributing to viral egress.

Recently a study has shown that murine MBL has an anti-inflammatory function by interfering with the interaction between LPS and TLR4 on mast cells (Ma,

Kang et al. 2013). In agreement with this, our work supports further testing of supraphysiologic MBL therapy against Ebola virus infection which could be a potential therapeutic agent against Ebola virus and may offer a novel antiviral approach. However, since MBL is activated by shed GP, treatment should be done with recombinant MBL, or synthetic MBL derivatives, not containing MASP-1 in order to avoid the impairment of coagulation. Although MASP-2 should be included in order to keep the opsonophagocytic functions of MBL. A treatment with MBL combined with anti-TLR4 antibodies and anti-coagulation modulators would prevent an exacerbated release of pro- and anti-inflammatory cytokines that could allow an early and well-regulated inflammatory response, which would prepare the host to control viral replication and coagulation impairment and induce specific immunity.

It has been shown that endothelial cell function is impaired during Ebola virus infection in humans (Zaki and Goldsmith 1999) and non-human primate models (Ryabchikova, Kolesnikova et al. 1999, Ryabchikova, Kolesnikova et al. 1999). Although endothelial cells are considered secondary targets, vascular dysregulation and instability are thought to be crucial symptoms during Ebola virus infection. However, proinflammatory cytokines such as TNF α activate the endothelium, increasing expression of cell adhesion molecules and affecting endothelial barrier integrity (Royall, Berkow et al. 1989). In vitro studies have demonstrated that Ebola virus-like particles expressing the transmembrane glycoprotein GP1,2 at the surface were able to activate endothelial cells and decrease the barrier function. In contrast sGP seem to have a protective role in endothelial barrier integrity when cells were treated with TNF- α (Wahl-Jensen, Afanasieva et al. 2005). Our data shows that shed GP itself but also supernatants resulting from shed GP activation of macrophages are able to increase vascular permeability. Endothelial cells are susceptible EBOV targets in vitro (Yang, Duckers et al. 2000, Wahl-Jensen, Afanasieva et al. 2005), but are considered to be rather late virus targets in vivo (Geisbert, Young et al. 2003, Hensley and Geisbert 2005) suggesting an indirect effect of EBOV infection on these cells, but suggesting a preponderant role for shed GP. These results also demonstrate that cross-talk between inflammation and endothelial

permeability may direct immune responses in the vascular endothelium to recruit and activate immune cells.

It has been suggested that there is a relationship between the procoagulant and permeability pathways; in fact, endothelial cell (EC) expression of TF and extrinsic clotting factors are critical in augmenting capillary leakage (Friedl, Puhlmann et al. 2002). It has been shown that treatment of EC with anti-TF antibodies blocks permeability, thus implicating an essential role for EC surface expression of TF and the extrinsic clotting cascade in increasing capillary leakage (Friedl, Puhlmann et al. 2002). The importance of TF in triggering the coagulation abnormalities that typify EBOV infection is supported by a study where rhesus monkeys treated with nematode anticoagulant protein c2 (rNAPc2), a potent inhibitor of tissue factor-initiated blood coagulation, significantly improved survival. Moreover, survival was associated with reduced activation of coagulation and proinflammatory response (Geisbert, Hensley et al. 2003). Our data suggests that shed GP is not only involved in the coagulation cascade, but would also play an important role in endothelial permeability. We have been able to detect increased pro-coagulant and pro-permeability marker tissue factor on the surface of HUVEC cells but also on the surface of immune cells such as monocytes and macrophages, data that correlates with previous studies that showed that EBOV infected monocytes/macrophages can trigger endothelial leakage (Feldmann, Bugany et al. 1996).

We have previously seen that soluble glycoproteins are able to induce cytokine production. In this study we also showed that soluble glycoproteins can also be involved in the last steps of leukocyte-endothelial adhesion. Soluble glycoprotein shed GP is able to induce the surface expression of ICAM and VCAM on the surface of endothelial cells, thus contributing to high-affinity adhesive interaction between leukocytes and endothelium but also inducing this phenomena in further cells throughout the bloodstream.

Importantly, we have also been able to show that pro-permeability markers such as ICAM-1, VCAM-1 and E-selectin are also released in a soluble form. The release of adhesion molecules in a soluble form may simply arise as a

consequence of inflammation or may have immune modulating properties by having inhibitory effects on neutrophil and monocyte extravasation. The soluble character of these molecules allows them to disseminate easily by traveling through the bloodstream, increasing their potential cellular targets and affecting permeability integrity. We have also studied the presence of pro-coagulant markers released from HUVEC in a soluble form, including tissue factor, also implicated in the permeability process as explained above. There are two forms of soluble TF: one anchored to membrane of microvesicles released from cells expressing TF on the surface (active form), and soluble TF that is not associated to membranes and generated by alternative splicing, asTF (inactive form). Soluble tissue factor (asTF) was thought to have a procoagulant role; however, it has recently been shown that the procoagulant activity of asTF is very low (Bogdanov, Balasubramanian et al. 2003, Eisenreich and Rauch 2010) and membrane anchored TF - on cells or on vesicles -, rather than asTF, was the main source of procoagulant TF activity (Yu and Rak 2004), whereas asTF has recently been linked more closely to proangiogenic processes and cell proliferation (van den Berg, van den Hengel et al. 2009, Eisenreich, Zakrzewicz et al. 2013). We have observed that shed GP also contributes to the induction of coagulation through sTF. Surprisingly, the soluble form of thrombomodulin, a protein expressed on the surface of endothelial cells and that has an anticoagulant role, was not shown to be affected by shed GP or other supernatants compared to $\text{TNF}\alpha$, which as expected, decreased its release into the medium.

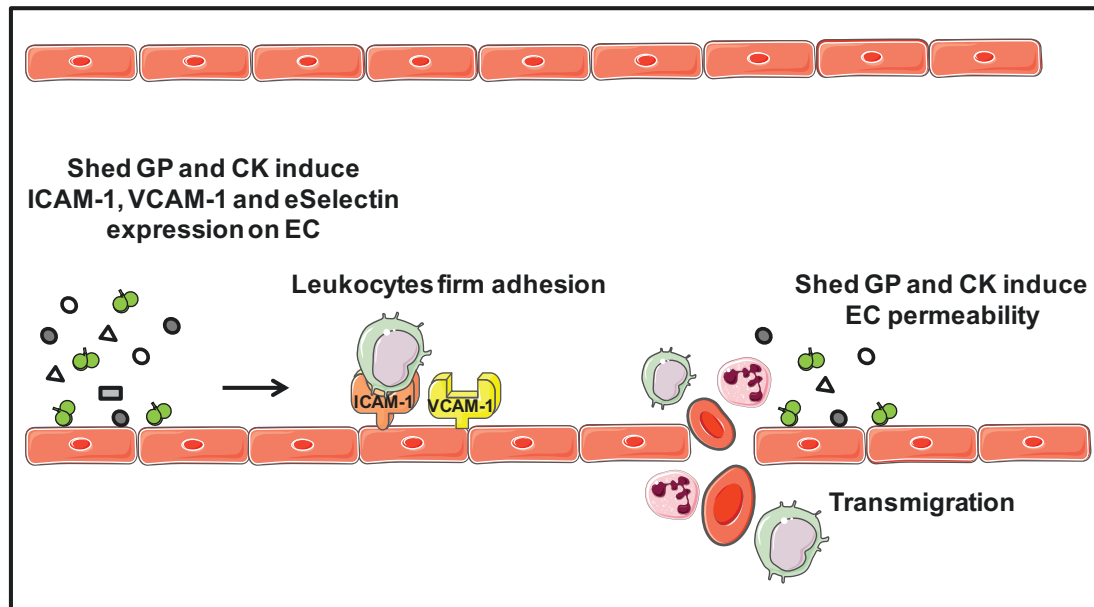


Figure 28. Role of shed GP inducing endothelial cell permeability.- Ebola virus shed GP and released cytokines induce both expression of adhesion molecules such as ICAM-1 and VCAM-1, which will bind leukocytes through integrins. At the same time, shed GP itself but also cytokines released due to shed GP binding to DCs and macrophages are capable to directly induce endothelial permeability.

The soluble nature of shed GP would also facilitate its dissemination through the bloodstream. This would explain the rapidity and the systemic behavior of the impairment of cellular integrity in vessels and the coagulation pathway, that are both observed during EBOV infection. However, it is important to take into account that coagulopathy and the impairment of vascular permeability noted during Ebola haemorrhagic fever could be caused by several factors including the massive release of pro-and anti-inflammatory cytokines observed during EBOV infection, and that we have shown can also be caused by shed GP.

In conclusion, in this second part of our study, we showed that shed GP is able to (i) directly interact with MBL and induce MASP-1 and MASP-2 activation, which are implicated in coagulation and complement pathways respectively, (ii) shed GP is capable of inducing the expression of coagulation and permeability markers on the surface of innate immune cells and on human endothelial cell

surfaces but is also released in a soluble form into the medium and (iii) shed GP seems to play an important role in the induction of adhesion molecule expression on the surface of human endothelial cells thus potentially contributing to the recruitment of leukocytes as possible new cellular targets. Therefore, even if shed GP is not the single factor affecting coagulopathy and vascular leakage, it seems reasonable to speculate that it could contribute in an important way to their impairment.

We have also determined another important role for shed GP. Current results suggest that shed GP, due to its almost identical structure and glycosylation pattern compared to viral surface GP, is capable to bind to similar receptors and help the virus to not re-infect the same cells, or neighbouring cells in close proximity, and thus spread to cells further afield. We thus hypothesize that shed GP is involved in the establishment of viremia. Our results provide a key for better understanding the role of soluble glycoprotein shed GP. Understanding mechanisms of filoviruses spread throughout the organism may shed light on how EBOV invades the bloodstream to disseminate and cause disease in infected hosts.

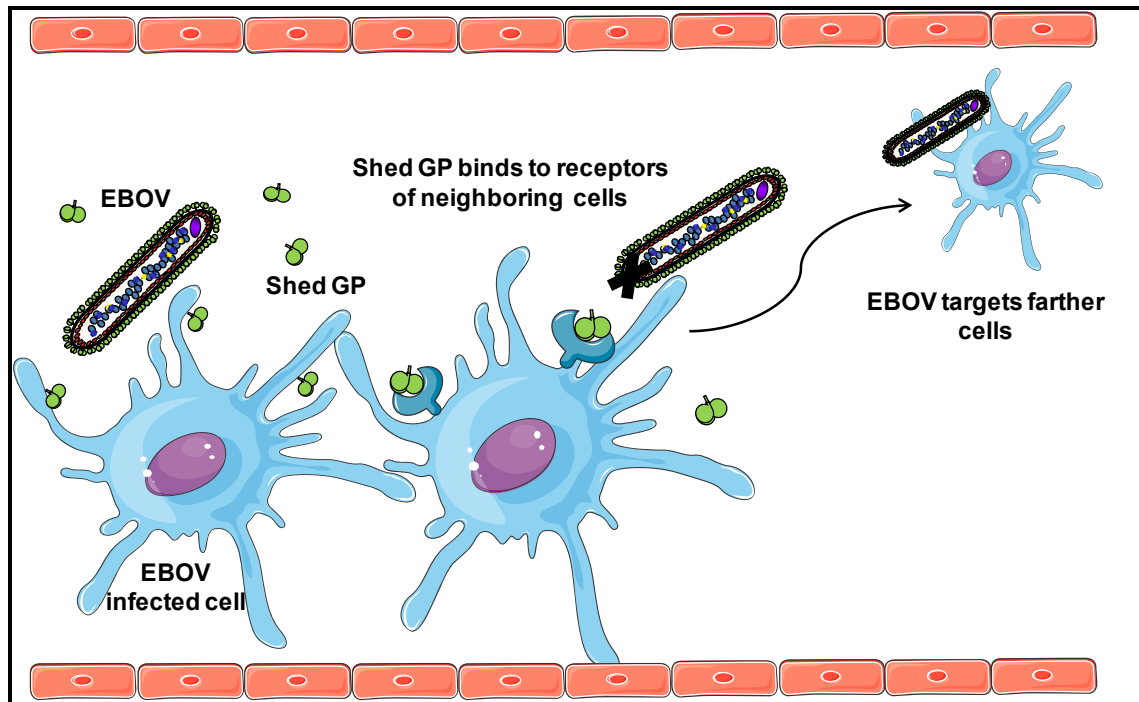


Figure 29.- Role of shed GP on virus spreading.- During EBOV infection, virions and soluble glycoprotein shed GP are released into the medium. Since shed GP shares receptors with surface GP present on virions, there is a competition established between them that will prevent some virus particles from binding to neighboring cells, which already have some of the receptors coupled to shed GP. This will contribute to virus spreading and dissemination into the bloodstream thus targeting more distant cells.

Shed GP as a key factor in EBOV pathogenicity

Ebola virus GP has been very studied due to its importance in viral entry and its potential as a target for vaccine development. Moreover, it has been shown to have a very important role in Ebola virus pathogenesis. First of all, surface GP is responsible for targeting cells that are important for EBOV pathogenesis, affecting them in several ways. It is capable not only to lead to immunosuppression by downregulating important molecules on cell surfaces which are important for lymphocyte binding and antigen presentation (Simmons, Wool-Lewis et al. 2002, Sullivan, Peterson et al. 2005, Reynard, Borowiak et al. 2009), but surface GP has also been shown to inflict direct cytotoxicity in

vascular cells by causing cell rounding and detachment, followed by cell death (Yang, Duckers et al. 2000). Other cell lines, such as 293T and macrophages have shown to be affected by this phenomenon (Simmons, Wool-Lewis et al. 2002, Alazard-Dany, Volchkova et al. 2006). These effects are mediated by the mucin-domain of GP, which is heavily glycosylated. However, it is important to take into account that infection can be influenced by multiple mechanisms such as pattern recognition, cytokine production and other factors such as adherence to host cells and growth rate and that the different glycosylation patterns observed in the different strains of EBOV could imply a difference in pathogenicity. One study has shown that GP is a major pathogenicity factor (Groseth, Marzi et al. 2012), as when they exchanged surface glycoproteins of Zaire EBOV and Reston EBOV (more and less pathogenic strains respectively) they observed that recombinant RestonGP-Zaire EBOV was less lethal for mice when compared to wild type Zaire EBOV. However, ZaireGP-Reston EBOV did not render the virus more lethal when compared with wild type Reston EBOV meaning that GP, even if it does contribute to filovirus virulence, is not the only pathogenic factor of the virus and other viral elements are needed for the acquisition of full virulence.

Our data demonstrates several common characteristics shared between surface GP and shed GP. Even if, differently from surface GP, shed GP does not produce cell rounding and death, it is able to target similar cells to surface GP including monocytes, macrophages, DCs and endothelial cells and activate them to produce pro- and antiinflammatory cytokines and vascular markers of endothelial permeability. This indicates that in vivo shed GP must surely contribute to EBOV pathogenicity. It is important to stress that shed GP, differently from surface GP, would target cells that are still non-infected. This is a very important phenomenon because (i) it allows the virus to affect the immune system without the need for infection, (ii) it allows the recruitment of new possible targets such as neutrophils, monocytes, DCs... and (iii) that shed GP can easily spread through the bloodstream reaching farther and different targets (other immune cells in other places in the body, other organs such as kidney, liver...). Therefore, shed GP probably triggers inflammation processes that on one side allow the recruitment of new possible targets and on the other

side induces migration of already infected cells to new areas, spreading infection.

Activation of the innate immune system is initiated by soluble pattern recognition molecules, which are expressed either bound to the extracellular matrix on innate immune cells or circulating in the blood as soluble molecules. One such soluble pattern recognition molecule is MBL, which is mostly (>95%) synthesized in the liver and secreted to circulate in the blood (Uemura, Saka et al. 2002, Takahashi, Ip et al. 2006). The innate immune response generates signals to activate appropriate effector molecules and cells of the adaptive immune system. MBL, as a pattern recognition molecule of the innate immune system, provides a surveillance system both locally and systemically. In this manner MBL can contribute both to immunity from pathogens as well as maintenance of tissue integrity and homeostasis. It has been recognized that there is significant interaction among the regulatory and effector systems of inflammation, complement activation, coagulation and vascular permeability (Dugina, Kiseleva et al. 2002, Levi, Keller et al. 2003, Takahashi 2011); the discoordination of these systems can have important pathologic consequences (including systemic inflammation, coagulopathy such as DIC and organ failure amongst others). MBL plays a very important role in inflammation, coagulation and immunity, and is a crucial point of connection for these systems. Their coordination is essential to maintain blood fluidity and preserve homeostasis throughout the body, and dysregulation by shed GP may lead to the systemic complications associated with EBOV infection.

Further experiments should be done with shed GP in vivo in order to fully ascertain its role in EBOV pathogenicity. Since differences in the amino acid sequence of the TACE cleavage site have been found between different strains of EBOV (**Table 7**), it would be interesting, for instance, to generate recombinant Ebola viruses where only the TACE site has been modified by exchanging TACE cleavage site sequence between the different strains of Ebola virus and to compare if infection of cells and guinea pigs results in differences in infection and pathogenicity.

TACE cleavage site								
	P5	P4	P3	P2	P1	P1'	P2'	P3'
ZEBOV	K	T	L	P	D	Q	G	D
SEBOV	N	P	L	P	N	Q	D	N
CIEBOV	N	N	L	P	D	Q	N	D
BEBOV	K	P	L	P	D	Q	D	T
REBOV	N	P	L	P	D	H	G	D

Table 7.- Sequence of the TACE cleavage site in the different EBOV strains. P1-P5 correspond to positions 1 to 5 up-stream of the cleavage site. P1'-P3' correspond to positions 1 to 3 down-stream of TACE cleavage site. Black arrow and dotted line indicate TACE cleavage site.

Our current observations broaden our view of the functional importance of shed GP by demonstrating a biological activity for it. Together, the data so far suggest that shed GP induces the release of a cocktail of proinflammatory cytokines and other mediators, such as TF, from non-infected macrophages and DCs, that as consequence damage the vascular endothelium, but also activates the coagulation cascade. The impairment of these systems could lead to multiorgan failure and a syndrome that in some ways resembles septic shock. Moreover it should be taken into account that besides the impairment of these systems, the activation of MBL by shed GP in these processes probably also represents a defective activity of the opsonic function of MBL and therefore affects a host's capacity to neutralize the virus. Finally, the fact that shed GP shares receptors with EBOV might increase the virus presence in bloodstream and assist its spread into further zones of the body thus facilitating the access to new cellular targets. Altogether, our data provides firm evidence that shed GP affects several linked processes including immune dysregulation, impairment of coagulation and vascular systems, and potentially increase of viremia thus meaning that shed GP could be a major factor contributing to EBOV GP virulence and/or pathogenesis.

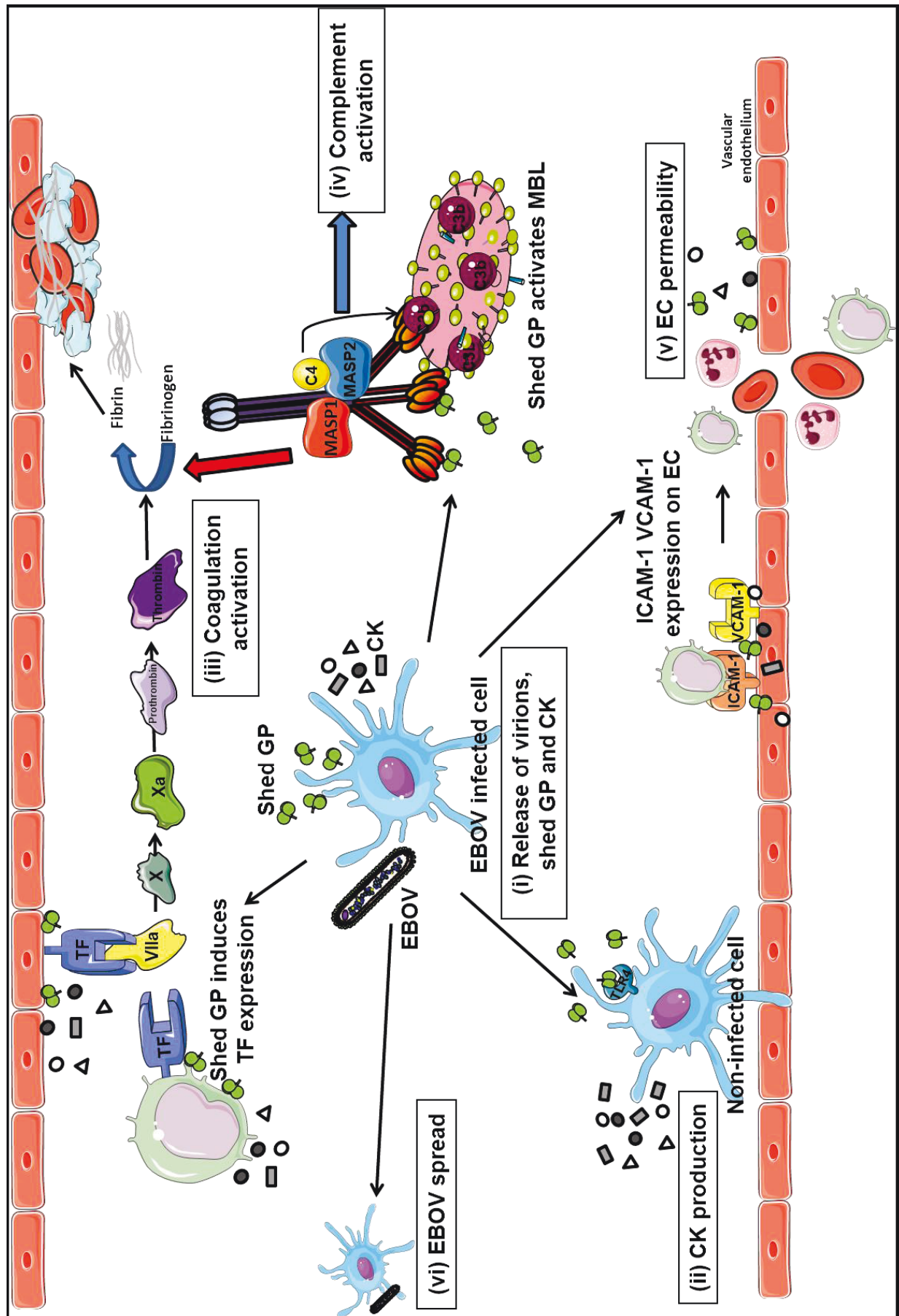


Figure 30.-Role of shed GP during EBOV pathogenesis.- In our model, EBOV-infected cells release both virions, soluble shed GP and cytokines (CK) **(i)**. Shed GP binds to DCs and macrophages via TLR4 and activates these cells for release of pro- and anti-inflammatory cytokines **(ii)**. The soluble nature of shed GP enables triggering of additional and remote targets which are still not infected. Release of cytokines from these cells will further amplify inflammation by recruiting and activating other APCs, lymphocytes, endothelial cells, and other cell types including hepatocytes. Overall the release of shed GP is likely to contribute to increased permeability, disseminated intravascular coagulation, dysregulated inflammation and impairment of cell function and organ failure **(iii, iv, v)**. Shed GP itself and the CKs released due to its binding to primary targets, are able to directly induce expression of tissue factor (TF) on monocytes, macrophages and endothelial cells that will initiate the coagulation pathway **(iii)**. Shed GP and CKs can also activate MBL and MASP-1 will contribute to coagulation activation and clot formation **(iii)** and MASP-2 will promote C4 fixation and complement pathway activation **(iv)**, thus preventing pathogen opsonisation by phagocytic cells. Since shed GP and CKs are both able to induce expression of adhesion molecules such as ICAM-1 and VCAM-1, they may increase leukocyte rolling and firm fixation to the endothelium; moreover shed GP itself and CKs can also induce EC permeability **(v)** thus probably contributing to vascular impairment observed during EBOV infection. Finally, binding of shed GP to similar receptors as surface GP could contribute to virus spreading **(vi)** through the bloodstream by preventing EBOV from binding neighboring cells. Altogether, our model indicates that shed GP contributes to the high pathogenicity of Ebola virus by altering essential systems to the detriment of host homeostasis and survival.

