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## ■■■ REPORT ■■■

## ➡■ Virus Like Particle: VLP/ SC2, and Gain Of Function. ■

## 1) Virus Like Particles (VLPs) represent an emerging technology with numerous biomedical applications, such as drug delivery, or vaccine development but also, due to the double

2) gain-of-function principle (GOF), it is possible to develop, in addition to a vaccine, also a virus.

➡■The VLP particles constitute bio-synthetic nanostructures that emulate the conformation of viruses but, normally, are totally free of infectivity, unless a

3) dedicated project is developed, almost always of a military nature, in which the VLP plates are "loaded" with pathogens.

➡■ This preface is intended to explain the BIO-SYNTHETIC / ZOONOTIC “nature” of SARS CoV 2 (SC2), a chimeric RNA virus (bio-weapon class) developed in

4) the BSL3 / 4 Laboratory.

➡■ SC 2 has an “armored” membrane (M) with spyke glycoprotein (S) protuberances, whose inner core (N) hosts a highly pathogenic viral RNA.

■But let's proceed in order ...

➡■These bio-synthetic VLP particles rely on the ability of both

5) Spike proteins (loaded with pathogens) and viral capsid proteins to penetrate cells and release their contents.

➡■The use of a container / membrane (M), achievable thanks to the use of bio-synthetic VLP particles, has allowed the construction and encapsulation of

6) the genomic sequence of both the wild SC2 virus and its recombinant vaccine.

➡■ But we begin to analyze a recombinant vaccine, made with VLP particles for SC2, and we would get its complete composition, as an answer.

7) A recombinant VLP SC2 vaccine is suitable for people with weakened immune systems, especially frail and elderly people, while its corresponding wild SC2 virus will look for those older people with fragile immune systems (this for laboratory

8) testing on one. .. "population of humanized, elderly mice" ... / R. Baric).

➡■VLPs contain large amounts of viral surface proteins that can elicit strong immune responses from B and T cells.

Since RNA viruses are prone to mutation, rendering all vaccines

9) ineffective, VLP vaccines are an option to combat virus mutations.

➡■ Lately, VLP particles have been produced to respond to a wide range of virus families, in particular:

■Parvoviridae (adenovirus-associated),

■Retroviridae (HIV),

■Flaviviridae (hepatitis C) ...

10) these characteristics of "particular" pathogens can be found, all together, within the SC 2 genome.

➡■This is a first clue that highlights how the "construction" of SC2 is not of a natural type,

11) The creation of the VLP vaccine can use different types of "scaffolding", let's start analyzing the one made with mammalian cells ...

12) Construction of SARS-CoV-2 virus-like particles by Mammalian Expression System

https://t.co/ohTGveW45A

➡■ In this study, the mammalian expression system is used, which was found to be useful in maintaining correct protein glycosylation patterns.

13) VLP SC2 exhibits a crown-like structure in Vero E6 cells that is more stable and unified than those in HEK-293T cells.

➡■ SARS-CoV-2 virions consist of four structural proteins, namely Spike glycoprotein (S), small envelope protein (E), membrane (M) and nucleocapsid (N)

14) The sequences were optimized for human codons of genes encoding structural proteins S, M, E and N, of SC2, then cloned into the double restriction sites Nhe I and NotI of the pcDNA3 expression vector.

➡■The HEK-293T human embryonic kidney cell line and the

15) African green monkey kidney cell line Vero E6, Cell culture medium contained 10% fetal bovine serum, penicillin and streptomycin.

➡■ To gain a better understanding of the secretory characteristics of four