Overall shed GP would contribute to the high pathogenicity of EBOV in very diverse ways, all linked to EBOV pathogenesis: i) by inducing an exacerbated pro-inflammatory response, ii) by triggering MBL and preventing it from neutralizing the virus and iii) by preventing MBL from blocking TLR4 and from preventing cytokine activation by both virus and shed GP itself, iv) by inducing coagulation activation through MBL, v) by increasing directly and indirectly endothelial cells permeability and by vi) favoring virus spreading.

Since shed GP seems to contribute to the virulence of ZEBOV, its administration could result in an effective therapeutic strategy that may induce an appropriate innate and adaptative immune response before infection. A proper inflammatory response combined with the production of antibodies against EBOV may result in a prophylactic treatment of EBOV infection. However, since it is also able to activate endothelial leakage and to impair the coagulation pathway, its production and administration should still be studied and optimized in the guinea pig model that, after the NHP model, most accurately reflects coagulation impairment during EBOV infection.

Beyond shed GP's application as a treatment at controlled levels and prior to contact with the virus, the immunogenicity of shed GP in human DCs and macrophages described herein also points toward its role during Ebola virus high pathogenicity and also provides a better understanding about the mechanism responsible for the interaction with different components of the host immune system during Ebola virus infection.

MATERIAL AND METHODS

Cell lines and plasmids

Both HEK 293T cells and Vero E6 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, PAA laboratories) supplemented with 10% fetal calf serum (PerbioHyclone) and without antibiotics. For transient protein expression 293T cells were seeded into 150 cm² flasks 24 h prior to transfection and were cultured in DMEM supplemented with 10% fetal calf serum (PerbioHyclone) and without antibiotics. Human umbilical vein endothelial cells (HUVECs) (Lonza) were maintained at 37°C with 5% CO₂ in EGM-2 Media (Lonza) enriched with EGM-2 Single Quots containing FBS, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, hEGF, GA-1000 and heparin. Cells were cultured for 3-4 days with EGM-2 medium and were passaged for no more than 5 generations for the experiments described.

We have previously demonstrated that the substitutions L635V and D637V dramatically alter the efficacy of EBOV GP shedding in a transient expression system (Dolnik, Volchkova et al. 2004). In this study to increase the efficiency of GP shedding in a transient expression system we introduced the substitutions D637A and Q638V using site-directed mutagenesis of a plasmid encoding wild-type GP. The resulting plasmid was designated phCMVGP-HS. Plasmid expressing GP with a substitution L635V (phCMVGP-LS) was described earlier (Dolnik, Volchkova et al. 2004). Plasmid expressing transmembrane-anchor truncated mutant GP_{ΔTM} was generated by introducing a stop codon at position 651 using site-directed mutagenesis of a plasmid encoding wild-type GP.

Viruses

Recombinant EBOV-HS (with mutation D637L) and EBOV-LS (with mutation L635V) and EBOV-WT (with no mutations) were used in order to produce supernatants containing respectively high, low and wild type quantities of shed GP in supernatants. For production, Vero E6 were grown for 24h to a confluence of ~40% in 150 cm² flasks; cells were then infected with 1:500 dilution of these recombinant viruses and after 5 days in culture, supernatants were collected, centrifugated 10 minutes at 1000 rpm and then ultracentrifuged with a 20% sucrose cushion at 25000 rpm for 50 minutes at 4°C using SW32

rotor. The clarified supernatants were aliquoted and stored at -80°C . Viruses were then titrated using TCID₅₀ technique.

Transient expression of EBOV GP

293T cells were grown for 24h to a confluence of ~60%. Transfection of cells with recombinant plasmids was performed using TurboFect reagent (Thermo). Briefly, 20 μg of respective plasmids (HS, LS, GP _{ΔTM} , sGP and GFP) were added to 500 μl of DMEM with no serum in a ratio 1:4 (plasmid:turbofect); the mixture was incubated for 20 minutes at room temperature and added to 150 cm^2 flasks containing 60% confluent 293T cells in 15 ml of VP-SFM medium (Gibco). Three hours later 25 ml of VP-SFM were added in each flask. During GP expression the cells were kept in VP-SFM medium containing no serum. Culture supernatants were harvested 36h post-transfection, clarified by centrifugation at 1000 rpm for 10 min at 4°C and then by centrifugation at 28,000 rpm at 4°C for 2 h using a SW28 rotor with a Beckman Optima L-70K ultracentrifuge. The clarified supernatants were concentrated using Concentrator tubes (Pierce) with a molecular weight cut off of 20,000 MWCO. All samples were aliquoted and stored at -80°C .

Culture of primary human monocyte-derived-DCs and macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by centrifugation onto a lymphocyte separation medium (Ficoll) cushion; briefly, 16 Leucosep tubes (Dutscher) were filled with 15ml of Ficoll and centrifuged at 1700 rpm for 1 minute. Then 31 ml of blood were added and Leucosep tubes were centrifuged for 30 minutes at 1700rpm with no acceleration nor brakes. PBMC rings were then collected in RPMI-1640 (Life Technologies) supplemented with 200 mM L-glutamine (Life Technologies), 10 mM HEPES buffer (Life Technologies), 10% de-complemented FCS and 8 $\mu\text{g}/\text{ml}$ of Gentamicine (Life Technologies) (complete RPMI). Tubes containing PBMCs in complete RPMI were washed by centrifugation three times at 1600 rpm for ten minutes. After each centrifugation supernatant was replaced by 40 ml of fresh complete RPMI medium. Then PBMCs were enriched by centrifugation through a Percoll density gradient. Briefly, 22,5 ml of Percoll, 2,5 ml of PBS 10x and 25ml of PBS 1x with CaCl₂ were mixed and 6ml were

deposited in 8 x 15ml tubes. Carefully, 3 ml containing 3×10^7 cells/ml were loaded on the 6ml of Percoll. Tubes were then centrifuged for 20 minutes at 2000rpm with no acceleration nor brakes. The recovered monocyte fraction, contained not in the pellet but in the ring between medium and percoll, was washed three times as explained before (1600rpm, 10 minutes) and were depleted of cell contaminants by positive selection (Miltenyi Biotec), ensuring purification rates of $\geq 95\%$. Briefly, the cell pellets recovered after washing centrifugations were resuspended in 80 μ l of Equilibration Buffer (0,5% Bovine Serum Albumin, 2mM EDTA and PBS) and 20 μ l of CD14 MicroBeads (Miltenyi Biotec) per 10^7 total cells. Mixture was incubated at 4°C for 15 minutes and then was centrifuged at 300xg for 10 minutes and resuspended in 3ml of Equilibrium Buffer. Columns with additional filters (to remove cell clumps) were then placed on the magnetic field and were rinsed with 3ml of Equilibrium buffer. Cell suspension was then applied to the column. Unlabeled cells were collected and three washings steps were performed by adding 3ml of Equilibrium buffer. Columns were then removed and 5ml of Equilibrium Buffer were added and placed on a collection tube. Magnetically labelled cells were flushed out by pushing the plunger into the column and the eluted fraction was resuspended in complete RPMI. Cells were then washed three times as before (1600rpm, 10 minutes) and resuspended in RPMI-1640. Immature DCs and monocyte-derived macrophages were obtained upon culturing of cells in complete RPMI-1640 for 5 days either with 40 ng/ml of granulocyte- macrophage colony-stimulating factor (GM-CSF) or 50 ng/ml of interleukin-4 (IL-4) and 40 ng/ml of macrophage colony-stimulating factor (M-CSF), respectively.

Endotoxin test

The *Limulus* amebocyte lysate test (LAL Chromogenic Endotoxin Quantitation Kit, Pierce) was used to determine endotoxin levels in culture supernatants used in this study. The Endotoxin Standard vial containing 26EU of lyophilized endotoxin was reconstituted by adding 1mL of room temperature endotoxin-free water to make Endotoxin Standard Stock. Then, from this initial tube, standard stock solutions were made at 1, 0,5, 0,25 and 0,1 EU/ml. Then the microplate was warmed for 10 minutes at 37°C. With the microplate maintained at 37°C, 50

μl of each standard or unknown sample replicate were added and incubated for 5 minutes at 37°C. Then, 50 μl of LAL were added to each well, gently shaken and incubated at 37°C for 10 minutes. After exactly 10 minutes, 100 μl of substrate solution was added to each well and incubated for 6 minutes at 37°C. After that, 50 μl of Stop Reagent (25% acetic acid) were added, maintaining the same pipetting order as at the beginning. The absorbance was then measured at 405-410nm on Tecan 200. All values were less or equal to those of VP-SFM medium (<0.25 endotoxin units per ml).

Sedimentation analysis

The oligomeric structures of EBOV shed GP and transmembrane-anchor truncated mutant GP_{1,2Δ} were compared using a sedimentation assay. 293T cells were transfected with plasmids phCMVGP-HS or phCMVGP_{1,2Δ} and culture medium was collected 36h post-transfection. Medium was clarified by low-speed centrifugation and subjected to ultracentrifugation through a linear 5–25% (w/w) sucrose gradient prepared in co-IP buffer (1% NP40, 0.4% deoxycholate, 5 mM EDTA, 100 mM NaCl, 20 mM Tris–HCl, pH 7.5, 25 mM iodacetamide) on a Beckman Optima L-70K ultracentrifuge using a SW60 rotor for 21 h at 40 000 rpm, 10°C. Fractions were collected from the bottom to the top, proteins were separated by SDS–PAGE and analyzed by Western blot using anti-GP antibodies.

Binding assay

5×10^5 monocyte-derived DCs or macrophages per well were placed in conic-bottom 96-well plates and plates were centrifuged at 2000 rpm for 2 min. The culture medium was removed and replaced by medium containing EBOV GPs (in VP-SFM medium, Gibco). Cells were incubated for 1 hour at 4°C, washed twice with ice-cold phosphate-buffered saline (PBS) and then incubated in FACS buffer (PBS containing 2% FBS, 2% normal rat serum, 2% normal hamster serum, 2% normal mouse serum and 2% human FcR blocking solution, Milteny Biotec) at 4°C for 30 min. Cells were then incubated with mouse anti-GP₁ antibody for 1 hour at 4°C, washed twice with PBS and incubated with anti-mouse IgG1 coupled to Alexa 488 for DCs and MØ or to Allophycocyanin (APC)

for lymphocytes for 30min at 4°C. All mAbs were purchased from BD Pharmingen except those indicated otherwise. After incubation with antibodies, cells were washed and fixed with 1% PFA solution. Cells were analyzed using a Becton Dickinson LSRII flow cytometer (BD) and FlowJo software. In some experiments samples of GP were incubated with human sera containing MBL (150ng/ml) or MBL- deficient sera (Statens Serum Institute, Denmark) for 1 hour at 4°C prior to addition to immune cells. In TLR4-related experiments, cells were incubated for 10 min at room temperature with 30 µg/ml of anti-TLR4 antibodies (HTA125) or unspecific isotype antibody prior to addition of EBOV GPs.

Treatment of cells with EBOV GPs

5×10⁵ of monocyte-derived DC or macrophages per well were placed in 48-well plates and after 5 days differentiation, media was replaced with 600 µl of fresh RPMI 1640 containing 200 mM L-glutamine, 10 mM HEPES buffer, 5 mM CaCl₂ and 400 µl of culture supernatants containing soluble EBOV glycoproteins (in VP-SFM medium) or 500 pg/ml of LPS as positive control. Cells were incubated for 4, 8, 12 and 24h at 37°C and culture supernatants were collected, clarified from cell debris by centrifugation (1600 rpm at 4°C for 10 min), aliquoted, and stored at -80°C until analysis. The cells were also used for RNA extraction and real time RT-PCR analysis. Experiments using either anti-TLR4 antibodies or sera containing MBL were performed as described above.

In all assays except deglycosylation experiments, shed GP was used at a final concentration of 2 µg/ml. In deglycosylation experiments shed GP was used at a concentration of 0.5 µg/ml.

Real time RT-PCR quantitative analysis

RNAs from monocyte-derived DCs and macrophages treated with EBOV GPs were isolated using Total RNA Isolation kit (Machery Nagel). Briefly, 300 µl of RLT buffer were used to lyse cells contained in each well (5×10⁵ cells) and purified RNA was eluted in 100 µl of water. Then cDNAs were generated using iScript cDNA Synthesis kit (Bio-Rad); reaction was setup by adding 2 µl of RNA template to 8 µl of iScript reaction mix, 2 µl of iScript reverse transcriptase and

28 µl nuclease-free water. Complete reaction mix was then incubated at 5 minutes at 25°C, then 30 minutes at 42°C and finally 5 minutes at 85°C.

PCR products were then synthesized from cDNA samples by real-time PCRs using FastStart Universal SYBR Green Master (ROX) in triplicate following manufacturer's recommendations. Briefly, each reaction was performed in a total volume of 50 µl containing: 25 µl of FastStart Universal SYBR Green Master (ROX), 0,5 µl of forward primer and 0,5 µl of reverse primer at a final concentration of 300 µM each, 23 µl of RNase/DNase free water and 1 µl of template cDNA. Parameters used to program STEPONE Plus machine (Roche) were 10 minutes at 95°C in order to activate the FastStart Taq DNA Polymerase and then 40 cycles comprising: 10 seconds at 95°C and 30 seconds at 60°C in order to amplify PCR products. Validated RT-PCR primers specific for human TNF α (5'-CCTGCCCCAATCCCTTTATT and 5'-CCCTAAGCCCCCAATTCTCT), IL-6 (5'-TGCAATAACCACCCCTGACC and 5'-TGCGCAGAATGAGATGAGTTG), IL10 (5'-GAGGCTACGGCGCTGTCAT and 5'-CCACGGCCTTGCTCTTGTT), IL12p40 (5'-CCAGAGCAGTGAGGTCTTAGGC and 5'-TGTGAAGCAGCAGGAGCG) and GAPDH (5'-CCATGTTTCGTCATGGGTGTG and 5'-GGTGCTAAGCAGTTGGTGGTG) were used to quantify mRNA levels. The real-time PCR analysis was performed on STEPONE Plus apparatus using Roche software. Relative quantification was made by normalization to GAPDH mRNA levels. All samples were also normalized to mock.

Cytokine release

Culture supernatants from monocyte-derived DCs or macrophages treated with 2 µg of soluble EBOV GPs for 24 hours were assayed for the presence of TNF- α , IL-6, IL-8, IL12p40, IL1 β , IL10 and IL1-RA using Multiplex cytometric bead array (Bio-Rad) in Luminex MAGPIX. The assay was performed according to the manufacturer's instructions. First, each lyophilized cytokine standard (1 and 2) were reconstituted by adding 500 µl of RPMI not containing serum, then were vortexed for 5 seconds and incubated on ice for 30 minutes. During this time, frozen samples at -80°C were slowly thawed in ice. After 30 minutes of incubation, eight serial dilutions (S1-S8) were made by adding 72 µl of standard

1 and 72 μ l of standard 2 in S1 and then, four-fold serial dilution were made from S2 to S8 by adding 50 μ l in 150 μ l of RPMI with no serum in each tube.

Magnetic beads specific for each cytokine were then vortexed and 575 μ l of each (TNF- α , IL-6, IL-8, IL12p40, IL1 β , IL10 and IL1-RA) were added to 1725 μ l of assay buffer (provided with the kit). Then, 50 μ l of beads were added per well and 50 μ l samples and standards were also added in triplicate. Incubation of the plate, previously covered with sealing tape and aluminium, was carried out in the dark on a shaker at 300 rpm for 1 hour at room temperature. Then wells were washed with 100 μ l of washing buffer (provided in the kit) using a magnet to keep beads attached during washing and 300 μ l of each specific antibody against beads were added to 900 μ l of Bio-Plex detection antibody diluent (provided in the kit). Incubation was done as before: shaking, in the dark for 1 hour. Finally, after washing with 100 μ l of washing buffer, 60 μ l of streptavidin-PE (provided in the kit) were added to 5940 μ l of assay buffer and 50 μ l of this mixture was added per well and incubated for 10 minutes at room temperature. Then wells were washed with 100 μ l of washing buffer and add 125 μ l of assay buffer per well. Plate was then read using Luminex MAGPIX.

Expression of co-stimulatory molecules

Both monocyte-derived DCs and macrophages treated with 2 μ g soluble EBOV GPs for 48 hours were placed in conic-bottom 96-well plates and plates were centrifuged at 2000 rpm for 2 min. Cells were then washed in cold PBS supplemented with 2% fetal bovine serum and pre-blocked at 4°C for 30 min in FACS buffer (PBS containing 2% FBS, 2% normal rat serum, 2% normal hamster serum, 2% normal mouse serum and 2% human FcR blocking solution, Milteny Biotec). Cells were then washed with PBS and cell-surface staining was performed using 1 μ l of the following antibodies: FITC-conjugated anti-CD80, PE-conjugated anti-CD40 (Beckman Coulter) and APC-conjugated anti-CD83, Peridinin Chlorophyll Protein Complex Vio 700 (PerCP-Vio700)-conjugated anti-CD86 (Miltenyi Biotec). After incubation with antibodies for 1 hour at 4°C, cells were washed three times with PBS, fixed with 1% PFA solution and analyzed using a Becton Dickinson LSRII flow cytometer (BD) and FlowJo software. A minimum of 10,000 events were collected and analyzed for each sample.

Deglycosylation assay

For GP deglycosylation, supernatants containing EBOV GPs were treated for 48 hours using EDEGLY kit (Sigma) containing PNGase F, α -(2 \rightarrow 3,6,8,9)-Neuraminidase, O-Glycosidase, β (1 \rightarrow 4)-Galactosidase and β -N-Acetylglucosaminidase. Shed GP mixed with deglycosylation enzymes inactivated at 80°C for 30 min were used as a control. Monocyte-derived DCs and macrophages were treated with 100 μ l of deglycosylated shed GP samples for 24 hours and culture supernatants were analysed by western blot in non-reducing conditions and assayed for the presence of TNF α using a Multiplex cytometric bead array (Bio-Rad) in Luminex MAGPIX.

HUVEC permeabilization assay

HUVEC were grown in collagen-coated inserts of permeability assay chambers (Millipore) for 72h until confluence. The media was replaced with 600 μ l of fresh EBM (endothelial cell basal medium without phenol red, Lonza) and 400 μ l of culture supernatants containing soluble EBOV glycoproteins (VP-SFM medium, Gibco) or 400 μ l of culture supernatants from MØ previously stimulated with 2 μ g of soluble EBOV glycoproteins for 24h. After 22 h of incubation, FITC-Dextran was added to each insert and FITC-Dextran leaking out into the bottom chamber was measured. Monolayer permeability was assessed both on the basis of fluorescence intensities measured using a Tecan fluorometer and the integrity of the HUVEC monolayer using a MacroFluo microscope.

EBOV replication in human monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors as explained before. After 24 hours, supernatant was removed by centrifugation at 1500 for 5 minutes. Then, 400 μ l of supernatants from recombinant viruses EBOV-HS, EBOV-LS and EBOV-WT were added together with 600 μ l of RPMI medium with no serum for 2 hours at 37°C. After that, 1 ml containing of RPMI 5% of FBS was added per well (to a final concentration of 2.5% of serum). After 48 hours at 37°C, monocytes were centrifuged three times at 1500 rpm for 5 minutes and supernatants were removed and replaced

by RPMI with no serum. Monocytes were then infected with recombinant EBOV expressing GFP (rEBOV-GFP) at a MOI of 1 for 2 hours at 37°C. Monocytes were then washed and incubated with RPMI medium containing 2,5% of serum for 48 hours at 37°C and fluorescence was then analyzed by flow cytometry.

Surface Plasmon Resonance/Fluid-phase interaction between MBL and Ebola soluble glycoproteins

Real time surface plasmon resonance spectroscopy was performed using a Biacore T100 biosensor. Briefly, recombinant MBL, kindly provided by Jens Christian Jensenius, was covalently bound to CM5 biosensorchip as ligand according to the manufacturer's instructions. Briefly, the sensorchip was activated by injection of 80 µl of NHS/EDC (Amine Coupling Kit, GE Healthcare) at 10µl/min for 7 minutes. Then, 200 µg/ml of rhMBL in 120 µl of Acetate 10mM pH 4 was coupled to the surface of the sensorchip by injection into one channel at 10 µL/minute flow rate to a refractive unit (RU) value of 7000. Another channel was used as a negative control. Deactivation of unreacted NHS-esters was done by injection of 80 µl of ethanolamine 1M pH 8.5 (Amine Coupling Kit, GE Healthcare) at 10µl/min for 7min. Analytes containing soluble glycoproteins in running buffer with no detergent (TBS 10 mM HEPES buffer [pH 7.4], 150 mM NaCl, 10mM CaCl₂) were then were flowed onto the coated surface (10 µl/minute for a 7-minute association time), after which the channels were rinsed with the running buffer (TBS 10 mM HEPES buffer [pH 7.4], 150 mM NaCl, 10mM CaCl₂, 0.05% surfactant P-20 10%) to analyze the dissociation phase. Sensorchip was regenerated by the injection of 100 µl of 2M NaCl at 10 µl/ml for 7 minutes. Affinities were determined by analysis of the association using BIAevaluation software.

Measurement of shed GP binding to rMBL by ELISA

Shed GP bound to rMBL was evaluated by ELISA. Briefly, flat-bottomed maxisorp polystyrene tissue culture plates (Nunc) were coated in triplicate overnight at 4°C with recombinant MBL (20 µg/well) in TBS containing 10 mM CaCl₂. Wells were then incubated with supernatants containing VSV GP (as positive control), VSV G (as negative control) and supernatants containing 1 µg

of HS, 500 ng of HS, 250 ng of HS, GP_{ΔTM}, LS, sGP, mock (supernatant from GFP expressing cells) from transiently transfected cells. After 1 hour of incubation at room temperature, wells were blocked for 1 hour at room temperature with 3% milk in TBS buffer containing 10 mM CaCl₂. Wells were then washed three times with 200 μl of TBS 0,1% Tween containing 10 mM CaCl₂ and incubated with 1:20000 dilution of α-GP antibody 3357 in TBS containing 10 mM CaCl₂. After consecutive washing as before, wells were incubated for 30 minutes at room temperature with 100 μl containing secondary antibody α-mouse-HRP at 1:20000 dilution in TBS 10 mM CaCl₂. Wells were then washed three times as before and were developed using 100 μl of TMB according to manufacturer's instructions; reaction was stopped with 100 μl of 10% phosphoric acid in water and absorbance was read at 405 nm.

Activation of MASP-1

Flat-bottomed maxisorp polystyrene tissue culture plates (Nunc) were coated in triplicate for 1 hour at room temperature with 2ug per well of α-GP antibody in TBS supplemented with 10mM CaCl₂. Wells were washed with 40mM HEPES, 2M NaCl, 10mM CaCl₂ pH 7,4 (buffer 1) and incubated by shaking overnight at 4°C with 100 μl of supernatants and mannan (1 mg/ml) in mock supernatant containing 5mM CaCl₂. Mannan was used as a representative ligand of MBL. Wells were washed with buffer 1 three times. Human serum containing 30 μg/ml of recombinant MBL was 1:1 diluted in buffer 1 and 100 μl were added in the coated plate for 1 hour in ice. After consecutive washing twice with 20 mM HEPES, 1 M NaCl, 5mM CaCl₂, 0,1% Tween-20 pH 7,4 (buffer 2) and three times with 20 mM HEPES, 140mM NaCl, 5mM CaCl₂, 0,1% Tween-20 pH 7,4 (buffer 3), 100 μl of 0,2mM VPR-AMC (R&D systems) in 20 mM HEPES, 5mM CaCl₂ pH 8,5 (buffer 4) was added. The samples were excited at 355nm and emission read at 460nm every 60s for 1h using a TECAN 200 plate reader. Experiments in BSL-4 conditions were done similarly but soluble glycoproteins were obtained from recombinant Ebola virus infected cells where virus had been clarified by ultracentrifugation as explained above. Supernatants came from EBOV-HS, EBOV-LS and EBOV-WT viruses.

The competition assay was done similarly but samples containing 2 µg of soluble glycoproteins were previously incubated with human serum containing 150 µg of MBL.

Activation of MASP-2/measurement of lectin complement pathway activity

MBL-bound-MASP-2 activity was evaluated by C4 fixation assay. Flat-bottomed maxisorp polystyrene tissue culture plates (Nunc) were coated in triplicate overnight at 4°C with 200 µl of supernatants and mannan (1 mg/ml) in mock supernatant containing 5mM CaCl₂. MBL-MASP complexes in buffer 1 were bound to mannan-coated wells as for MASP-1 assay above. Wells were then blocked with 20% gelatin in warm TBS buffer for 2 hours at 37°C and then were washed with TBS containing 0,05% Tween 20 and 10 mM CaCl₂. Human serum containing 25 µg/ml of MBL was 1:1 diluted in buffer 3 and incubated for 1 hour at 37°C. Following two washes with buffer 2 and three with buffer 3, 0,1 µg/well of purified C4 (Hycult biotech) in buffer 3 was added to each well and incubated for 1 hour at 37°C. Wells were then washed with buffer 3 and goat α-C4 (Abcam) was added to 3µg/ml in buffer 3 for 1 hour at 37°C. After washing three times with buffer 3, secondary antibody α-goat HRP was added to 1:2000 in buffer 3 and incubated 30 minutes at 37°C. Wells were then washed three times with buffer 3 and developed using TMB according to manufacturer's instructions; reaction was stopped with 10% phosphoric acid in water and absorbance was read at 405 nm. Experiments in BSL-4 conditions were done similarly but soluble glycoproteins were obtained from Ebola virus recombinant infected cells where virus had been clarified by ultracentrifugation as explained before. Supernatants came from EBOV-HS, EBOV-LS and EBOV-WT viruses. Competition assay was done similarly but samples containing 2 µg of soluble glycoproteins were previously incubated with human serum containing 150 µg of MBL.

Induction and detection of Tissue factor in monocytes, macrophages, DCs and HUVEC

Dendritic cells, macrophages and HUVEC were plated at 1x10⁶ cells/ml in 6-well plates. Medium in cells was removed and 600 µl of RPMI (for immune cells)

or 600 μ l of EGM-2 medium (for HUVEC) with no serum but supplemented as explained before was added. 400 μ l of soluble glycoproteins (shed GP, sGP, Low shedding, shed GP+MBL) and positive control TNF α (20ng/ml) was added per well and incubated for 24 hours. Cells were then trypsinized, centrifuged at 1200 rpm and resuspended in PBS in conic-bottom 96-well plates. Cells were then antibody blocked at 4°C for 30 min in FACS buffer (PBS containing 2% FBS, 2% normal rat serum, 2% normal hamster serum, 2% normal mouse serum and 2% human FcR blocking solution (Milteny Biotec). All mAbs were purchased from BD Pharmingen except where noted. Cells were then incubated with α -tissue factor for 1 hour at 4°C, washed twice with PBS and incubated with Alexa 488 for 30min at 4°C. After incubation with antibodies, cells were washed and fixed with 1% PFA solution. Cells were analyzed using a Becton Dickinson LSRII flow cytometer (BD) and FlowJo software.

Detection of soluble coagulation and permeability marker release in culture medium

Supernatants containing soluble glycoproteins and their respective controls were assayed in MAGPIX in order to determine soluble levels of tissue factor, thrombomodulin, ICAM-1, VCAM-1 and E-selectin using Multiplex cytometric bead array Milliplex kits Human Cardiovascular Disease Panel 2 and 4 (Millipore). Assay was performed according to the manufacturer's instructions. First, antibody-bead vials were sonicated for 30 seconds on ice at 10 kHz to avoid clumping. Then, 150 μ l from each antibody bead vial (ICAM-1, VCAM-1, E-selectin, sTissue Factor and sThrombomodulin) were added to the Mixing Bottle and were brought to a final volume of 3 mL with Bead Diluent (provided in the kit). Quality Control 1, Quality Control 2 and standards were each reconstituted with 250 μ l of deionized water, vortexed and left to sit for 10 minutes. Working standards were prepared by adding 250 μ l of Assay Buffer (provided in the kit) to each of 6 polypropylene tubes. Serial dilutions were prepared by adding 125 μ l of Reconstituted Standard to the 250 μ l of the next tube; after mixing, 125 μ l were transferred consecutively from tube 1 to 6. A 0 pg/ml Standard (Background) was made with Assay Buffer. Sixty milliliters of

Wash buffer 10x (provided in the kit) were diluted into 540 mL of deionized water.

Assay Buffer, 100 μ l, was added into each well of the plate and incubated at room temperature for 10 minutes. After its removal, 25 μ l of Assay Buffer and 25 μ l of culture medium (EGM-2) were added per well; then, 25 μ l of standards, Controls, background and samples were added into the appropriate wells. Twenty-five microliters of sonicated Mixed Beads were added to each well and incubated on a plate shaker overnight at 4°C. The content of the plate was then removed and wells were washed 3 times with wash buffer, using the magnet so not to lose beads during washing. Next, 50 μ l of Detection Antibodies (provided in the kit) were added to each well and incubated with agitation on a plate shaker for 1 hour at room temperature (20-25°C). Without washing, 50 μ l Streptavidin-Phycoerythrin were added to each well containing the 50 μ l of Detection Antibodies and the plate was incubated for 30 minutes at room temperature. Well contents were then removed, washed as explained before and 100 μ l of Drive Fluid was added to all wells. Beads were resuspended on a plate shaker for 5 minutes and the plate was run on a MAGPIX machine.

Plaque size assay

Confluent Vero cells in 12 well plates were incubated with supernatants containing HS, sGP and LS from transiently transfected cells for 1 hour at 37°C. Cells were then washed three times with room temperature PBS containing Ca^{+2} and Mg^{+2} and infected with recombinant VSV pseudotyped with Ebola virus GP (rVSV-GP) or wild-type VSV for 1 hour at 37°C. Cells were then washed three times with room temperature PBS containing Ca^{+2} and Mg^{+2} and medium was replaced by 3 milliliters of 2,5% medium containing soluble glycoproteins and avicell (final concentration 0,6%). Plaque size was analysed by counting plaques pixels with AxioVision software.

Binding and replication of rVSV GP-R18 in the presence of shed GP

VSV-containing cell culture supernatants were stained with 1 μ g/ml R18 for 1 h on a shaker at RT and then centrifuged (10 min, 3000 \times g at 4 °C). Supernatants were diluted in up to 3 ml of PBS containing Ca^{+2} and Mg^{+2} , loaded onto a 1 ml

20% sucrose cushion and ultra-centrifuged for 1 h at 50000 rpm at 6 °C (Himac CS 150GX ultra-centrifuge with an S80473 rotor). Pellets were washed with PBS containing Ca^{+2} and Mg^{+2} and re-ultracentrifuged. Pellets were finally resuspended in 200 μL PBS containing Ca^{+2} and Mg^{+2} . Vero cells were treated with HS or LS supernatant from transiently transfected cells for 1 hour at 4°C, washed with PBS Ca Mg three times and R18-labeled rVSV GP were added and binding was determined by flow cytometry.

For time course studies of rVSV-GP replication in the presence of HS or LS and mock as controls, Vero cells were incubated with HS, LS and mock (from GFP expressing cells) supernatants as explained before, and were then infected with rVSV-GP. Supernatants and cells were collected at 4, 8 and 12 hours post rVSV-GP infection and the presence of M protein of the virus was analysed by Western blot.

In BSL-4 conditions, Vero cells were incubated with mock supernatant or supernatants from recombinant viruses releasing high, low or normal amounts of shed GP into the media (named EBOV-HS, EBOV-LS and EBOV-WT respectively) for 1 hour at 37°C. Then cells were washed three times with PBS containing Ca^{+2} and Mg^{+2} and were infected with recombinant Ebola virus expressing GFP during infection (rEBOV-GFP). Replication was determined by flow cytometry 48 hours post infection.

PUBLICATIONS AND OTHER COMMUNICATIONS

Publications

- **Shed GP of Ebola Virus Triggers Systemic Immune Activation and Increased Vascular Permeability.** Escudero-Perez B., Volchkova V., Volchkov V., Submitted to PLoS Pathogens, May 2014.
- **Surface glycoproteins of the recently identified African Henipavirus promote viral entry and cell fusion in a range of human, simian and**

bat cell lines. Lawrence P, Escudero-Perez B. Drexler JF, Corman VM, Müller MA, Drosten C, Volchkov V., Virus Research, January 2014.

- **Dérégulation de l'hémostase dans l'infection à filovirus.** Reynard O, Escudero-Pérez B., Volchkov V., Submitted to Médecine/sciences, July 2014.

International Conferences attended

Oral presentations

- 2013 Oral presentation: **Soluble proteins of Ebola virus activate dendritic cells and macrophages causing release of pro- and anti-inflammatory cytokines.** Escudero Pérez B., Lawrence P., Volchkov V., Presented at 15th International Negative Strand Virus meeting, Palacio de Congresos, Granada, Spain
- 2013 Oral presentation: **Soluble proteins of Ebola virus activate dendritic cells and macrophages causing release of pro- and anti-inflammatory cytokines.** Escudero Pérez B., Lawrence P., Volchkov V., Presented at 5th European Congress of Virology, Centre de Congrès de Lyon, Lyon, France

Posters

- 2014 Poster: **Ebola virus shed GP binds and activates dendritic cells and macrophages for release of pro- and anti-inflammatory cytokines.** Escudero Pérez B., Lawrence P., Volchkova V., Reynard O., Volchkov V., Presented at European meeting on Viral zoonoses 2014, Saint-Raphael, France

- 2013 Poster: **Surface proteins of the recently identified African Henipavirus are able to promote viral entry, cell fusion and cytopathic effects in a range of cell lines from Human, Simian and Bat hosts.** Lawrence P., Drexler JF, Corman VM, , Escudero B., Volchkova V., Müller M, Drosten C., Volchkov V., Presented at International Negative Strand Virus meeting, Palacio de Congresos, Granada, Spain
- 2012 Poster: **Role of soluble proteins of Ebola virus in dendritic cell deregulation.** Escudero Pérez B., Reynard O., Lawrence P., Volchkov V., Presented at CFCD annual congres 2012 : « Diversité et Plasticité des Cellules Dendritiques », Institut Pasteur, Paris, France

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ANNEXES

GLYCOSYLATION IN EBOV

Glycosylation process

Glycosylated proteins (glycoproteins) are found in almost all living organisms including eukaryotes, eubacteria and archae.

Approximately half of all proteins typically expressed in a cell undergo glycosylation, which entails the covalent addition of sugar moieties to specific amino acids. Lipids and proteoglycans can also be glycosylated. Glycosylation takes place in the endoplasmic reticulum (ER) and Golgi apparatus (Wormald, Petrescu et al. 2002).

Protein glycosylation has multiple functions in the cell. In the ER, glycosylation is used to monitor the status of protein folding, acting as a quality control mechanism to ensure that only properly folded proteins are trafficked to the Golgi. Sugars on soluble proteins can be bound by specific receptors in the *trans* Golgi network to facilitate their delivery to the correct destination. These sugars can also act as ligands for receptors on the cell surface to mediate cell attachment or stimulate signal transduction pathways. Because they can be very large and bulky, oligosaccharides can affect protein-protein interactions by either facilitating or preventing proteins from binding to cognate interaction domains. Because they are hydrophilic, they can also alter the solubility of a protein (Wormald, Petrescu et al. 2002).

Glycoprotein diversity

Glycosylation increases the diversity of the proteome to a level unmatched by any other post-translational modification. The cell is able to facilitate this diversity, because almost every aspect of glycosylation can be modified, including:

- **Glycosidic linkage** – the site of glycan (oligosaccharide) binding
- **Glycan composition** – the types of sugars that are linked to a particular protein
- **Glycan structure** – branched or unbranched chains
- **Glycan length** – short- or long-chain oligosaccharides

Glycosylation is one of the most complex post-translational modification because of the large number of enzymatic steps involved. Glycosylation process includes linking monosaccharides together, transferring sugars from one substrate to another and trimming sugars from the glycan structure.

Glycosylation is non-templated, and thus, all of these steps do not necessarily occur during every glycosylation event. Instead of using templates, cells rely on a host of enzymes that add or remove sugars from one molecule to another to generate the diverse glycoproteins seen in a given cell. The different mechanisms of glycosylation are highly-ordered, step-wise reactions in which individual enzyme activity is dependent upon the completion of the previous enzymatic reaction. Because enzyme activity varies by cell type and intracellular compartment, cells can synthesize glycoproteins that differ from other cells in glycan structure (Spiro 2002).

Types of Glycosylation

Glycopeptide bonds can be classified in 5 groups (N-, O- and C-linked glycosylation, glypiation and phosphoglycosylation) based on the nature of the sugar-peptide bond and the oligosaccharide attached. N- and O-glycosylation are the glycosylations present in EBOV.

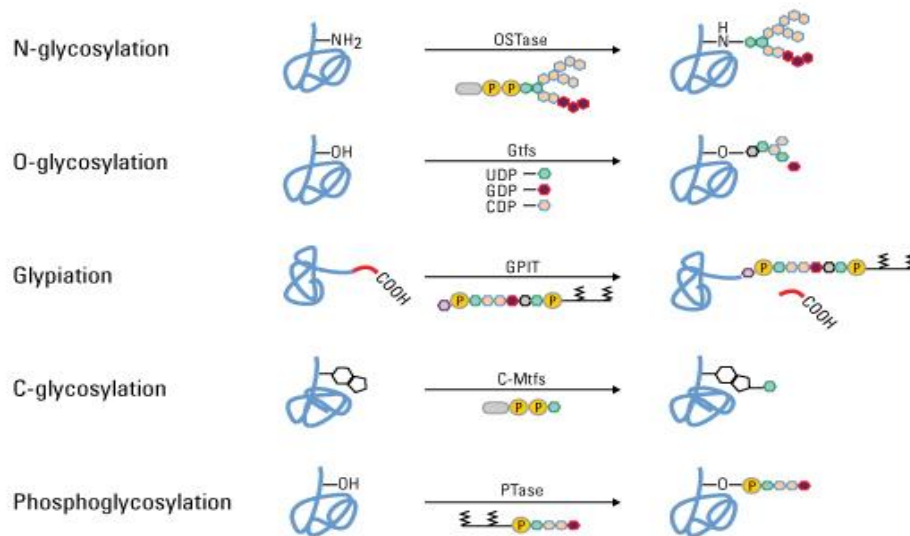
N-linked Glycan binds to the amino group of asparagine in the ER

O-linked Monosaccharides bind to the hydroxyl group of serine or threonine in the ER, Golgi, cytosol and nucleus

Glypiation Glycan core links a phospholipid and a protein

C-linked Mannose binds to the indole ring of tryptophan

Phosphoglycosylation Glycan binds to serine via phosphodiester bond



Proteins are not restricted to a particular type of glycosylation. Indeed, proteins are often glycosylated at multiple sites with different glycosidic linkages, which depends on the enzyme availability to glycosylate, the amino acid sequence and the protein conformation.

Glycoproteins are unique from other post-translationally modified proteins, because they are a combination of a protein portion and a glycan portion, both of which can comprise a significant proportion of the molecular weight of the molecule.

Glycosylation types in EBOV

N-Glycosylation

Glycosylation is often characterized as a post-translational modification. While this is true with other types of glycosylation, N-glycosylation often occurs co-translationally, in that the glycan is attached to the nascent protein as it is being translated and transported into the ER. The "N" in the name of this type of glycosylation denotes that the glycans are covalently bound to the carboxamido nitrogen on asparagine (Asn or N) residues.

Because the ER is the site of translation and processing of most membrane-bound and secreted proteins, it is not surprising that most of these are N-linked glycoproteins. Besides being the most common type of glycosylation (90% of glycoproteins are N-glycosylated), N-linked glycoproteins also have large and often extensively branched glycans that undergo multiple processing steps after being bound to proteins (Elgaard and Helenius 2003, Trombetta 2003).

O-Glycosylation

While N-glycosylation is the most common glycosidic linkage, O-glycoproteins also play a key role in cell biology. This type of glycosylation is essential in the biosynthesis of mucins, a family of heavily O-glycosylated, high-molecular weight proteins that form mucus secretions. O-glycosylation is also critical for the formation of proteoglycan core proteins that are used to make extracellular matrix components.

O-glycosylation occurs post-translationally on serine and threonine side chains in the Golgi apparatus. N-glycosylation does not preclude the other from occurring, as O-glycosylation commonly occurs on glycoproteins that were N-glycosylated in the ER. Besides the different linkage, O-glycosylation also differs in the method of glycosylation. While a precursor glycan is transferred *en bloc* to Asn via N-glycosylation, sugars are added one-at-a-time to serine or threonine residues. O-glycosylation can also occur on oxidized forms of lysine and proline. Additionally, O-linked glycans usually have much simpler oligosaccharide structures than N-linked glycans (Gemmell and Trimble 1999, Dell and Morris 2001).

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PUBLICATIONS

Shed GP of Ebola Virus Triggers Systemic Immune Activation and Increased Vascular Permeability.

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Short Title: Shed GP of Ebola Virus: Systemic Immune Activation and Vascular Permeability.

Abstract

During Ebola virus (EBOV) infection a significant amount of surface glycoprotein GP is shed from infected cells in a soluble form due to cleavage by cellular metalloprotease TACE. Shed GP and non-structural secreted glycoprotein sGP, both expressed from the same GP gene, have been detected in the blood of human patients and experimentally infected animals. In this study we demonstrate that shed GP plays a particular role during EBOV infection. In effect it binds and activates non-infected dendritic cells and macrophages inducing the secretion of pro- and anti-inflammatory cytokines (TNF α , IL1 β , IL6, IL8, IL12p40, and IL1-RA, IL10). Activation of these cells by shed GP correlates with the increase in surface expression of co-stimulatory molecules CD40, CD80, CD83 and CD86. Contrary to shed GP, secreted sGP activates neither DC nor macrophages while it could bind DCs. In this study, we show that shed

GP activity is mediated through cellular toll-like receptor 4 (TLR4) and is dependent on GP glycosylation. Treatment of cells with anti-TLR4 antibody completely abolishes shed GP-induced activation of cells. We also demonstrate that shed GP activity is negated upon addition of mannose-binding sera lectin MBL, a molecule known to interact with sugar arrays present on the surface of different microorganisms. Furthermore, we highlight the ability of shed GP to affect endothelial cell function both directly and indirectly, demonstrating the interplay between shed GP, systemic cytokine release and increased vascular permeability. In conclusion, shed GP released from virus-infected cells could activate non-infected DCs and macrophages causing the massive release of pro- and anti-inflammatory cytokines and effect vascular permeability. These activities could be at the heart of the excessive and dysregulated inflammatory host reactions to infection and thus contribute to high virus pathogenicity.

Author Summary

Ebola virus, a member of the Filoviridae family, causes lethal hemorrhagic fever in man and primates, displaying up to 90% mortality rates. Viral infection is typified by an excessive systemic inflammatory response resembling septic shock. It also damages endothelial cells and creates difficulty in coagulation, ultimately leading to haemorrhaging, organ failure and death.

A unique feature of EBOV is that following infection high amounts of truncated surface GP, named shed GP, are released from infected cells and are detected in the blood of patients and experimentally infected animals. However the role of shed GP in virus replication and pathogenicity is not yet clearly defined.

Here we show that shed GP released from virus-infected cells binds and activates non-infected DCs and macrophages causing the massive release of pro- and anti-

inflammatory cytokines and also effects vascular permeability. These activities could be at the heart of the excessive and dysregulated inflammatory host reactions to infection and thus contribute to high virus pathogenicity.

Introduction

Ebola virus (EBOV) is the causative agent of a haemorrhagic fever in humans responsible for sporadic outbreaks in several African countries including the current epidemic in Guinea, Liberia and Sierra Leone [1,2] and for which there is currently no vaccine or approved treatment [3]. Fatal EBOV infection involves massive disseminated viral replication and host immune dysregulation with uncontrolled cytokine secretion (for review, see [4]) and a disseminated intravascular coagulation syndrome resembling septic shock [5,6].

EBOV is a member of the *Mononegavirales* order *Filoviridae* family. Its genome consists of seven genes and encodes seven structural and at least one non-structural protein [7-9]. Due to RNA editing phenomenon, transcription of the GP gene results in the synthesis of several GP gene specific mRNAs coding for viral glycoproteins including non-structural sGP and surface virion GP [10,11]. Both glycoproteins are synthesized as a precursor molecule that is proteolytically cleaved by the cellular protease furin during intracellular processing [12]. sGP forms dimers [10,13] whereas the cleaved carboxy-terminal fragment, termed delta peptide, is a monomer [14]. Viral surface spikes are formed as a trimer of GP_{1,2}, made up of two subunits: GP₁ and GP₂ linked by a disulfide bond [15,16]. GP₁ is known to mediate virus attachment to host cells whereas GP₂ is involved in membrane fusion [11,17].

GP_{1,2} is a type I glycoprotein containing multiple N- and O-linked glycans [7,18]. The majority of O-glycans are grouped in a region termed mucin-like domain [18-20].

A unique feature of EBOV is that following infection, virus encoded glycoproteins are released from cells in soluble forms. High amounts of sGP and truncated surface GP are detected in the blood of patients and experimentally infected animals [11,21]. Release of sGP is explained by its secretion via the classical secretory pathway due to the lack of a transmembrane anchor [11,22]. Cleavage of surface GP by the cellular metalloprotease TACE (TNF α -converting enzyme), a member of the ADAM (*a disintegrin and metalloproteinase*) proteinase family, is responsible for GP shedding [21]. In this manner soluble shed GP resembles virion GP_{1,2}, and has been shown to bind and sequester virus-neutralizing antibodies directed against surface GP [21]. Aside from this antibody-neutralizing activity the role of shed GP in virus replication and pathogenicity has not yet been clearly defined.

Following EBOV infection, primary virus replication occurs in dendritic cells (DCs) and macrophages and is then followed by massive replication in hepatocytes and splenocytes [23-26]. It has recently been revealed that monocytes are also involved in virus replication albeit in an entry-delayed and differentiation-dependent fashion [27]. Massive release of cytokines, chemokines and vasoactive substances follows the course of infection thus promoting the development of inflammatory disorders and playing an important role in EBOV pathogenesis [24-26]. Overall, excessive inflammatory responses to infection contribute to massive infiltration of virus target cells to sites of infection but also result in the observed septic shock-like syndrome. Remarkably, little cytokine secretion is detected following EBOV infection of DCs which can be explained by their impaired maturation upon infection [26,28-30]. Monocytes also

appear to secrete inflammatory cytokines when infected, but at lower levels [27]. Based on in vitro data macrophages are the only cells that produce cytokines at relatively high levels following EBOV infection [26,28]. Using EBOV VLPs it has been demonstrated that cytokine and chemokine secretion from macrophages and DCs is likely to be dependent on GP [19,30,31] whereas the question remains as to how these host responses are triggered during infection.

In this study we identify the cellular targets for EBOV shed GP and answer the question if soluble EBOV glycoproteins could trigger the release of cytokines from non-infected immune cells. We show that shed GP but not sGP activates human-derived DCs and macrophages and induces the secretion of pro- and anti-inflammatory cytokines. We demonstrate that this activation can be abolished by anti-TLR4 antibodies or by addition of sera lectin MBL. Moreover, we show that the glycosylation pattern of shed GP is important for the activation of cytokine release. We also demonstrate the ability of shed GP to affect endothelial cell permeability both directly and indirectly through cytokine release. Our results indicate that shed GP plays an important role in viral pathogenicity.

Results and Discussion

Production and characterization of recombinant EBOV soluble glycoproteins.

During EBOV infection significant amounts of soluble glycoproteins, including shed GP and secreted sGP, are released from virus-infected cells [21]. The role that these proteins might play in virus replication and pathogenicity has not yet been clearly defined. In effect, EBOV shed GP is structurally identical to virion surface GP except for the lack of its carboxy-terminal part consisting of 13 amino acids upstream of the membrane anchor, the anchor itself and a short cytoplasmic tail [21,32]. Consequently,

it was of interest to investigate whether shed GP and potentially sGP, that shares 295 amino-terminal amino acids with surface GP, can interact with and affect the function of DCs and macrophages that constitute an important and primary line of immune defense but that are also the major primary targets for viral infection *in vivo*. While transient expression of sGP from plasmid provides sufficient amounts of the protein secreted into the medium, the low efficiency of GP shedding seen with transient expression systems has hampered production of large enough quantities of this protein equivalent to those observed during EBOV infection. Of note, TACE, an enzyme responsible for GP shedding, is activated during EBOV infection [33] and the efficiency of shedding from virus-infected cells appears to be significantly higher. On the other hand the extent of glycosylation and post-translational modifications of EBOV GP prevents the use of more efficient expression systems such as bacteria or baculovirus. To surmount these difficulties we substituted two amino acids immediately surrounding the TACE cleavage site at positions D637A and Q638V and thus expected to increase efficiency of GP shedding as this newly generated sequence resembles that previously shown to be recognized by TACE for efficient release of TNF α [34]. The plasmid expressing this mutant was designated phCMVGP-HS and GP expressed from this plasmid as GP-HS (high shedding). Shed GP expressed by this mutant contains a single amino acid substitution at the carboxy-terminal end of shed GP. According to structural predictions it is highly unlikely that this single amino acid change would alter the structure or function of shed GP. Another difficulty faced in the production of recombinant GP of EBOV results from its strong cytotoxic effects [20,35,36] causing cell rounding, loss of cell attachment, and possibly the release of cellular factors due to eventual cell death. In order to address solely the potential functions of shed GP and to allow for possible effects caused by cytotoxicity, an EBOV GP mutant lacking shedding properties was

used in this study as a negative control. This mutant contains a substitution L635V (GP-LS, low shedding) that was previously shown to prevent TACE cleavage and result in increased surface GP expression [21]. Of note, expression of this mutant is expected to result in similar, if not higher, cytotoxicity than that caused by wild-type GP (GP-WT) and would provide culture supernatants containing the same potential extracellular factors released from GP expressing cells whilst displaying undetectable amounts of shed GP.

In this manner, 293T cells were transfected with the plasmids expressing mutated GPs, GP-WT and sGP as well as GFP as a non-relevant control. Western blot analysis of cell lysates and culture supernatants revealed as expected, an important difference in efficacy of GP shedding between GP-WT and mutated GPs (Figure 1A). Of note, due to the removal of the region spanning the transmembrane anchor, after cleavage by TACE the GP₂ within shed GP showed a lower molecular mass and faster migration on SDS-polyacrylamide gels compared to GP₂ in intracellular GP_{1,2} (compare lines 1 and 7; lines 2 and 8). While similar amounts of intracellular GP (represented by endoplasmic reticulum GP precursor - preGPer and mature GP_{1,2}) were seen in cells expressing either GP-WT or GP-LS (compare lines 1 and 3), GP-LS did not show detectable levels of shed GP in the medium when compared with GP-WT. Notably, relatively low amounts of intracellular GP were detected with GP-HS whilst increased levels of shed GP were detected in the medium. As expected EBOV sGP is efficiently secreted from 293T cells transfected with corresponding plasmid (Figure 1A, lane 10). Using Vivaspin concentrators the volume of culture supernatants was reduced by 10 fold to increase the concentration of shed GP in supernatant samples. Of note, no significant differences were detected in total amount of proteins between samples (2 mg/ml), as also illustrated in Figure 1B by Coomassie staining. The presence of shed

GP in concentrated samples from GP-HS expressing cells and its absence in samples from GP-LS expressing cells was confirmed by Western Blot analysis (Figure 1B, left panel, line 3).

Using serial dilutions of a commercially available recombinant baculovirus-expressed EBOV GP lacking the transmembrane domain we were able to estimate the amounts of shed GP and sGP in concentrated supernatants by western blot analysis and quantification software. The standard curve was linear over the range used for quantification and the GP-HS sample was found to contain $\sim 1 \mu\text{g/ml}$ and sGP $\sim 18 \mu\text{g/ml}$ (Figure S1 A and B). In subsequent experiments with immune cells shed GP (GP-HS) was typically used at a concentration of $\sim 0.4 \mu\text{g/ml}$ and sGP at a concentration of $\sim 7.2 \mu\text{g/ml}$.

To avoid using the produced recombinant shed GP in subsequent experiments at concentrations superior to those observed with infection the quantity of shed GP in concentrated samples was compared with that present in the blood of experimentally infected animals. For this purpose groups of guinea pigs were inoculated intraperitoneally with 500 infectious units (TCID_{50}) of guinea pig-adapted recombinant EBOV [37]. Infected guinea pigs were monitored for clinical manifestations and showed an increase in temperature starting by day 3 post-infection. When animals reached the ethical end point of infection they were euthanized. Blood was collected at different intervals post infection and the sera were assayed for the presence of shed GP in comparison with recombinant-produced shed GP. As demonstrated in Figure 1C and 1D the amounts of shed GP present in the blood of animals significantly exceed those used in our study. Quantification of shed GP in the sera based on amounts of truncated GP_2 reveals approximately 85 times higher concentrations of shed GP ($34 \mu\text{g/ml}$) than

those used in this study. Furthermore the analysis of sera samples collected at different intervals post infection showed that the amounts of shed GP in the blood of animals significantly increased during the course of infection (Figure 1D), which is likely to be explained by both virus-induced activation of TACE [34] and also by spreading of infection that increases the number of virus infected cells.

Of note, our GP construction and production approach differed from that used in an earlier study investigating role of soluble EBOV glycoproteins [32]. In the previous study to address the role of shed GP the authors had used purified amino-terminal HA-tagged soluble glycoprotein produced through the addition of a stop codon immediately after the TACE cleavage site that allows efficient secretion of this truncated GP into the extracellular media. However, we have observed that when GP is produced through truncation of its transmembrane anchor it does not appear to wholly represent shed GP. Whilst truncated forms of GP had been previously used for crystal structure analysis of surface GP, and were principally shown to form trimers [18], the stability of such truncated oligomeric structures is suspected to be significantly lower in cell culture conditions. Indeed, as evidenced by our sedimentation analysis the transmembrane-anchor truncated GP mutant is mostly a monomer in solution (Figure S2). In contrast shed GP produced in our study is a trimer as was also observed during EBOV infection [21] and is in agreement with the trimeric structure of surface GP. Of note the process of shedding includes proper GP oligomerization, maturation, and transport to the cell surface where the mature GP spikes are cleaved by cellular TACE and shed from the cells.

In addition, in the previous study the soluble recombinant glycoproteins were produced in conditions containing human sera [32] which are known to contain sera

lectins, including human MBL [38-40]. Indeed, MBL is a C-type lectin capable of acting as a pattern recognition molecule for pathogens by recognizing heavily glycosylated proteins, particularly those containing a high-mannose type of glycosylation such as that seen with EBOV surface GP [41-45]. Importantly, MBL has recently been shown capable of binding EBOV surface GP [42,46,47]. The presence of such sera lectins during production of soluble GP could hamper the investigation of the interaction of this protein with its cellular targets and therefore the expression and production of shed GP, sGP and also of all control proteins used in this study was performed in cells cultured in FCS free medium (VP-SFM, Gibco).

EBOV soluble glycoproteins bind to DC and macrophages

Next, human monocyte-derived DCs and macrophages (Mø) as well as peripheral blood lymphocytes (PBL) were incubated with the samples of culture medium containing the soluble EBOV GPs described above. To assess the ability of shed GP to bind to DCs, Mø or specific fractions of lymphocytes, flow cytometry was performed using anti-GP specific antibodies. DCs were shown to bind both shed GP and sGP while Mø could only bind shed GP (Figures 2A and S3A). As expected, incubation of the cells with GP-LS failed to evidence any GP specific binding in agreement with undetectable levels of shed GP in samples. It appears that neither B nor T lymphocytes (Figures 2A and S3A) bind shed GP or sGP in agreement with earlier data revealing an absence of surface GP binding to these cells [48,49].

In light of data revealing an interaction between surface GP and MBL [42,46], we also investigated whether MBL present in human sera is capable of affecting the binding of shed GP to cells. Of note, normal human plasma contains MBL concentrations ranging from 10 to 5000 ng/ml [50,51]. Using commercially available

human sera (Statens Serum Institute, Denmark) we demonstrate that pre-incubation of shed GP with such sera (final MBL concentration of 150 ng/ml) blocked shed GP binding to both DCs and Mø (Figures 2A and S3A).

Previously it was demonstrated that surface GP of EBOV interacts with toll-like receptor 4 (TLR4) [52], a cellular surface receptor that is known to recognize highly glycosylated molecules containing O-linked mannosyl residues such as those present on GP. It was thus of interest to investigate if shed GP could also mediate its activity through TLR4. To address this question DCs and Mø were pre-treated with mouse anti-TLR4 antibody (HTA125) and then incubated with shed GP. As a control, the cells were first incubated with mouse isotype control antibodies and then with shed GP. Binding of shed GP to both DCs and Mø was dramatically reduced upon treatment of the cells with anti-TLR4 antibody whereas no effect on GP binding was observed when control antibodies were used (Figures 2B and S3B). These results suggest that shed GP binding to DCs and Mø is likely to be explained by its interaction with TLR4. In this regard an absence of shed GP binding to PBLs is in agreement with little to no surface TLR4 expression on lymphocytes from healthy donors [53-55].

It is worthy to note that MBL can also bind TLR4 [56-58]. In this regard the MBL-mediated reduction in shed GP binding to the cells could be explained either by the interference of MBL in shed GP binding to TLR4 and/or by sequestering shed GP via direct interaction with MBL. In an attempt to clarify this question we performed experiments where cells were pre-treated with sera containing MBL with subsequent washing and incubation with shed GP. As demonstrated in Figures 2C and S3C, shed GP binding to the cells was also blocked under these conditions, in this case most likely

via interaction of TLR4 with MBL. In agreement, pre-incubation of cells with MBL deficient sera did not affect shed GP binding.

Thus far data obtained clearly indicate an interaction between shed GP and DCs and Mø. This is contrary to a previous publication in which no evidence of an interaction was found between shed GP and immune cells [32]. In this regard, the avoidance of sera lectins during GP production appears to be of vital importance as we demonstrate that the addition of human sera containing MBL abrogates binding of shed GP to target cells in vitro. Of note, as demonstrated in Figure 1C significantly higher amounts of shed GP are present in the blood of EBOV-infected guinea pigs than in our experimental conditions. Furthermore the continuous and massive release of shed GP during infection (Figure 1D) most likely would reduce or counteract the inhibitory effect of sera lectins in vivo even further.

Importantly, the data obtained in this study suggest that TLR4 is likely to be the key cellular partner for binding of shed GP to DCs and Mø. As TLR4 is abundantly expressed on macrophages and DCs [59-61] our findings led us to investigate further the role that such an interaction might play, in terms of activation of these cells and the induction of the release of pro-inflammatory cytokines that is known to be a characteristic of EBOV pathogenesis [62,63].

EBOV shed GP induces upregulation of cytokine transcription in both DCs and Mø

To address whether shed GP binding to immune cells results in their activation, monocyte-derived DCs and Mø were incubated with samples of culture supernatants containing the soluble EBOV glycoproteins detailed above and expression of TNF α , IL6, IL10 and IL12p40 mRNA was measured by real-time PCR at 4, 8, 12 and 24h

post-treatment. In controls, cells were treated either with culture supernatants from cells expressing GFP (Mock) or LPS (500 pg/ml). Culture supernatants from cells expressing GP-LS were used as an additional negative control. Prior to experiments, all culture supernatants were tested for the absence of endotoxin and showed endotoxin concentrations below 0.25 units/ml and were thus considered to be endotoxin-negative. In a sharp contrast to all negative controls used in this experiment, treatment of DCs and Mø with either shed GP or LPS results in transcriptional activation of a number of analysed genes (Figure 3A and B). Upregulation of TNF α and IL6 occurred as early as 4h post treatment whereas IL10 and IL12p40 were activated only 8h and 12h post treatment, respectively. The increase in mRNA levels appears to be higher in Mø than in DCs and when compared to LPS, cell activation caused by shed GP appears to be more durable. Treatment of the cells with sGP did not reveal any significant effects on cytokine mRNA synthesis. In agreement with experiments concerning shed GP binding, pre-treatment of both DCs and Mø with anti-TLR4 antibody significantly reduced shed GP-induced activation of TNF α mRNA synthesis (Figure 3C). A similar or even complete neutralizing effect of anti-TLR4 antibody was observed in the case of LPS treatment, in agreement with previous publications demonstrating that LPS acts on cells via TLR4 [57,64]. Furthermore, as shown in Figure 3D, pre-treatment of shed GP with human sera containing MBL results in the abrogation of shed GP-dependent TNF α activation. Importantly experiments with dose-dependent inhibition of shed GP activity by MBL-containing sera clearly support the conclusion that the inhibitory effect of sera lectins will be countered by the continuously growing amounts of shed GP in vivo (Figure 3D). A two-fold decrease in MBL amount led to a significant reduction of the MBL inhibitory effect on the activation of both DCs and macrophages by shed GP. No

blocking effect was observed after pre-treatment of shed GP with MBL-deficient sera. As expected, both human sera used did not themselves activate DCs or Mø.

EBOV shed GP induces release of pro- and anti-inflammatory cytokines from DCs and Mø

Given the transcriptional activation observed above it follows that upon stimulation DCs and Mø would release a range of cytokines into the medium. Accordingly, culture supernatants of DCs and Mø collected 24h after addition of shed GP (as detailed above) were assayed using a Multiplex cytometric bead array (Bio-Rad) against a panel of cytokines previously shown to be upregulated during EBOV infection [62,65]. As expected, shed GP induced the secretion of TNF α , IL6, IL10, IL12, IL8, IL1 β and IL1RA in both DCs and Mø (Figure 4A). Comparable levels of these cytokines were also observed in the medium when cells were treated with LPS. All negative controls used in this study including samples of medium from GP-LS expressing cells did not show any significant release of the cytokines. Incubation of the cells with the sGP-containing sample also did not result in detectable cytokine secretion. Expectedly, pre-treatment of shed GP-containing samples with MBL resulted in a complete block in cytokine release that correlates well with the absence of activation of cells, as demonstrated in figure 3A and 3B. In accordance with the results presented above, pre-treatment of cells with an anti-TLR4 antibody considerably reduced the release of TNF α , IL6, IL10, IL12, IL8, IL1 β and IL1RA (Figure 4B). Again a similar or even stronger neutralizing effect of anti-TLR4 antibody was observed in the case of LPS treatment. Importantly, shed GP produced in 293T cells cultured with 5% FCS also failed to show activation of immune cells (Figure S4) supporting the importance of using sera-free medium for shed GP production.

In a separate experiment we determined that shed GP activity is dose-dependent. Several dilutions of shed GP from GP-HS expressing cells as well as from wild-type GP-expressing cells were used to treat immune cells and culture supernatants from DCs and Mø collected 24h after addition of shed GP were assayed (as detailed above) using a Multiplex cytometric bead array (Bio-Rad) against a panel of cytokines. As shown in figure 4C the activity of shed GP released from wt GP- and GP-HS expressing cells is comparable when the concentration of the proteins is adjusted. This also demonstrates that whilst increasing GP shedding efficacy the single amino acid exchange at the carboxy-terminal end of GP-HS does not affect the protein's activity in comparison to the wild-type sequence.

As activation of DCs and Mø is usually associated with an increase in expression of phenotypic activation markers such as the co-stimulatory molecules CD86, CD80, CD83 and CD40, we investigated whether the binding of shed GP or sGP to these immune cells could induce the expression of these surface molecules. As demonstrated in figure 5 A and B and figure S5 A and B, exposure to shed GP but not to sGP induced the expression of co-stimulatory molecules on both DCs and Mø. Similar expression of surface markers was observed upon treatment of the cells with LPS. Samples of culture medium from GP-LS expressing cells, used here as a negative control, did not reveal any significant effect on immune cells. Pre-treatment of cells with MBL prevents cell activation as demonstrated by the absence of any increase in co-stimulatory molecule expression.

Glycosylation of shed GP is vital to activate DCs and Mø

Since anti-TLR4 antibodies were capable of blocking shed GP binding and also of preventing immune cell activation we speculated that GP glycosylation might serve

as a pathogen associated molecular pattern (PAMP) recognized by TLR4. Indeed, the glycosylation pattern of EBOV GP is complex and composed of mature and immature N-glycans but also a dense cluster of O-glycosylated serine and threonine residues referred to as mucin-like domain [18,19]. In order to verify this idea shed GP was treated under non denaturing and non-reducing conditions with a mix of N- and O-glycosidases and then used for incubation with immune cells, as detailed above. As shown in figure 6A the treatment with deglycosylation enzymes reduced the molecular weight of shed GP as expected, in comparison to shed GP treated with the same mix of deglycosidases that were previously inactivated by heating for 30 min at 80°C (Figure 6A, compare lanes 1 and 2). DCs and Mø were incubated with shed GP samples or LPS alone for 12h, and then culture supernatants were analyzed by ELISA for amounts of secreted TNF α . As an additional control cells were subjected to incubation with LPS following incubation with deglycosylated shed GP. Strikingly, as shown in Figure 6B, deglycosylation of shed GP considerably decreased the release of TNF α in both DCs and Mø, confirming the importance of the glycosylation pattern of shed GP for its activity. Cells incubated with deglycosylated shed GP remained viable and capable of responding to LPS stimuli ruling out a possible negative effect of deglycosydases on cells.

Taken together these results suggest that the particular glycosylation pattern of shed GP is responsible for its functions as an inducer of immune cell activation and that this function is primarily mediated via shed GP binding to TLR4 present on DCs and Mø acting as a pathogen pattern recognition receptor. This would appear consistent with the hypothesis that shed GP is acting in a manner somewhat similar to that seen for LPS in its ability to cause systemic inflammation.

Modulation of endothelial permeability by EBOV shed GP

Vascular dysregulation and instability are thought to be crucial symptoms during EBOV infection [24,66]. While endothelial cells are susceptible EBOV targets in vitro [35,67], they are considered to be rather late virus targets in vivo [68,69] suggesting an indirect effect of EBOV infection on these cells. A dysregulated inflammatory host response to EBOV infection is believed to facilitate increased permeability of endothelial barriers that would eventually cause haemorrhaging [67,70]. In this regard proinflammatory cytokines, in particular TNF α , released from immune cells activated by shed GP could affect endothelial barrier integrity [68,70]. Furthermore, as we demonstrated that shed GP is capable of activating immune cells for release of different cytokines, we also sought to determine if shed GP could directly affect endothelial cell permeability. To answer these questions, an *in vitro* monolayer cell permeability assay was performed using HUVECs. Firstly, HUVEC monolayers grown on semi-permeable membranes in cell culture plate inserts were treated for 22h with samples containing soluble EBOV glycoproteins. Following treatment FITC-dextran was added to the apical part of the insert and the permeability of the monolayer (Figure 7A) was assessed by measuring fluorescence in the basal compartment. As controls, cells were treated with samples of culture medium from GFP- (Mock), sGP- or GP-LS-expressing cells and also with LPS (as above), MBL-containing sera, MBL-deficient sera and TNF α (10ng/ml). The integrity of HUVEC monolayers was verified with a fluorometer both before and after 22h of incubation with supernatants containing soluble glycoproteins. HUVEC monolayers were also photographed under a light microscope (Figure 7C). As seen in Figure 7A and 7C, samples containing GP-HS, LPS and TNF α significantly increased the permeability of the HUVEC monolayer when compared with negative controls and sGP. As expected, culture supernatants containing GP-HS in the presence

of MBL-containing sera were significantly less capable of inducing an increase in permeability of the HUVEC monolayer compared to MBL-deficient sera. Treatment with human sera alone did not show any significant effect on permeability either with or deficient for MBL. Secondly, we also assayed whether the levels of soluble modulators released from macrophages treated with samples of shed GP and all controls (as above) were sufficient to contribute to the decreased barrier function of the endothelium. As seen in figure 7B and D, the supernatants from shed GP-treated macrophages significantly increased the permeability of the HUVEC monolayer when compared to samples of culture medium from GFP- (Mock) or sGP-expressing cells, in a manner comparable with that seen with LPS-treated macrophages or when $\text{TNF}\alpha$ was used as a control. Somewhat surprisingly given the absence of detectable activation in previous assays, a moderate increase in permeability was observed when culture medium from GP-LS-expressing cells was used, probably explained either by residual amounts of shed GP present in GP-LS samples used to treat macrophages or by the presence of another undetermined factor released during cell treatment with medium upon GP expression. Culture supernatants from macrophages treated with shed GP in the presence of MBL containing sera were significantly less capable of inducing an increase in permeability of the HUVEC monolayer compared to MBL-deficient sera. Treatment with human sera alone did not show any significant effect on permeability either with or deficient for MBL. The ability of shed GP to affect endothelial cells function both directly and indirectly highlights the interplay between shed GP, systemic cytokine release and increased vascular permeability.

Taken together the data obtained support a role for EBOV shed GP in the creation of excessive and dysregulated host inflammatory responses and an increased vascular permeability. Based on the results presented here we postulate that once

released from the surface of infected cells shed GP could be responsible for the induction and release from non-infected macrophages and DCs of a battery of pro- and anti-inflammatory cytokines. This is especially interesting as elevated levels of pro- and anti-inflammatory cytokines have been shown to be present in fatal EBOV infections in man [62,71]. Importantly, a clear link has been demonstrated between clinical status and inflammatory responses in that fatal outcome of infection is often associated with aberrant innate immune responses [71,72].

This study provides a potential explanation as to the cause of these dysregulated host responses, since it has been demonstrated that neither the whole virus nor viral replication in immune cells could explain an excessive activation of immune cells for release of these cytokines [30,62,71,73]. We now show that cultured primary DCs and macrophages are activated upon treatment with EBOV shed GP, resulting in release of several mediators including pro- and anti-inflammatory cytokines such as TNF α , IL6, IL10, IL12, IL8, IL1 β and IL1RA independently of virus replication in these cells.

The apparent similarity in the response of immune cells to shed GP and LPS highlights a likely common triggering of TLR4 and shared downstream pathway for activation and cytokine release. Intriguingly, a common feature of both LPS as a bacterial component and EBOV infection is their ability to trigger a similar physiological syndrome typified by elevated cytokine release, which for EBOV infection closely resembles the septic shock seen with bacterial infection. Indeed, when presented in VLPs EBOV GP has been shown to interact with TLR4, leading to the induction of proinflammatory cytokines as well as SOCS1 in both a human monocytic cell line (THP-1) and 293T cells expressing a functional TLR4/MD2 receptor [52]. Furthermore, a recent study by Lubaki et al. [30] provided clear evidence that an unrelated viral vector carrying EBOV

GP could effectively cause maturation of dendritic cells. However, during EBOV replication in dendritic cells interferon antagonism mediated by virus-encoded proteins VP35 and VP24 strongly reduces both the expression of maturation markers and secretion of cytokines and chemokines from these cells. On the contrary, EBOV shed GP as a soluble mediator is able to activate non-infected immune cells, making it distinct from a number of other viruses whose surface glycoproteins have been shown to act as ligands for TLR4 recognition but that either do not shed their surface glycoproteins into the extracellular environment or do not spread systemically and thus only cause local inflammatory disorders [74-78].

In conclusion, in the present study we have identified the primary targets of EBOV soluble glycoprotein shed GP. In our model, EBOV infected cells release both virions and shed GP into extracellular environment. Shed GP, most likely via interaction with TLR4 on the surface of macrophages and DCs induces activation of these cells and the release of pro- and anti-inflammatory cytokines. The soluble nature of both shed GP and cytokines enables them to trigger new, remote targets such as non-infected APCs, which will then amplify inflammation by recruiting and/or activating other cell types including lymphocytes, endothelial cells and other cell types including hepatocytes, leading to the impairment of cell functions and organ failure (Figure 8). Our results strongly suggest that the target cell range for EBOV infection is considerably broader than previously imagined. In effect, activation of non-infected immune cells by shed GP could play an important role in the establishment of systemic inflammation during infection. Furthermore the data obtained with shed GP provide a potential link between systemic inflammation and increased endothelial permeability that together contribute to the disseminated intravascular syndrome seen with lethal EBOV infection. Overall, our data contribute to a better understanding of the way

EBOV might provoke the excessive cytokine storm that appears to be detrimental to survival of infection and provide new insights with which to develop therapeutic strategies to combat this newly defined role for shed GP in high viral pathogenicity. In this regard, it is intriguing to speculate that treatment with anti-TLR4 antibodies could be used to reduce the inflammatory reaction caused by shed GP in a similar way than has been demonstrated for the dramatically successful treatment of septic shock in mice after lethal LPS challenge [79]. Similarly, it is conceivable that neutralizing antibodies targeting shed GP could also help to alleviate the systemic shock-like syndrome seen with EBOV infection.

Materials and methods

Virus

The work with infectious EBOV belonging to *Filoviridae* family, *ebolavirus* genus, species *Zaire Ebolavirus* was performed at INSERM “Laboratoire Jean Mérieux” within biosafety level 4 containment at Lyon, France. The virus was propagated on Vero E6 cells. Culture supernatants were collected, clarified, frozen and kept at -80°C before virus titration by TCID₅₀ [80]. The virus was generated using a reverse genetics system previously described in [81]. Amino acid substitutions M71I, L147P, T187I were introduced into the VP24 gene to increase the pathogenicity in recombinant EBOVs for guinea pigs [37,82]. Mutations introduced into the virus were confirmed by sequencing of complete genomic RNA isolated from virus stocks.

Cell lines and plasmids

293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, PAA laboratories) supplemented with 10% fetal calf serum (PerbioHyclone). Human

umbilical vein endothelial cells (HUVECs) (Lonza) were maintained at 37°C with 5% CO₂ in EGM-2 Media (Lonza) enriched with EGM-2 Single Quots containing FBS, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, hEGF, GA-1000 and heparin. Cells were cultured for 3-4 days with EGM-2 medium and were passaged for no more than 5 generations for the experiments described.

We have previously demonstrated that the substitutions L635V and D637V dramatically alter the efficacy of EBOV GP shedding in a transient expression system [21]. In this study to increase the efficiency of GP shedding in a transient expression system we introduced the substitutions D637A and Q638V using site-directed mutagenesis of a plasmid encoding wild-type GP. The resulting plasmid was designated phCMVGP-HS. Plasmid expressing GP with a substitution L635V (phCMVGP-LS) was described earlier [21]. Plasmid expressing transmembrane-anchor truncated mutant GP_{1,2Δ} was generated by introducing a stop codon at position 651 using site-directed mutagenesis of a plasmid encoding wild-type GP.

Transient expression of EBOV GP

293T cells were grown for 24h to a confluence of ~60%. Transfection of cells with recombinant plasmids was performed using TurboFect reagent (Thermo). During GP expression the cells were kept in VP-SFM medium (Gibco) containing no serum. Culture supernatants were harvested 36h posttransfection, clarified by centrifugation at 1000 rpm for 10 min at 4°C and then by centrifugation at 28,000 rpm at 4°C for 2 h using a SW28 rotor with a Beckman Optima L-70K ultracentrifuge. The clarified supernatants were concentrated 10 fold using Concentrator tubes (Pierce) with a molecular weight cut off of 20000. All samples were aliquoted and stored at -80°C.

Culture of primary human monocyte-derived-DCs and macrophages

Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by centrifugation onto a lymphocyte separation medium (Ficoll) cushion and then were enriched by centrifugation of PBMCs through a Percoll density gradient. The recovered monocyte fraction was depleted of cell contaminants by positive selection (Miltenyi Biotec), ensuring purification rates of $\geq 95\%$. Immature DCs and monocyte-derived macrophages were obtained upon incubation of cells in complete RPMI-1640 (Life Technologies) supplemented with 200 mM L-glutamine (Life Technologies), 10 mM HEPES buffer (Life Technologies), 10% de-complemented FCS and 8 $\mu\text{g/ml}$ of Gentamicine (Life Technologies) for 5 days either with 40 ng/ml of granulocyte- macrophage colony-stimulating factor (GM-CSF) or 50 ng/ml of interleukin-4 (IL-4) and 40 ng/ml of macrophage colony-stimulating factor (M-CSF), respectively.

Endotoxin test

The Limulus ameocyte lysate test (LAL Chromogenic Endotoxin Quantitation Kit, Pierce) was used to determine endotoxin levels in culture supernatants used in this study. The values were less or equal to those of VP-SFM medium (<0.25 endotoxin units per ml).

Sedimentation analysis

The oligomeric structures of EBOV shed GP and transmembrane-anchor truncated mutant GP_{1,2 Δ} were compared using a sedimentation assay. 293T cells were transfected with plasmids phCMVGP-HS or phCMVGP_{1,2 Δ} and culture medium was collected 36h post-transfection. Medium was clarified by low-speed centrifugation and subjected to ultracentrifugation through a linear 5–25% (w/w) sucrose gradient prepared on co-IP buffer (1% NP40, 0.4% deoxycholate, 5 mM EDTA, 100 mM NaCl, 20 mM Tris–HCl,

pH 7.5, 25 mM iodoacetamide) on Beckman Optima L-70K ultracentrifuge using a SW60 rotor for 21 h at 40 000 rpm, 10°C. Fractions were collected from the bottom to the top, proteins separated by SDS–PAGE and analyzed by Western blot using anti-GP antibodies.

Binding assay

5×10^5 monocyte-derived DCs or macrophages per well were placed in conic-bottom 96-well plates and plates were centrifuged at 2000 rpm for 2 min. The culture medium was removed and replaced by medium containing EBOV GPs (in VP-SFM medium, Gibco). Cells were incubated for 1 hour at 4°C, washed twice with ice-cold phosphate-buffered saline (PBS) and then incubated in FACS buffer (PBS containing 2% FBS, 2% normal rat serum, 2% normal hamster serum, 2% normal mouse serum and 2% human FcR blocking solution, Milteny Biotec) at 4°C for 30 min. Cells were then incubated with mouse anti-GP1 antibody for 1 hour at 4°C, washed twice with PBS and incubated with anti-mouse IgG1 coupled to Alexa 488 for DCs and Mø or to Allophycocyanin (APC) for lymphocytes for 30min at 4°C. All mAbs were purchased from BD Pharmingen except those indicated otherwise. After incubation with antibodies, cells were washed and fixed with 1% PFA solution. Cells were analyzed using a Becton Dickinson LSRII flow cytometer (BD) and FlowJo software. In some experiments samples of GP were incubated with human sera containing MBL (150ng/ml) or MBL-deficient sera (Statens Serum Institute, Denmark) for 1 hour at 4°C prior to addition to immune cells. In TLR4-related experiments, cells were incubated for 10 min at room temperature with 30 µg/ml of anti-TLR4 antibodies (HTA125) or unspecific isotype antibody prior to addition of EBOV GPs. The statistical significance of the differences in median fluorescence intensity (MFI) values for each donor were evaluated by paired-

sample *t* test and GraphPad Prism 6 software (GraphPad Software, La Jolla California, USA).

Treatment of cells with EBOV GPs

5×10^5 of monocyte-derived DCs or macrophages per well were placed in 48-well plates and after 5 days differentiation, media was replaced with 600 μ l of fresh RPMI 1640 containing 200 mM L-glutamine, 10 mM HEPES buffer, 5 mM CaCl₂ and 400 μ l of culture supernatants containing soluble EBOV glycoproteins (in VP-SFM medium). Cells were incubated for 4, 8, 12 and 24h at 37°C and culture supernatants were collected, clarified from cell debris by centrifugation (1600 rpm at 4°C for 10 min), aliquoted, and stored at -80°C until analysis. The cells were used for real time RT-PCR analysis. Experiments using either anti-TLR4 antibodies or sera containing MBL were performed as described above in Binding assay.

Unless otherwise stated shed GP was used at a final concentration of 0.4 μ g/ml and sGP at 7.2 μ g/ml. In deglycosylation experiments shed GP was used at a concentration of 0.2 μ g/ml.

Real time RT-PCR quantitative analysis

RNAs from monocyte-derived DCs and macrophages treated with EBOV GPs were isolated using Total RNA Isolation kit (Machery Nagel). cDNAs were generated using iScript cDNA Synthesis kit (Bio-Rad), followed by real-time PCRs using Sybr-Green-Master-Mix (Roche) in triplicate following manufacturer's recommendations. Validated RT-PCR primers specific for human TNF α (5'-CCTGCCCCAATCCCTTTATT and 5'-CCCTAAGCCCCCAATTCTCT), IL-6 (5'-TGCAATAACCACCCCTGACC and 5'-TGCGCAGAATGAGATGAGTTG), IL10

(5'-GAGGCTACGGCGCTGTCAT and 5'-CCACGGCCTTGCTCTTGTT), IL12p40 (5'-CCAGAGCAGTGAGGTCTTAGGC and 5'-TGTGAAGCAGCAGGAGCG) and GAPDH (5'-CCATGTTTCGTCATGGGTGTG and 5'-GGTGCTAAGCAGTTGGTGGTG) were used to quantify mRNA levels. The real-time PCR analysis was performed on Stepone Plus apparatus using Roche software. Relative quantification was made by normalization to GAPDH mRNA levels.

Cytokine release

Culture supernatants from monocyte-derived DCs or macrophages treated with soluble EBOV GPs for 24 hours were assayed for presence of TNF- α , IL-6, IL-8, IL12p40, IL1 β , IL10 and IL1-RA using Multiplex cytometric bead array (Bio-Rad) in Luminex MAGPIX. The assay was performed according to the manufacturer's instructions.

Expression of co-stimulatory molecules

Both monocyte-derived DCs and macrophages treated with soluble EBOV GPs for 48 hours were washed in cold PBS supplemented with 2% fetal bovine serum and pre-blocked at 4°C for 30 min in FACS buffer. Cell-surface staining was performed using the following antibodies: FITC-conjugated anti-CD80, PE-conjugated anti-CD40 (Beckman Coulter) and APC-conjugated anti-CD83, Peridinin Chlorophyll Protein Complex Vio 700 (PerCP-Vio700)-conjugated anti-CD86 (Miltenyi Biotec). After incubation with antibodies for 1 hour at 4°C, cells were washed with PBS, fixed with 1% PFA solution and analyzed using a Becton Dickinson LSRII flow cytometer (BD) and FlowJo software. A minimum of 10000 events were collected and analyzed for each sample. The statistical significance of the differences in median fluorescence intensity (MFI) values for each donor were evaluated by paired-sample *t* test and GraphPad Prism 6 software (GraphPad Software, La Jolla California, USA).

Deglycosylation assay

For GP deglycosylation, supernatants containing EBOV GPs were treated using EDEGLY kit (Sigma) for 48h according to the manufacturer's instructions. Shed GP mixed with deglycosylation enzymes pretreated at 80°C for 30 min were used as a control. Monocyte-derived DCs and macrophages were treated with deglycosylated shed GP samples for 24 hours and culture supernatants were assayed for the presence of TNF α using a Multiplex cytometric bead array (Bio-Rad) in Luminex MAGPIX.

HUVEC permeabilization assay

HUVEC were grown in collagen-coated inserts of permeability assay chambers (Millipore) for 72h until confluence. The media was replaced with 600 μ l of fresh EBM (endothelial cell basal medium without phenol red, Lonza) and 400 μ l of culture supernatants containing soluble EBOV glycoproteins (VP-SFM medium, Gibco) or 400 μ l of culture supernatants from Mø previously stimulated with soluble EBOV glycoproteins for 24h. After 22 h of incubation, FITC-Dextran was added to each insert and FITC-Dextran leaking out into the bottom chamber was measured. Monolayer permeability was assessed on the basis of both fluorescence intensities measured using a Tecan fluorometer and the integrity of the HUVEC monolayer using a MacroFluo microscope.

Animal experiments

Guinea pigs, strain Hartley (3 week-old females), were infected intraperitoneally with 500 TCID₅₀ of recombinant guinea-pig adapted EBOV as previously described [82]. Mock-infected controls were inoculated with DMEM. Animals were monitored for clinical manifestations and were euthanized when they reached an ethical end-point.

Retro-orbital sinus sampling of blood was performed after local anaesthesia at day 3, 6 and 9 and prior to euthanasia. The sera were purified using VenoSafe PET tubes (VenoSafe).

All animals were handled in strict accordance with good practices as defined by the French national charter on the ethics of animal experimentation. Animal work was approved by the regional ethical committee (CREEA), and experiments were performed in the INSERM Jean Mérieux BSL-4 laboratory in Lyon, France.

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Figure Legends.

Figure 1. Production of recombinant EBOV shed GP and analysis of shed GP release in the blood of infected guinea pigs. (A) 293T cells were transfected with plasmids expressing GP-WT (WT), GP-HS (HS), GP-LS (LS), sGP and GFP and 36h post-transfection cells and culture supernatants were collected and analyzed by Western Blot using anti-GP₁ and anti-GP₂ antibodies. GP₁, endoplasmic reticulum precursor GP (preGPer), sGP, GP₂, and truncated GP_{2Δ} are indicated. (B) Culture supernatants from 293T cells expressing EBOV glycoproteins were concentrated using concentrator tubes (Pierce) and analyzed by Coomassie staining following SDS-PAGE (right panel). Samples were also analyzed by Western blot with appropriate antibodies (left panel). (C). Guinea pigs (strain Hartley) were infected intraperitoneally with 500 TCID₅₀/animal of guinea pig-adapted EBOV [82]. Serial dilutions of sera collected at day 6 post-infection (5μl, dilutions 1/4, 1/8, 1/16 & 1/32, lanes 2-5), supernatant from EBOV-infected Vero E6 cells (20μl, EBOV Vero, lane 1) and a sample of concentrated recombinant shed GP (5μl, HS, lane 6) were analyzed by Western blot using anti-GP₂

antibodies. Estimation of shed GP amounts in the sera of animals in comparison with shed GP from GP-HS expressing cells was performed using ImageQuantTL software (GE Lifesciences). Sera of animals contained approximately 85 times higher amounts of shed GP than those used in experiments with immune cells. **(D)**. Samples of sera collected at different intervals post infection from Mock (day 3 p.i.) - and EBOV-infected guinea pigs (animal numbers 192, 193, 195 & 196) as indicated, were analyzed by Western blot using anti-GP2 antibodies. The release of shed GP dramatically increased during the course of infection. Sera of infected animals contains both virion GP and shed GP as demonstrated by the presence of full-length virion-associated GP₂ and truncated GP₂ (GP_{2Δ}).

Figure 2. EBOV shed GP binding to DCs and macrophages.

(A) Human monocyte-derived dendritic cells (DCs), monocyte-derived macrophages (MØ), and PBLs (shown B lymphocytes, B) were incubated with the concentrated culture supernatants described as well as shed GP in the presence of MBL-containing sera (150 ng/ml, HS+MBL⁺). Bound proteins were detected by subsequent incubation with mouse anti-GP1 antibodies and anti-mouse Alexa 488 coupled antibodies (DCs and MØ) and anti-mouse APC (B lymphocytes). Fraction of B lymphocytes was stained using CD20-FITC antibodies (Beckman Coulter). Shed GP binding to cells was analyzed by flow cytometry **(B)** DCs and MØ were either incubated with supernatants containing GP-HS (as above) or were pre-treated with anti-TLR4 antibody (Ab⁺) or isotypic control antibodies (Ab⁻) prior to shed GP treatment. Shed GP binding to cells was analyzed by flow cytometry. **(C)** DCs and MØ were incubated with serum containing 150 ng/ml of MBL-containing sera (MBL⁺), MBL-deficient sera (MBL⁻) or culture media alone before washing and incubation with shed GP (as above). Filled

histograms represent staining with an isotype control antibody. Data shown are representative of three independent experiments with at least three different donors. For quantitative data and statistics see figure S3.

Figure 3. Shed GP induces transcriptional activation of cytokines in DCs and MØ.

DCs and MØ (5×10^5 cells) were incubated with concentrated culture supernatants as above (as Figure 2) and with LPS (500 pg/ml). Cells were collected at 4, 8, 12 and 24h post-incubation as indicated. Expression levels of mRNA encoding TNF- α , IL-6, IL-10 IL-12p40, and GAPDH were measured by RT-Q-PCR in DCs **(A)** and MØ **(B)**. Relative levels of the cytokines were obtained by normalization to GAPDH and Mock as indicated. **(C)** DCs and MØ as above were treated with concentrated culture supernatants for 8h as follows : culture medium alone (Mock), 500 pg/ml LPS, shed GP (HS) or the cells were pre-incubated with anti-TLR4 antibody (Ab^+) and then treated with shed GP or LPS. Expression levels of mRNAs encoding TNF α and GAPDH were analyzed by RT-Q-PCR as above. Relative levels of TNF α mRNA were obtained by normalization to GAPDH and Mock. **(D)** DCs and MØ were treated with concentrated culture supernatants as above. In addition HS culture supernatants were pre-incubated with different concentrations of MBL-containing sera as indicated (150 ng/ml, 75 ng/ml and 7.5 ng/ml) before addition to DCs or MØ. An MBL-deficient serum (MBL $^-$) was used as a negative control. Cells were collected 8 hours post treatment and analyzed by RT-Q-PCR as above. Human sera did not itself activate DCs or MØ. **(A, B, C and D)** The data shown are representative of three independent experiments using three blood donors and presented as mean \pm sd of triplicates. Statistical significance (paired-sample *t* test) compared to GP-HS is shown as follows: * - $p < 0.05$ and ** - $p < 0.01$; n.s. – not significant.

Figure 4. Shed GP induces secretion of cytokines from DCs and MØ. (A) 5×10^5 of DCs (in white) and MØ (in gray) were incubated for 24h with concentrated culture supernatants as in Figure 2. (B) 5×10^5 of DCs (in white) and MØ (in gray) were incubated for 24h with concentrated culture supernatants as above and, in addition, the cells were pre-incubated with an anti-TLR4 antibody (Ab^+) followed by treatment with HS or LPS. (C) Shed GP induces secretion of cytokines from DCs and MØ in a dose-dependent manner. 5×10^5 of DCs (in white) and MØ (in gray) were incubated for 24h with concentrated culture supernatants, or LPS as above. Decreasing quantities of shed GP (HS) ranging from $0.4 \mu\text{g}$ to $0.1 \mu\text{g}$ were tested as indicated. (A, B and C) The concentration of each cytokine in duplicate was measured using Multiplex cytometric bead array (Bio-Rad) in Luminex MAGPIX. The data shown are representative of three independent experiments with three blood donors and presented as mean \pm sd of triplicates. Statistically significant differences (paired-sample *t* test) compared to GP-HS are shown as follows: * - $p < 0.05$.

Figure 5. Shed GP induces the phenotypic maturation of DCs and MØ. 5×10^5 of DCs (A) and macrophages (B) were incubated with concentrated culture supernatants as above. The cells were harvested at 48h post-incubation and expression of CD80, CD86, CD40 and CD83 was analyzed by flow cytometry. Filled histograms represent staining with an appropriate isotype-matched control antibody. Data shown are representative of three independent experiments using three blood donors. For quantitative data and statistics see figure S5.

Figure 6. Deglycosylation of shed GP affects activation of DCs and macrophages. (A) Concentrated culture supernatants of 293T cells expressing GP-HS were treated

either with inactivated deglycosylation enzymes (lane 1) or a mix of deglycosylases (lane 2). Samples were analyzed by Western blot under non-reducing conditions. Deglycosylated EBOV GP is indicated as *GP_{1,2Δ}. **(B)** 5×10^5 DCs (in white) and MØ (in gray) were incubated for 24h with concentrated culture supernatants of 293T cells expressing GP-HS (HS), GP-LS (LS), and GFP (Mock) after treatment with inactivated deglycosylation enzymes, LPS as a positive control and HS after treatment with a mix of deglycosylation enzymes (*HS). In addition the cells were incubated with *HS following LPS treatment (*HS+LPS). The concentration of TNF α in duplicate was measured using Multiplex cytometric bead array (Bio-Rad) in Luminex MAGPIX. The data shown are representative of three independent experiments with three blood donors and presented as mean \pm sd of triplicates. Statistical significance (paired-sample *t* test) compared to GP-HS is shown as follows: * - $p < 0.05$.

Figure 7. EBOV Shed GP increases cell permeability of HUVEC in vitro. HUVEC cells were seeded onto the culture inserts of permeability assay chambers and incubated for 22h with concentrated culture supernatants from 293T cells expressing GFP (Mock), sGP, GP-LS (LS), GP-HS (HS) and LPS (10ng/ml). Cells were also incubated with human sera containing MBL (MBL⁺), MBL-deficient sera (MBL⁻), a mixture of HS/MBL-containing sera (HS+MBL⁺) and HS/MBL-deficient sera (HS+MBL⁻). As a permeability control the cells were treated with TNF α (20 ng/ml) **(A and C)**. In parallel, HUVEC cells were also incubated for 22h with culture supernatants from macrophages previously stimulated with samples of concentrated culture supernatants and controls as above. As a permeability control the cells were treated with TNF α (100 ng/ml) **(B and D)**. In all cases following incubation, FITC-Dextran was added to each insert and FITC-Dextran leaking out into the bottom chamber was assayed using a fluorometer and presented as absorbance fluorescent units. Bars represent \pm SD of triplicates. The data

are representative of three independent experiments. * indicates statistically significant differences (paired-sample *t* test, * - $p < 0.05$ and ** - $p < 0.01$) or n.s. - statistically not significant (**A and B**). The integrity of HUVEC monolayers assayed in A and B was also observed with a MacroFluo microscope after fixation and coloration (**C and D**).

Figure 8. Role of shed GP during EBOV infection. In our model, EBOV-infected cells release both virions and soluble shed GP. Shed GP binds DCs and macrophages using TLR4 and activates these cells for release of pro- and anti-inflammatory cytokines. The soluble nature of shed GP enables triggering of additional and remote targets such as non-infected APCs. Release of cytokines from these cells will further amplify inflammation by recruiting and activating other APCs, lymphocytes, endothelial cells, and other cell types including hepatocytes. Overall the release of shed GP is likely to contribute to increased vascular permeability, disseminated intravascular coagulation, dysregulated inflammation and impairment of cell function and organ failure.

Figure S1. (A and B) Quantification of shed GP (A) and sGP (B) produced in GP-HS- and sGP-expressing 293T cells. Serial dilutions of recombinant EBOV GP as indicated, produced in insect cells (recGP, IBT Bioservices) were used to estimate amounts of HS and sGP in concentrated culture supernatants. Samples of GP and sGP were analyzed by Western blot using anti-GP1 antibodies and ImageQuantTL software (GE Lifesciences). As illustrated the standard curve was linear with an R^2 value > 0.99 over the range used for quantification (open diamonds). Note: the molecular mass of shed GP is ~ 160 kDa, sGP ~ 55 kDa, in comparison to recGP of ~ 110 kDa. Shed GP sample (closed square) corresponds to ~ 11.3 ng of recGP and thus contains ~ 1 μ g/ml in the concentrated

supernatants. sGP sample (closed square) corresponds to ~44 ng of recGP and thus contains ~18 $\mu\text{g/ml}$ of sGP.

Figure S2. (A) Schematic representation of EBOV surface GP, shed GP and a truncated GP mutant ($\text{GP}_{\Delta\text{TM}}$) containing a stop codon immediate upstream of the transmembrane anchor. (B) Sedimentation analysis. Samples of shed GP and $\text{GP}_{\Delta\text{TM}}$ were subjected to centrifugation through 5-25% sucrose gradients followed by analysis of gradient fractions using Western blot and anti-GP antibodies. Fractions 1-2 correspond to GP trimers and 5-7 to GP monomers. The orientation of the gradient is shown.

Figure S3. Quantitative data and statistical analysis of data presented in Figure 2. EBOV shed GP binding to DCs and macrophages. (A) Human monocyte-derived dendritic cells (DCs), monocyte-derived macrophages ($\text{M}\emptyset$), and PBLs (shown B lymphocytes, B) were incubated with shed GP as well as with shed GP in the presence of MBL-containing sera (150 ng/ml, $\text{HS}+\text{MBL}^+$), as described in Figure 2. Bound proteins were detected by subsequent incubation with mouse anti-GP1 antibodies and anti-mouse Alexa 488 coupled antibodies (DCs and $\text{M}\emptyset$) and anti-mouse APC (B lymphocytes). Fraction of B lymphocytes was stained using CD20-FITC antibodies (Beckman Coulter). (B) DCs and $\text{M}\emptyset$ were either incubated with supernatants containing GP-HS (as above) or were pre-treated with anti-TLR4 antibody (Ab^+) or isotypic control antibodies (Ab^-) prior to shed GP treatment. (C) DCs and $\text{M}\emptyset$ were incubated with serum containing 150 ng/ml of MBL-containing sera (MBL^+), MBL-deficient sera (MBL^-) or culture media alone before washing and incubation with shed GP (as above). (A, B and C) Shed GP binding to cells was analyzed by flow cytometry and shown as raw MFI data for at least three independent blood donors. Statistically

significant differences compared to HS are shown as follows: * - $p < 0.05$ and ** - $p < 0.01$; n.s. – not significant.

Figure S4. EBOV shed GP containing sera does not activate DCs and MØ. Human monocyte-derived dendritic cells (DCs) and monocyte-derived macrophages (MØ) were incubated with either shed GP as above (HS+0%) or with shed GP in the presence of 5% bovine sera (HS+5%). As control, the cells were incubated with LPS or concentrated culture supernatants from GFP expressing cells (Mock). Statistically significant differences (paired-sample t test) compared to GP-HS are shown as follows: * - $p < 0.05$.

Figure S5. Quantitative data and statistical analysis of data presented in Figure 5. Shed GP induces the phenotypic maturation of DCs and MØ. 5×10^5 of DCs (**A**) and macrophages (**B**) were incubated with concentrated culture supernatants. The cells were harvested at 48h post-incubation and expression of CD80, CD86, CD40 and CD83 was analyzed by flow cytometry. Shed GP binding to cells was analyzed by flow cytometry and shown as raw MFI data for at least three independent blood donors. Statistically significant differences compared to HS are shown as follows: * - $p < 0.05$ and ** - $p < 0.01$; *** - $p < 0.001$.

Figure 1

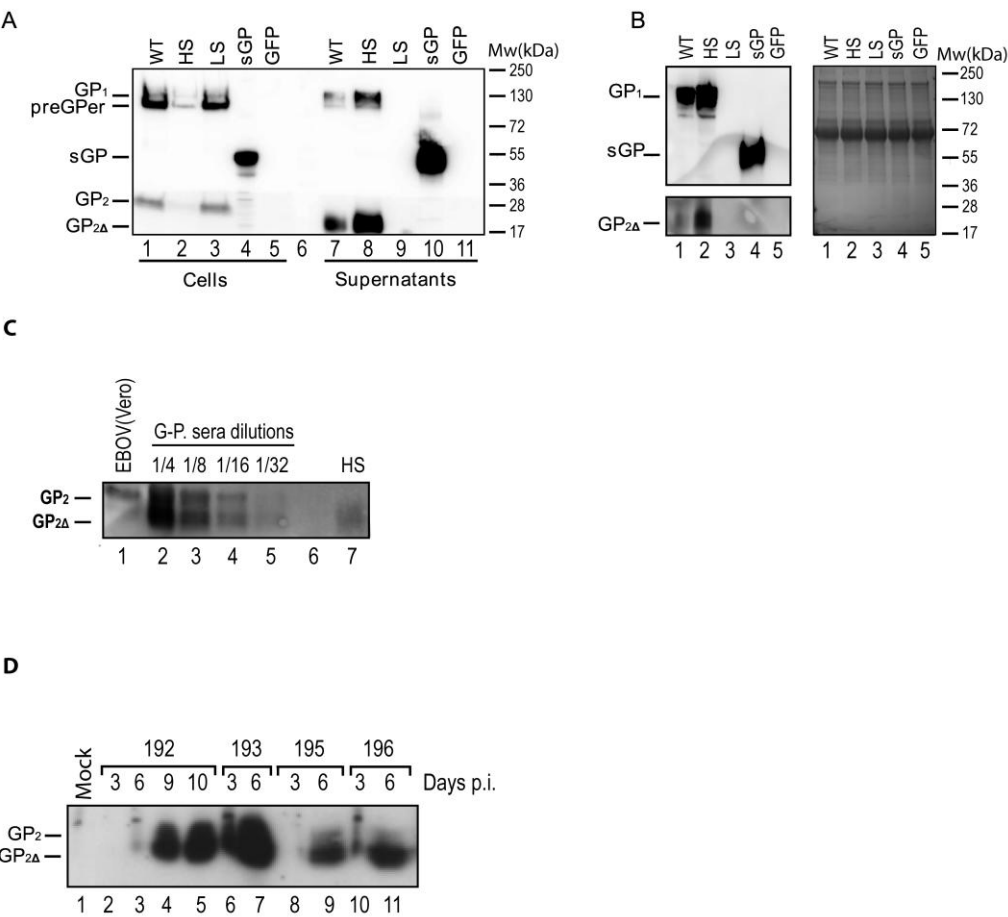
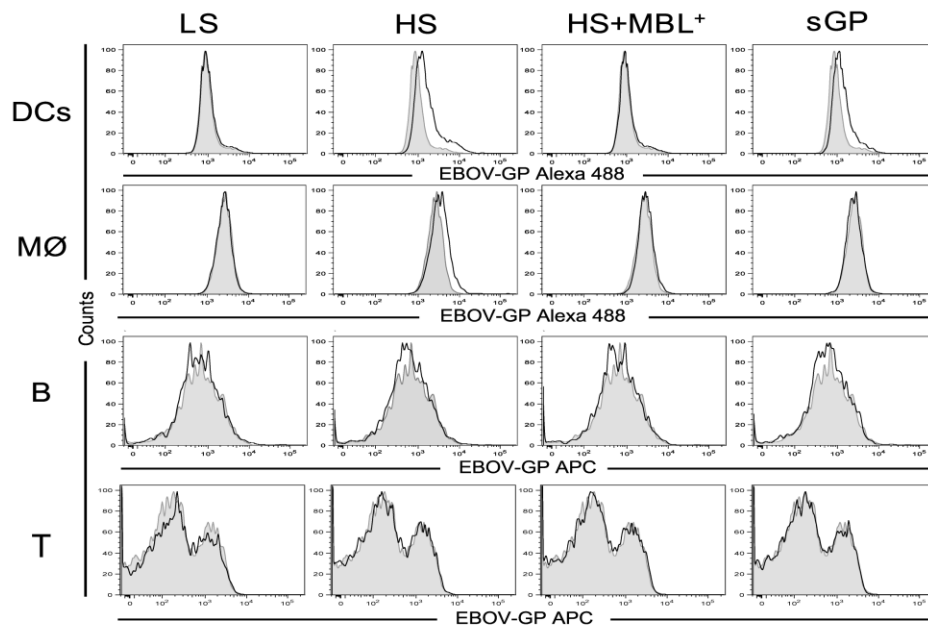
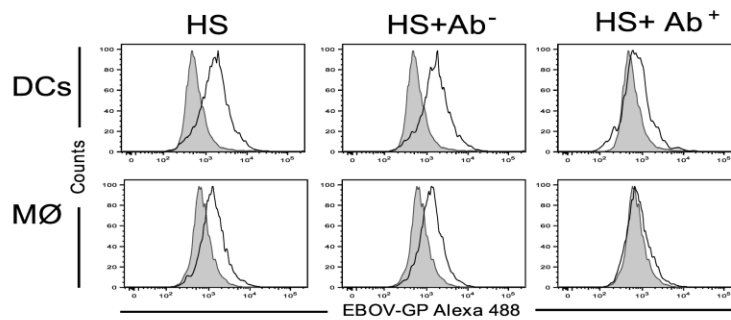


Figure 2

A



B



C

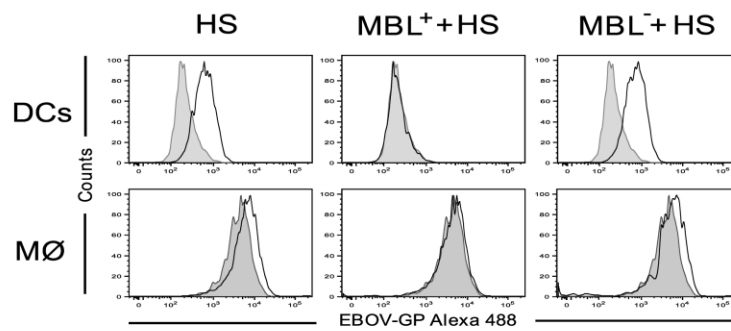


Figure 3

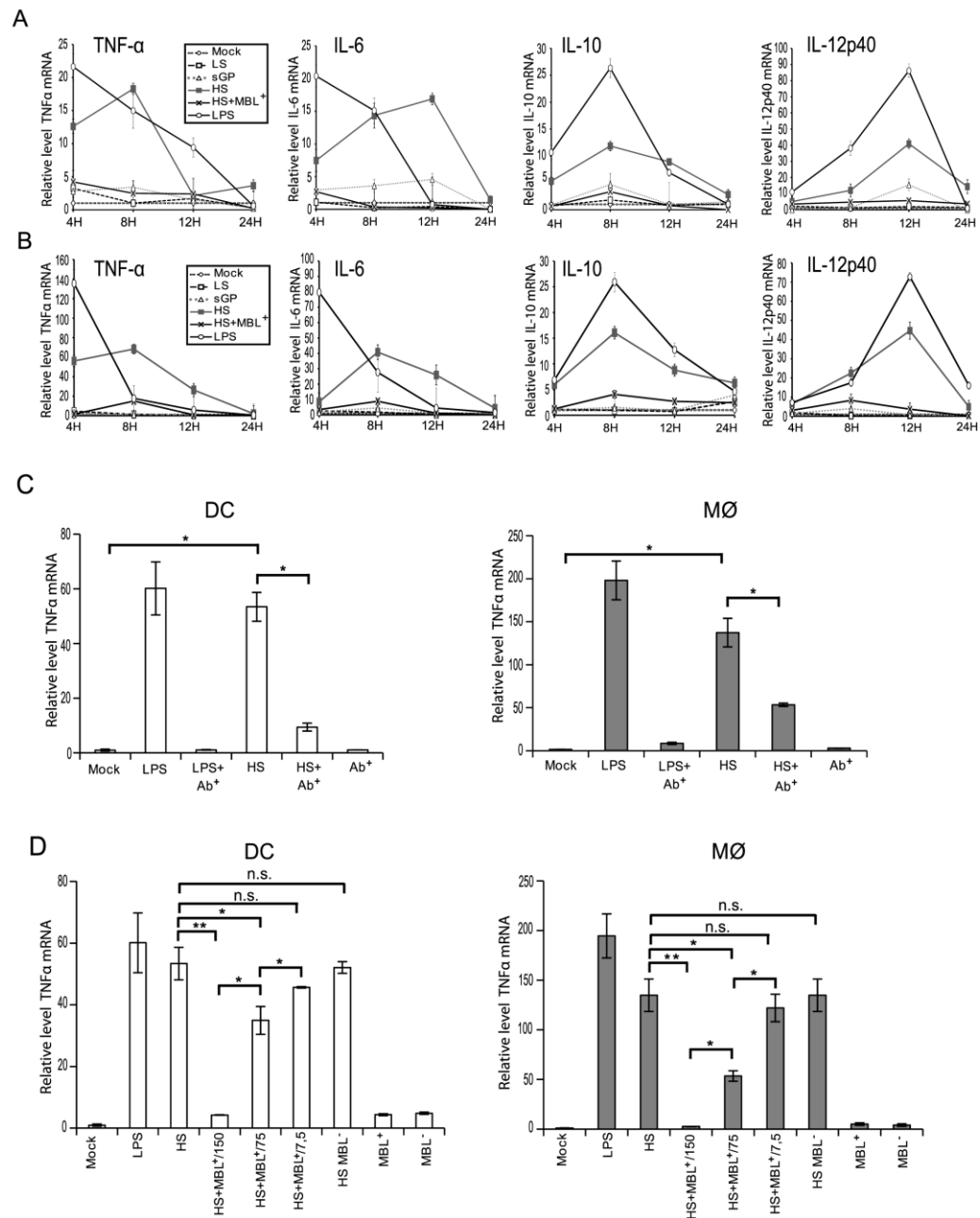


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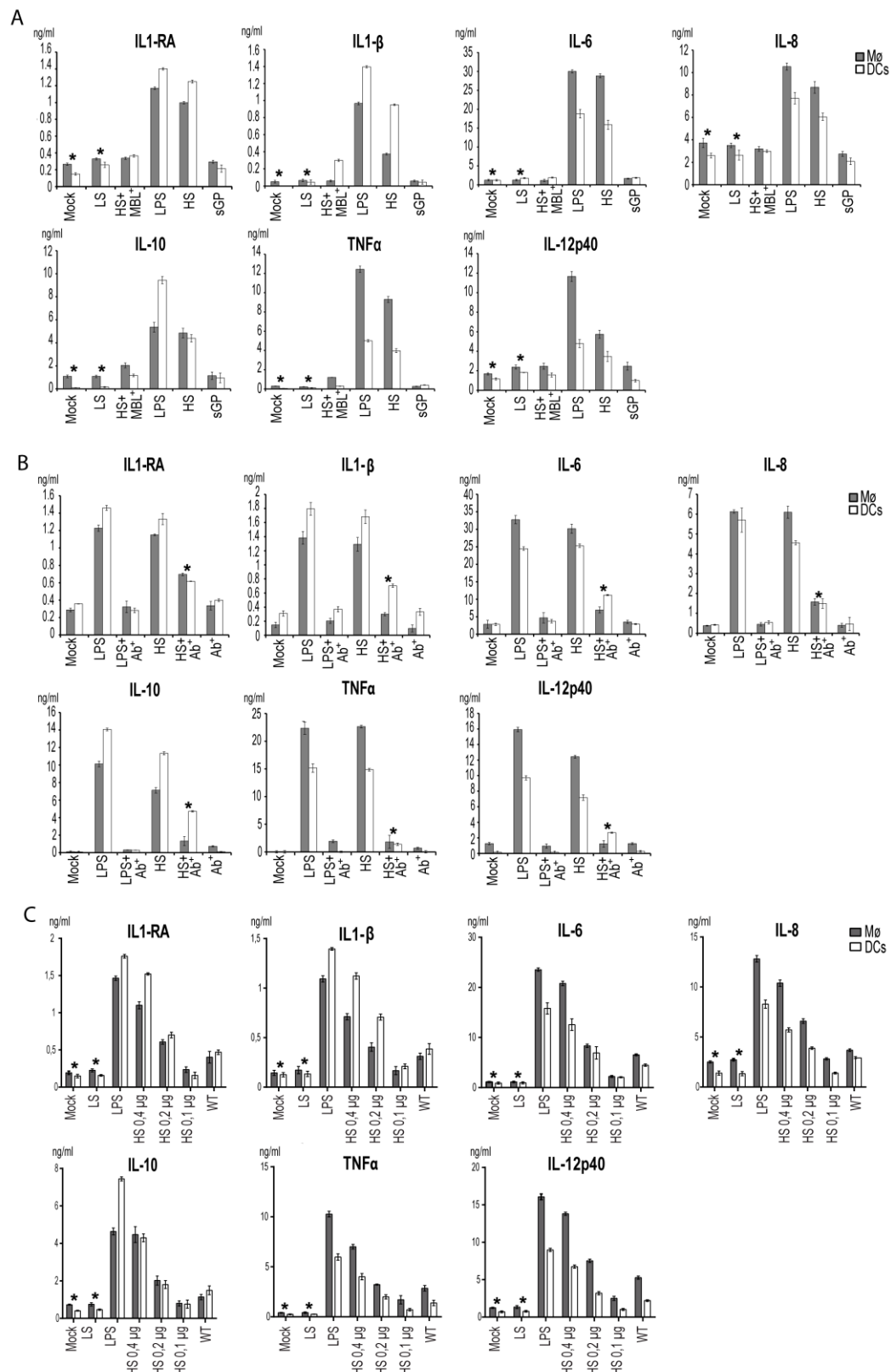


Figure 5

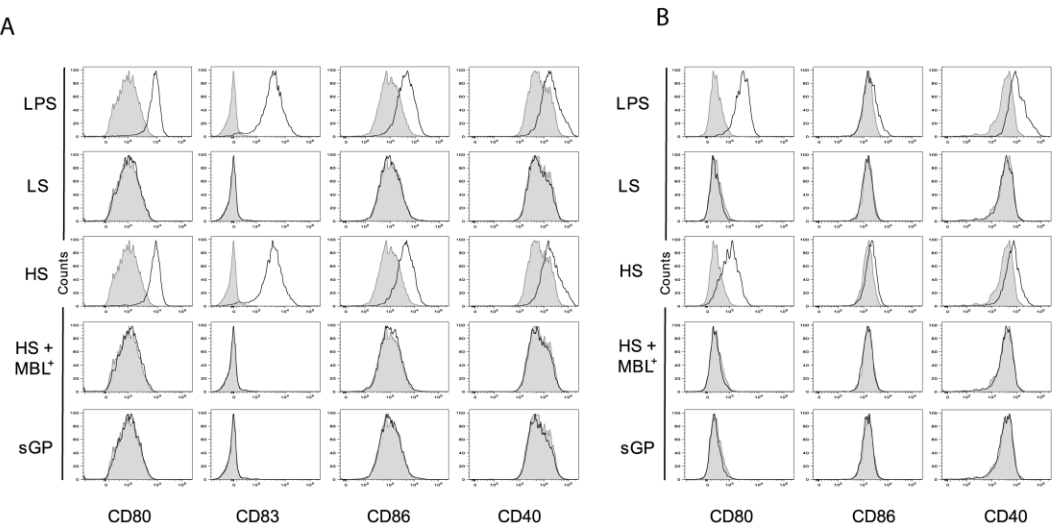


Figure 6

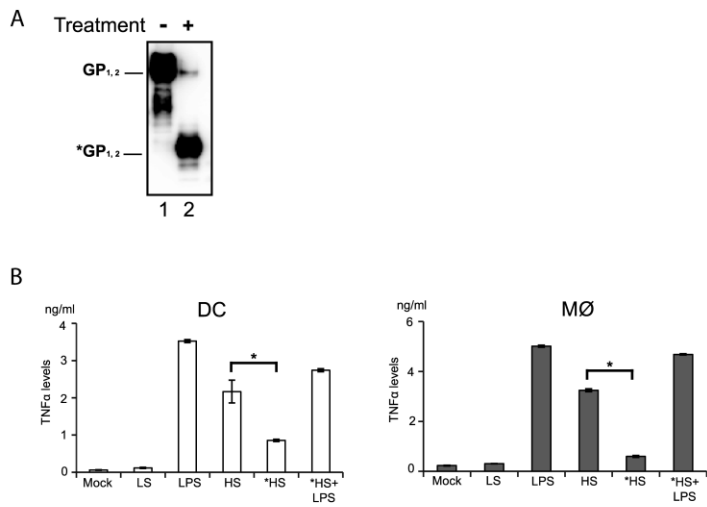


Figure 7

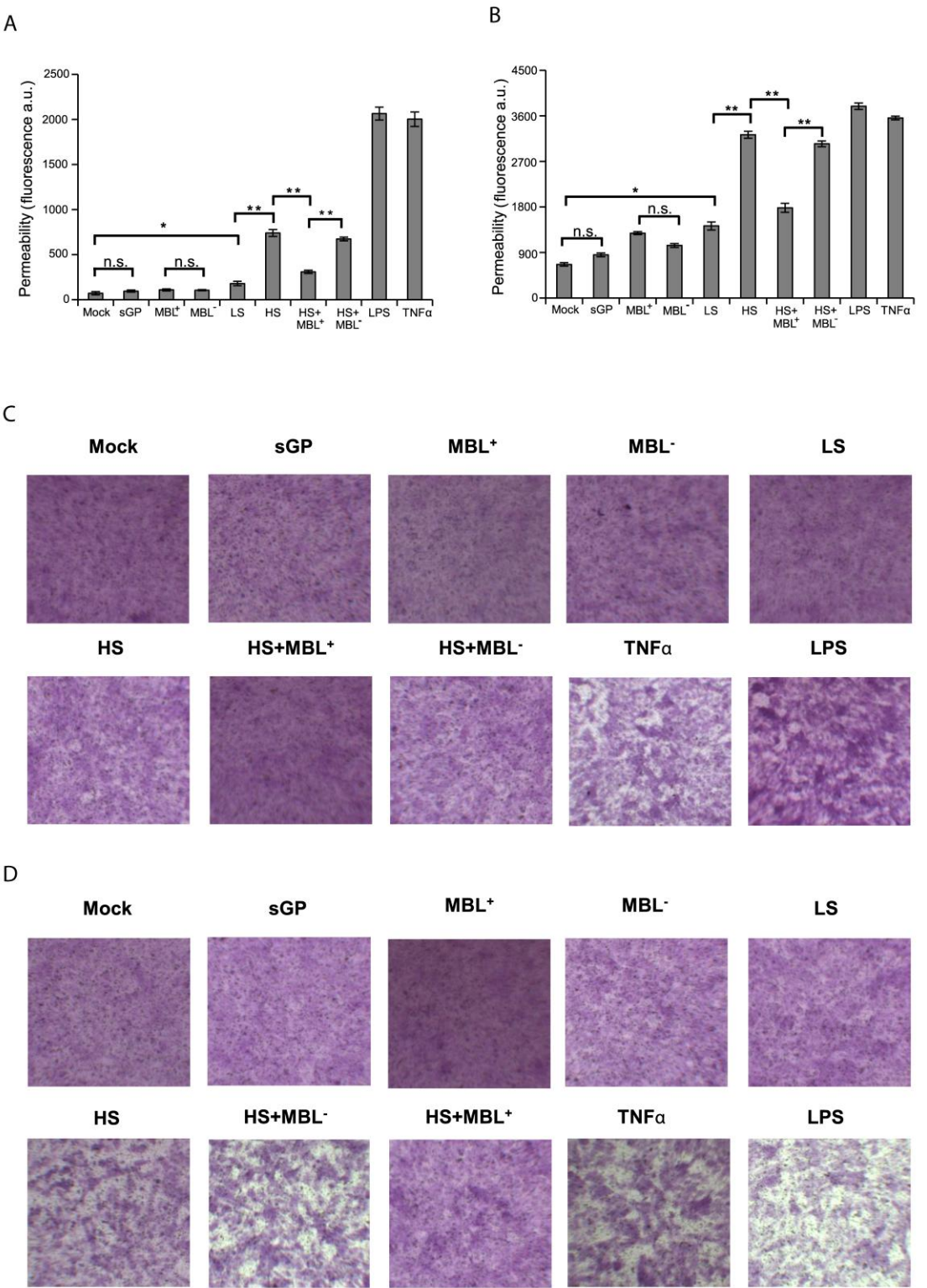


Figure 8

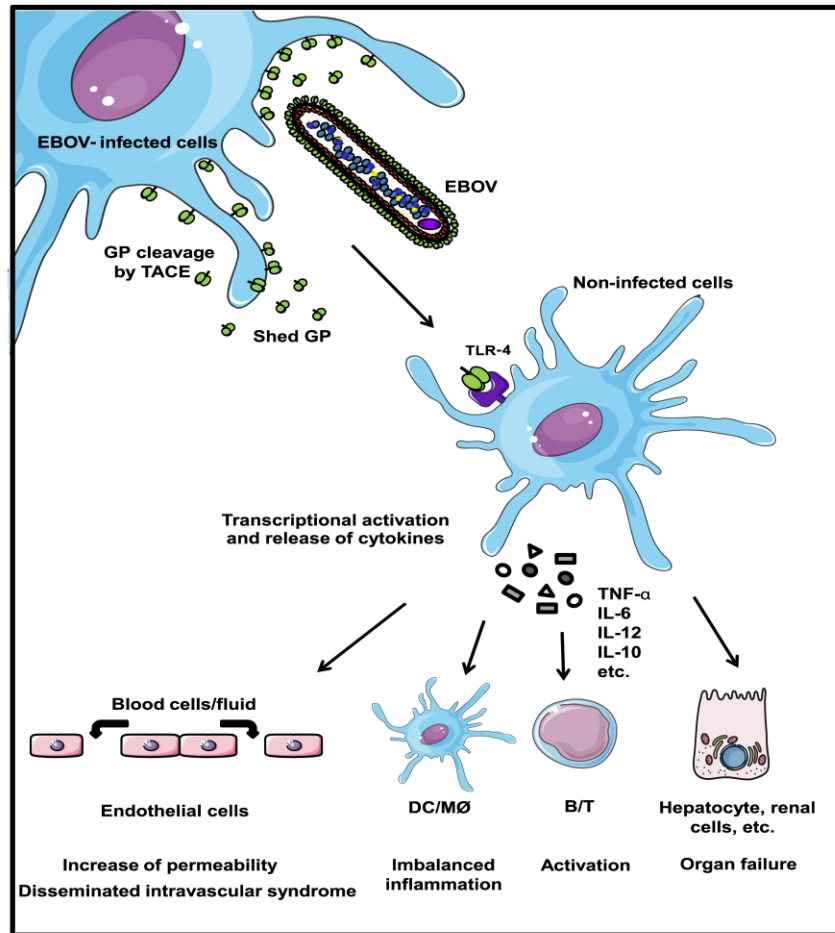


Figure S1

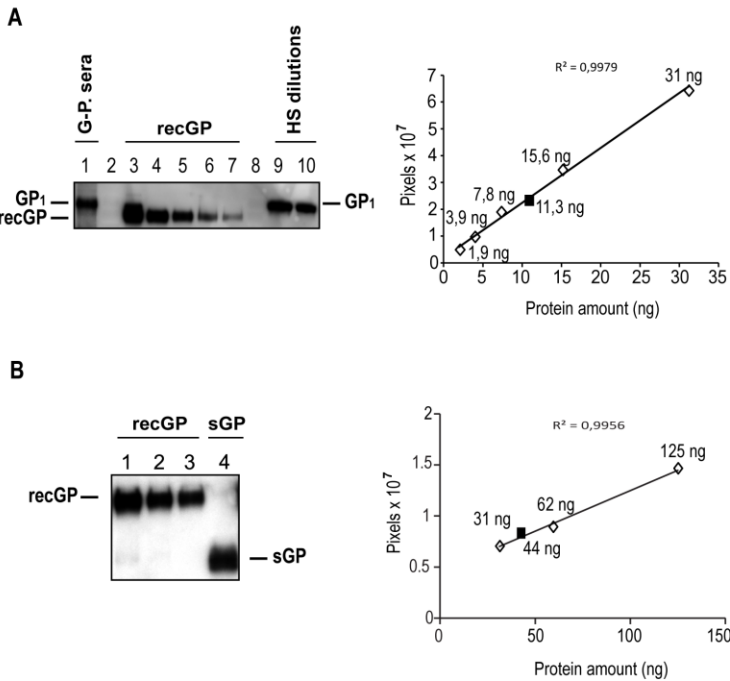


Figure S2

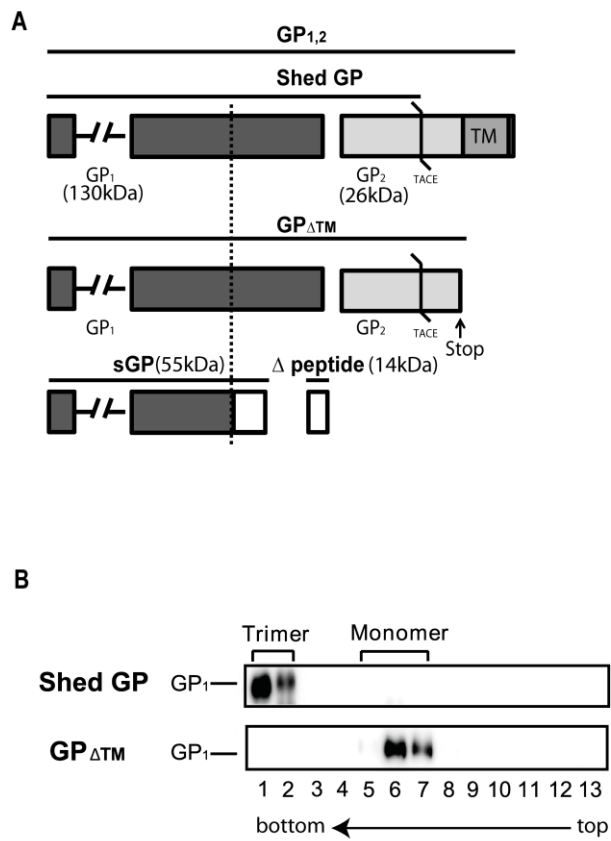


Figure S3

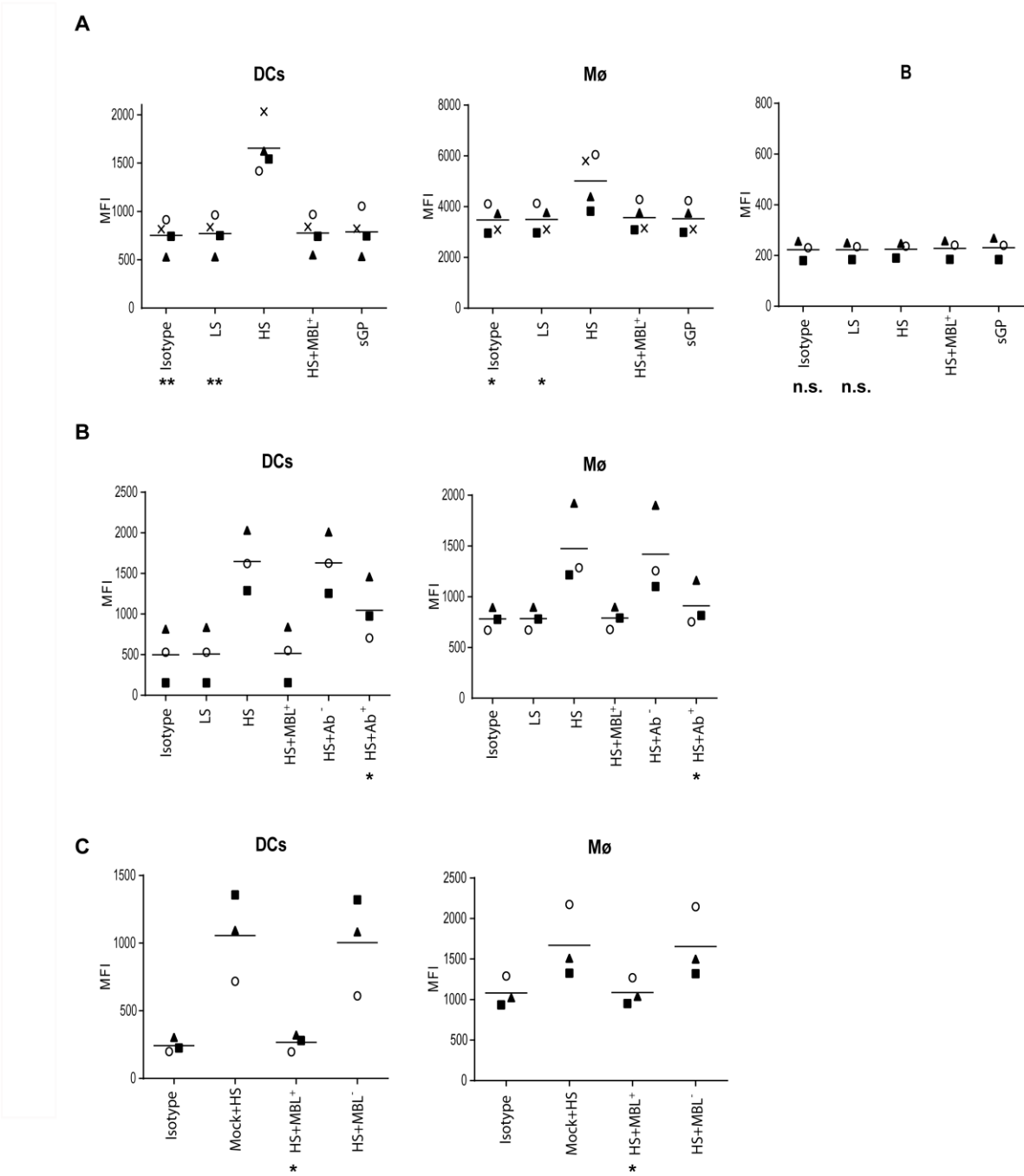


Figure S4

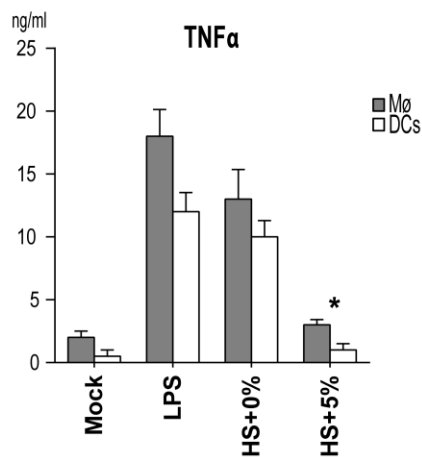
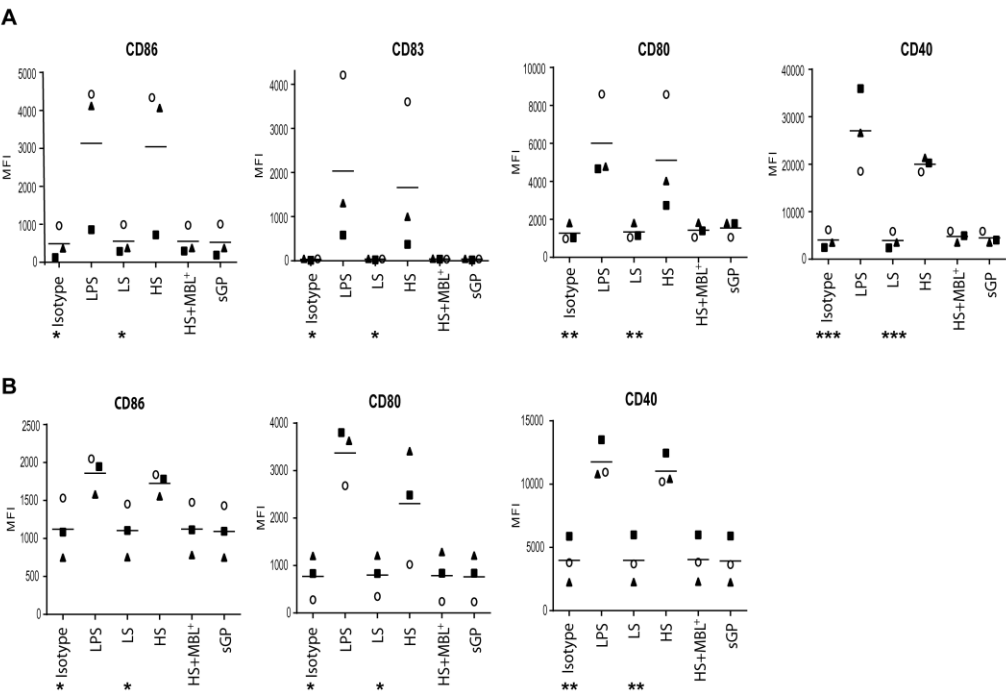


Figure S5



- **Surface glycoproteins of the recently identified African Henipavirus promote viral entry and cell fusion in a range of human, simian and bat cell lines.** Lawrence P, Escudero-Perez B. Drexler JF, Corman VM, Müller MA, Drosten C, Volchkov V., Virus Research, January 2014.

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Dérégulation de l'hémostase dans l'infection à filovirus

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Introduction

Les filovirus Ebola et Marburg sont des agents pathogènes de niveau 4 responsables de fièvres hémorragiques virales (FHV) souvent fatales chez l'homme et les primates non humains. Dans cette revue, nous nous attacherons à décrire l'aspect multi causal du syndrome hémorragique observé dans les infections à filovirus. Ces agents pathogènes entraînent des taux de mortalité chez l'homme pouvant atteindre 90% et pour lesquels aucun traitement thérapeutique ou prophylactique n'existe à ce jour.

Les filovirus appartiennent à l'ordre des Mononegavirales, qui regroupe les virus à ARN négatif simple brin non segmentés. Ils doivent leur nom à la morphologie filiforme des virions. Les genres *Marburgvirus*, *Ebolavirus* et *Cuevavirus* constituent la famille des Filoviridae. Il existe 5 espèces de virus Ebola : Zaïre, Reston, Bundibugyo, Sudan et Taï Forest ebolavirus, 2 espèces de virus Marburg : Marburg virus et Ravn virus et 1 seule espèce décrite de Cuevavirus, le virus Lloviu (22).

Les filovirus ont en commun la structure de leur génome. Celle-ci est composée d'une succession linéaire de gènes, séparée par de courtes régions intergénique, qui codent pour 7 protéines structurales : la nucléoprotéine NP, la protéine VP35, la protéine VP40, la glycoprotéine GP, la protéine VP30, la protéine VP24 et la polymérase L ainsi que pour une protéine non structurale sécrétée spécifique des virus Ebola, la protéine sGP. La glycoprotéine GP provient d'un second ARN messenger généré au cours de la transcription par l'édition du gène de la glycoprotéine (45). Les protéines NP, VP35, VP30 et L constituent avec l'ARN viral le complexe réplcatif permettant la synthèse des ARN messagers viraux et la production d'ARN génomique viral. Ce complexe, appelé ribonucléoprotéine, est transporté à la membrane plasmique via l'interaction avec la protéine de matrice VP40 qui initie le bourgeonnement des virions à la membrane plasmique. Ceux-ci sont ainsi enveloppés d'une bicouche lipidique et arborent à leur surface la glycoprotéine GP responsable de l'attachement aux cellules cibles. Fait intéressant, bien que les protéines soient très similaires entre les virus Ebola et Marburg, il existe des différences de fonction portées par celles-ci. Ainsi chez Ebola

la protéine VP24 bloque la réponse à l'interféron alors que cette fonction est portée par la protéine de matrice VP40 dans le virus Marburg (34, 43).

Depuis la découverte du premier filovirus (Marburg 1967), de nombreuses campagnes de recherche ont été entreprises afin de caractériser le réservoir de ces virus. Ces études ont permis d'identifier divers mammifères infectés par le virus Ebola et notamment des primates, des antilopes et des porcs-épics (36). En 2005, Eric Leroy et son équipe ont identifié la présence d'ARN viral dans des organes de chauve-souris frugivores ne présentant aucun symptôme visible (25). Trois espèces sont supposées être les réservoirs du virus Ebola : *Hypsignathus monstrosus*, *Epomops franqueti* et *Myonycteris torquata* (Figure 1). Cependant il n'a jamais été possible de confirmer formellement la présence de virus infectieux dans ces espèces. A l'inverse, la circulation du virus Marburg dans les populations de *Rousettus aegyptiacus* et un lien direct entre les souches isolées sur les animaux et celle responsable d'épidémie a pu être établi (42). Les animaux porteurs de ce virus ne présentaient pas de symptôme apparent montrant ainsi que cette espèce contrôle parfaitement la réplication du virus. Quant au virus Lloviu, son ARN a été découvert très récemment en Espagne lors d'une épidémie ayant décimé une population de minioptère de Schreiber en 2002 (29). Aucun élément ne permet cependant d'affirmer que ce virus est responsable de la mortalité observée chez ces chauves-souris. Cette observation est la première réalisée chez des chauves-souris insectivores, ce qui sous-entend que les filovirus pourraient être retrouvés à plus grande échelle et dans des espèces beaucoup plus diverses.



Figure 1 : Les chauves-souris, les réservoirs présumés des filovirus :

- a) *Hypsignathus monstrosus*, b) *Epomops franqueti*, c) *Myonycteris torquata*, d) *Rousettus aegyptiacus*, e) *Miniopterus Schreibersi*

Manifestation clinique de l'infection à Filovirus

Chez l'homme, après une phase d'incubation de 2 à 21 jours, les symptômes cliniques débutent par une série de symptômes non spécifiques comprenant une brusque poussée de fièvre et de forts maux de tête durant 5 à 7 jours (figure 2). Les patients souffrent le plus souvent de cachexie, de vomissements, de diarrhée, de douleurs abdominales et d'une altération de leur état mental. Dans 10 à 50% des cas, des symptômes hémorragiques apparaissent avec notamment des hémorragies pétéchiales, des saignements au niveau du tractus digestif, des conjonctives, et très souvent aux points d'injections (11, 32). Dans les infections fatales, les causes du décès sont liées de façon générale à une incapacité de la réponse immunitaire à contrôler la croissance virale ce qui induit une défaillance multi viscérale, une dérégulation du système cardiovasculaire (pression sanguine, hémostasie, bio distribution des fluides) et une dérégulation de l'hémostasie (1). En dépit de l'extrême pathogénicité du virus Ebola, des patients infectés survivent à la phase aiguë de la maladie et guérissent de l'infection en absence de tout traitement en contrôlant la réplication virale. Une réponse inflammatoire précoce et mesurée semble avoir un rôle dans le contrôle de l'infection par le virus Ebola (4), cependant les mécanismes moléculaires permettant la survie de ces patients sont peu connus. De plus, lors des épidémies à virus Ebola, des cas d'infections humaines asymptomatiques ont été identifiés pour des individus directement exposés à des patients ou du matériel contaminé par le virus Ebola (5, 24). Cette observation est rapproché du fait que la plupart des mammifères non primates, infectés expérimentalement développent également une infection asymptomatique (40) ainsi il existe probablement des facteurs génétiques, immunitaires restrictifs à l'infection.

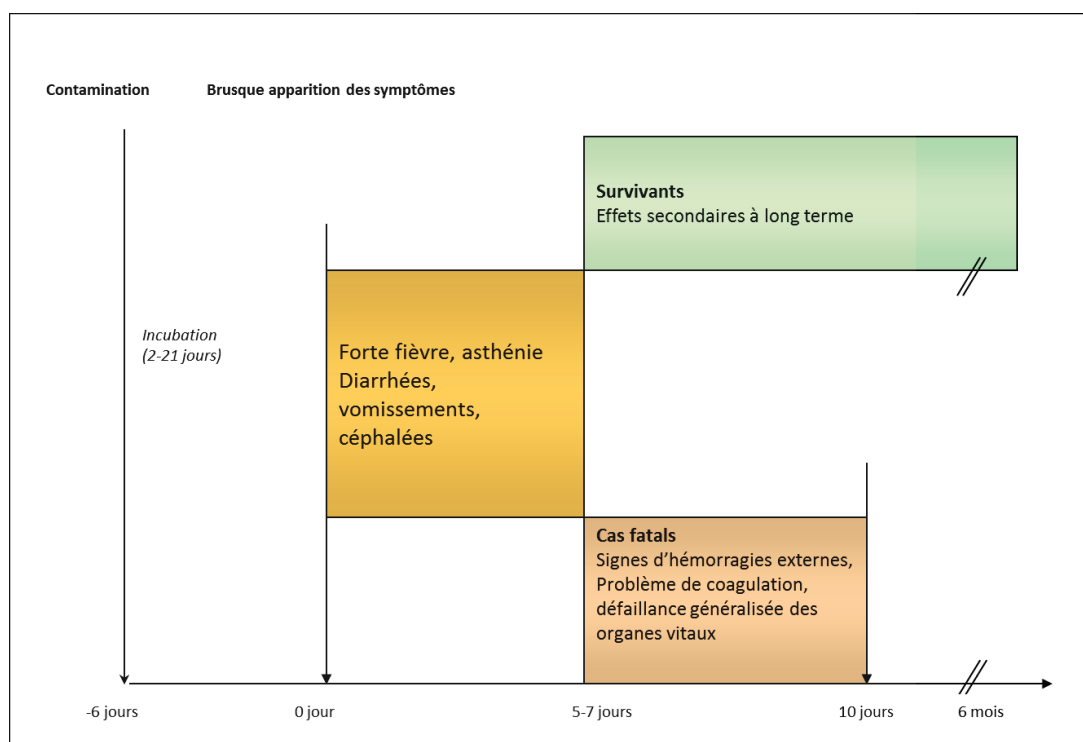


Figure 2 : Evolution de la maladie à virus Ebola

Schéma du développement des fièvres hémorragiques à virus Ebola à partir des données des épidémies de Mosango, de Yambuku et Gulu (données OMS et (26))

Des études détaillées des infections à filovirus ont montré que les cellules dendritiques et les macrophages sont les cellules cibles initiales (13, 15, 26). Ces cellules mobiles répliquent activement le virus et transportent l'agent pathogène vers les ganglions lymphatiques régionaux, d'où après d'autres cycles réplicatifs, des virions sont libérés par les voies lymphatiques et la circulation sanguine. Les organes primo infectés sont la rate et le foie. Dans ces tissus, le virus infecte les cellules parenchymateuses, y compris les hépatocytes et les cellules corticales surrénales, entraînant l'expansion de foyers de nécrose due à la mort cellulaire viro-induite. Les conclusions de quelques rapports d'autopsie disponibles de patients infectés font état d'une hémorragie limitée dans les muqueuses, la peau et les organes parenchymateux, y compris l'estomac et les intestins, associée à de vastes zones de nécrose du parenchyme hépatique (21, 31, 48, 49). Des nécroses similaires avec dépôt de fibrine, ont également été décrites dans la pulpe blanche et la pulpe rouge de la rate, modifiant souvent son architecture. De plus, bien que les cellules endothéliales en culture soient facilement infectées par le virus, il y a peu de preuves de la réplication virale dans ces cellules *in vivo* avant les dernières étapes de la maladie.

Une nécrose multifocale est observée dans les tissus lymphoïdes, celle-ci ne résulte pas directement de l'infection par le virus, les lymphocytes n'étant pas infectable mais provient d'un emballement du système immunitaire induisant l'apoptose massive des lymphocytes (3-5, 26).

Durant l'évolution de la maladie, les modifications hématologiques comprennent une lymphopénie, une hyperleucocytose des polynucléaires, et au niveau biochimiques une élévation des concentrations sériques d'aspartate et d'alanine aminotransférases caractéristique de l'atteinte hépatique (18). Une thrombocytopénie, associée à une augmentation des D-dimères (produit de dégradation de la fibrine) et une élévation du temps de céphaline activée sont retrouvées et sont les marqueurs du syndrome hémorragique et notamment de la coagulation intra vasculaire disséminé (CIVD). Cette CIVD est une caractéristique majeure de l'infection à filovirus.

La CIVD, une origine multifactorielle

La CIVD se caractérise par la formation localisée de multiples caillots dans la circulation sanguine ce qui a pour effet de consommer les facteurs de coagulation et les plaquettes. Ce syndrome induit des thromboses localisées entraînant des défaillances fonctionnelles et simultanément une perte de la coagulation sanguine par déplétion des facteurs de coagulations. Lors des fièvres hémorragiques à filovirus, la CIVD est retrouvée chez l'homme ainsi que dans les modèles infectieux de primates non-humains cependant elle n'est pas retrouvée chez les rongeurs à l'exception du hamster (35). La CIVD semble donc être dépendant de l'espèce, *a contrario*, la dérégulation du système immunitaire est retrouvée dans tous les modèles animaux et joue un rôle majeur dans l'absence de contrôle de la réplication virale. La CIVD trouve son origine dans plusieurs facteurs, certains étant liés directement aux virus, d'autres provenant des dommages induits et de l'emballement de la réponse inflammatoire.

Le facteur tissulaire (TF)

Les études sur le virus Ebola Zaïre dans un modèle primate ont montré que les anomalies de la coagulation sont initiées en tout début d'infection, lorsque les macrophages infectés expriment le facteur tissulaire (TF) à leurs surfaces (14). Cette glycoprotéine de 47kDa est un récepteur transmembranaire exprimé par les épithéliums, les muqueuses, la capsule des organes et de manière inductible par les macrophages, les monocytes et les cellules endothéliales. Le TF est exprimé dans des compartiments

n'étant pas en contact avec le sang et cette protéine joue un rôle de senseur des hémorragies, en induisant la coagulation du sang lorsque celui-ci entre en contact avec ces tissus. Cette protéine est également retrouvée, de façon pathologique dans la circulation sanguine, à la surface de microparticules membranaires et ce notamment dans les cas de CIVD. Ces microparticules sont notamment libérées par les monocytes. La stimulation des monocytes par des endotoxines, du TNF, de l'IL-1 β ou encore par MCP-1, induit l'expression à leurs surfaces du TF (7, 10). La libération de TF actif dans la circulation contribuent directement à l'élaboration de thrombose (9). En effet, le TF s'associe au facteur VIIa pour former un complexe protéolytique activant le facteur X, celui-ci étant l'acteur direct du clivage de la prothrombine en thrombine (Figure 3). De plus, le complexe TF-VIIa est un élément de signalisation connu notamment pour induire dans les cellules un afflux de Ca²⁺ et pour être pro-inflammatoire (induction d'IL6 et IL8) (33). La génération de dépôt de fibrine dû à l'action de la thrombine sur le fibrinogène résulte dans la formation de multiples thrombi et dans la consommation des facteurs de coagulation. Cette activation de la voie du TF conduit également à la consommation des plaquettes. Ainsi différentes protéines recombinantes (rNAPC2 et APC/Xigris) inhibitrices de la voie du TF ont montré leurs activités dans le traitement de l'infection à virus Ebola chez le singe (12, 17). Le rNAPC2, pour Recombinant nematode anticoagulant protein c2 inhibe directement l'activité du complexe TF/facteur VIIa en se fixant sur le facteur X. Le Xigris, lui, est une forme recombinante de la protéine C activée (APC). Cette protéine recombinante est indiquée dans le traitement des chocs septiques quelle qu'en soit la cause. La protéine C activée est un élément clef de la régulation de la coagulation et dans des modèles primates, il a pu être montré clairement une chute du taux d'APC au cours de l'infection (12, 14). Cependant, une récente étude a montré que pendant l'épidémie à *Sudan Ebolavirus* de 2000-2001, il n'y avait pas d'élévation du TF ni de corrélation entre son taux et la présence de signe hémorragique ou la létalité (28). Les différences observées entre le modèle primate et l'homme pourraient être de nature hôte spécifique ou virus spécifique (*Zaire ebolavirus* dans l'étude primate).

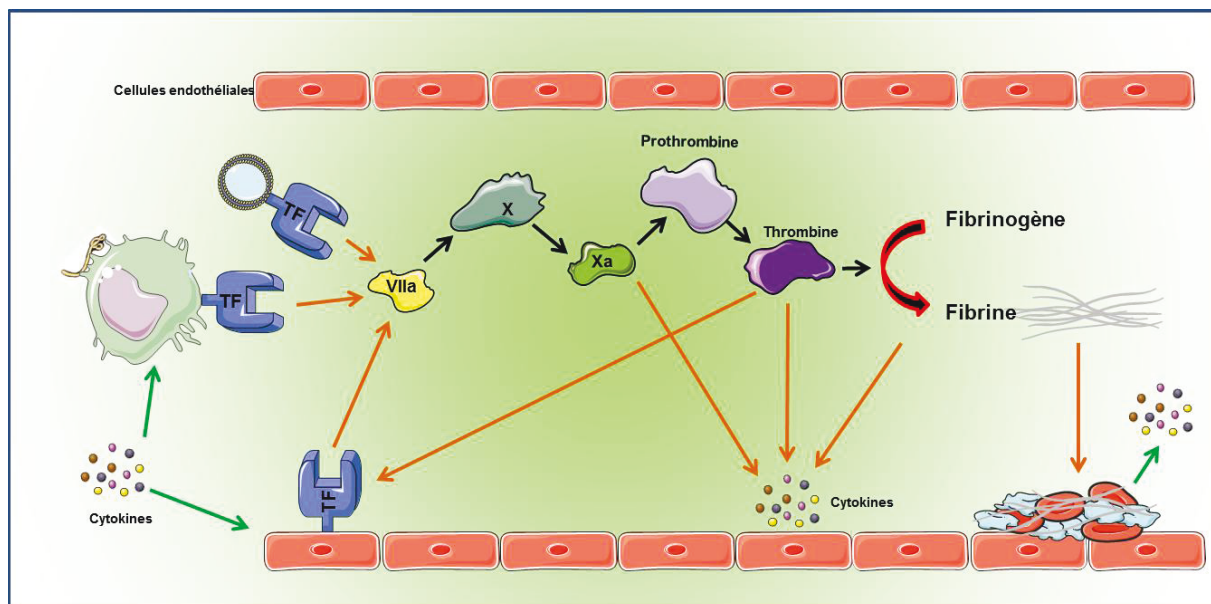


Figure 3 : Rôle du facteur tissulaire dans l'initiation de la coagulation

L'association du TF avec le facteur VIIa constitue l'élément initiateur de nombreuses voies d'activation et notamment celle de l'activation du fibrinogène.

Atteinte hépatique

Les filovirus sont des virus majoritairement hépatotropes. Les rares études histologiques sur des échantillons provenant de cas humains infectés par Zaïre ebolavirus ont montré une nécrose focale impliquant une à plusieurs cellules (2). La séquence de nécrose des hépatocytes implique tout d'abord une eosinophilie cytoplasmique avec un obscurcissement voire la dissolution des noyaux puis finalement une rarefaction des noyaux associée à une cytolysse, laissant la place à de grandes quantités de débris karyorrhéctiques. Fait intéressant, compte tenu de l'ampleur des zones de nécroses observées dans cette étude, très peu d'infiltration inflammatoire a été retrouvée dans les sinusoides. Dans les études menées sur des échantillons provenant de cas humains infectés par le virus Marburg, de grandes similarités ont été retrouvées mais la nécrose hépatique ne semble pas séquentielle (2). Ainsi, l'infection massive et la nécrose du parenchyme hépatique induit une modification de sa fonction et notamment de la production des facteurs de coagulation. La CIVD entraîne une diminution de ces facteurs et l'atteinte hépatique empêche très certainement de restaurer leurs valeurs physiologiques, contribuant ainsi à la dérégulation de l'hémostase.

Libération anarchique de cytokines

Une des caractéristiques des infections à filovirus est la libération désordonnée de cytokine pro et anti inflammatoire et notamment d'IFN de type I, de TNF α , d'IL-1, IL-2, IL-6, IL-8, IL-10 et d'IL1RA. Les données bibliographiques concernant le profil des cytokines sont toutefois encore incomplètes et parfois contradictoires (IFN et TNF α) et semblent varier selon l'espèce de virus Ebola (3, 23, 28, 44). De plus les données de concentrations de cytokines sériques représentent la somme des sécrétions par différents types cellulaires et dans différents compartiments, ceci pouvant expliquer partiellement les différences entre les observations *in vitro* et *in vivo*. Le TNF α est fortement sécrété par les macrophages infectés *in vitro* (16) et sa sécrétion est inhibée dans les cellules dendritiques dérivées de monocytes (19). Il est intéressant de noter qu'il existe plusieurs publications montrant *in vitro* le rôle de la glycoprotéine de surface (sous forme de VLP) dans l'induction de la sécrétion de TNF α par les cellules dendritiques (6, 47). Il constitue de plus un facteur essentiel de stimulation des cellules endothéliales, engendrant l'expression du TF à leur surface. De plus l'IL6 et l'IL1 ont montré leurs actions dans l'induction de la coagulation (20, 39). Une telle libération de cytokines devrait également libérer le facteur d'agrégation plaquettaire et des eicosanoides (thromboxane). La libération massive de cytokines inflammatoires et l'atteinte hépatique engendrent à leur tour la libération massive d'oxyde nitrique (NO), en effet lors de l'épidémie à *Sudan ebolavirus* de 2000, il a pu être mesurée à partir de 8 jours après le début des symptômes, des taux de plus de 150 μ M de NO (38). L'oxyde nitrique a pour principal effet le relâchement de la tunique de muscle lisse des vaisseaux sanguins, une libération importante comme celle observée en phase aiguë a pour effet une vasodilatation massive associée à une chute de la pression artérielle et une détresse cardiaque (des symptômes observés en phase aiguë de l'infection). Cependant l'oxyde nitrique est aussi connu pour son rôle protecteur de l'endothélium, sa présence en quantité importante pourrait aussi aider à préserver l'intégrité de l'endothélium et expliquerait l'absence d'altération vasculaire dans les infections à filovirus (41).

Cellules endothéliales

Les cellules endothéliales constituent la barrière entre le sang et les tissus, elles assurent l'échange contrôlé des molécules biologiques via un ensemble de jonctions pour l'eau et les sels (voie paracellulaire) et via une voie trans-cellulaire pour les macromolécules. Il existe des données contradictoires concernant le rôle des cellules

endothéliales dans les infections à filovirus. En effet celles-ci peuvent répliquer le virus et s'activer, cependant leur infection *in vivo* n'a été montrée chez le singe qu'à des stades très tardifs de l'infection. De plus, il n'a pas été noté post mortem chez le singe et l'homme de dommages vasculaires notables. L'activation de cette barrière naturelle par le TNF α produit par les cellules infectées devrait conduire à un remodelage de leur architecture, ceci entraînant une fuite de liquide vers l'interstitium. Étonnamment la sGP une protéine spécifique au virus Ebola, a montré un rôle protecteur contre les effets du TNF α sur ces cellules. La glycoprotéine structurale a quant à elle montré des propriétés inverses (46). De plus l'activation des cellules endothéliales induit l'expression à leurs surfaces du facteur tissulaire qui comme nous l'avons décrit précédemment, est initiateur de la coagulation. Ainsi ces cellules pourraient participer à la mise en place de la CIVD sans toutefois subir de modification majeure de leur architecture. Cependant la plupart des données disponibles sur ces cellules relèvent de modèle *in vitro* et des études complémentaires *in vivo* seraient nécessaires afin de mieux identifier leur rôle dans les fièvres hémorragiques à filovirus.

Charges virales

Lors de la phase aiguë de l'infection à virus Ebola on retrouve chez les patients des charges virales sanguines atteignant $10^{6.5}$ pfu/ml. Ces particules arborent à leur surface la glycoprotéine d'enveloppe (GP). Cette protéine fortement glycosylée a montré des pouvoirs activateurs sur les cellules dendritiques et les macrophages grâce à son domaine mucinique (27). Il a de plus été suggéré qu'elle pourrait se lier au récepteur TLR4 et ainsi être l'initiatrice d'une forte réponse inflammatoire (30). Dans le modèle cobaye, la forme soluble (shedGP) et la forme alternative (sGP) de la glycoprotéine virale sont également retrouvées en forte quantité. Ces formes non structurales pourraient également servir d'activateurs du système immunitaire (communication personnelle). En effet, le récepteur TLR4 initialement découvert comme senseur du LPS permet une transduction du signal via Myd88 et Trif qui a pour conséquence la libération de cytokines inflammatoires et d'interférons de type 1. Enfin sa coiffe glycosylée constitue un excellent ligand pour les lectines sériques (communication personnelle). Certaines collectines sont des acteurs majeurs de la voie d'activation du complément et de l'activation de la coagulation via le recrutement des sérines-protéases sériques (MASP).

Conclusion

Comme nous l'avons montré, les causes du dérèglement de l'hémostase observé dans les infections à filovirus sont multiples et interconnectées. Bien que les symptômes hémorragiques n'entraînent en général pas de perte volumique suffisante pour être la cause du décès, ils sont cependant la signature caractéristique de ce type d'infection et le dérèglement systématique de l'hémostase en phase aiguë de l'infection est un élément clef de l'instauration du choc septique observé pendant les FHV (Figure 4).

La récente étude de McElroy et collaborateurs a mis en évidence pour l'épidémie à *Sudan ebolavirus* que la présence de symptômes hémorragiques était liée statistiquement à une hausse de la ferritine, de la thrombomoduline et du facteur soluble d'adhésion intracellulaire (sICAM) mais qu'il n'existait pas de corrélation avec le taux de TF (28). Les différences observées entre les données *in vitro*, *in vivo* et épidémiologiques montrent à quel point il est important de clarifier les mécanismes exacts de la pathogénèse des filovirus.

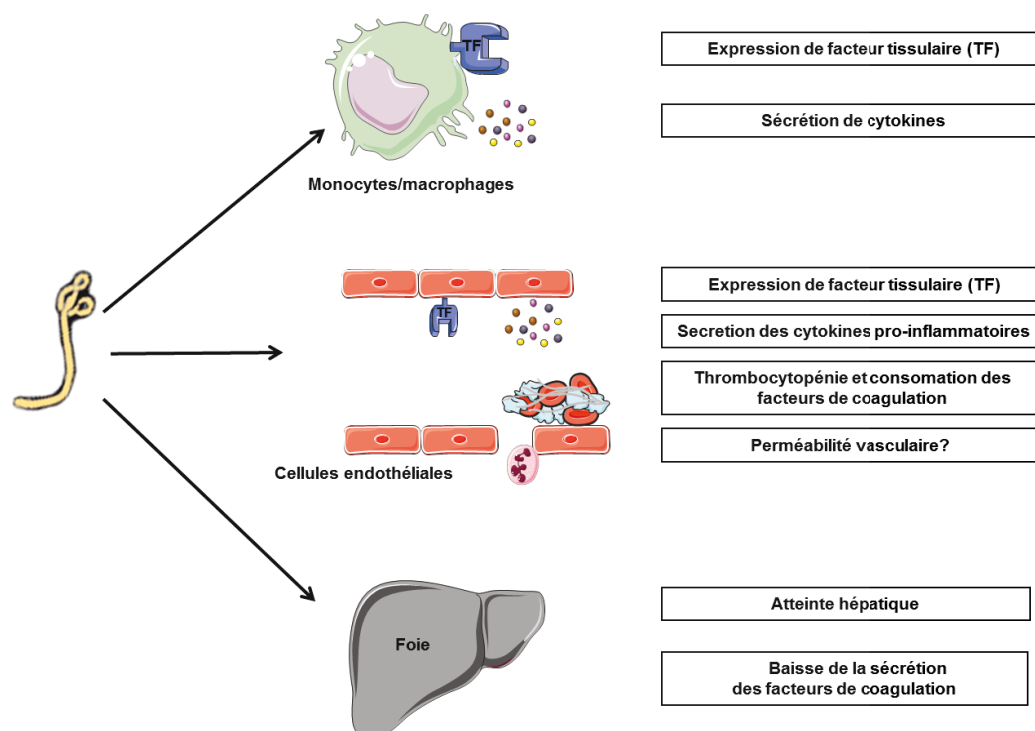


Figure 4 : Origine multifactoriels du dérèglement de l'hémostase dans l'infection à filovirus

L'infection virale conduit au dérèglement de nombreux mécanismes qui par synergie résulte dans l'instauration d'une CIVD et d'un état de choc.

Il semble que le traitement des infections à filovirus doive se baser sur trois approches parallèles, tout d'abord un traitement de support visant à rétablir les constantes vitales (hydratation, pression artérielle, NFS), un traitement antiviral (ARNi, inhibiteurs du cycle viral, anticorps monoclonaux) et enfin un traitement visant à réguler les mécanismes physiologiques (rNapC2, Xigris, heparine, ...). Cette dernière approche a montré des résultats encourageants dans des modèles primates (diminution de 30% de la mortalité avec le rNapC2). De plus, de nombreuses équipes médicales mènent des recherches dans les traitements des cas des fièvres hémorragiques dues à la Dengue, traitements de support qui pourront potentiellement être transposables aux infections à filovirus(8, 37).

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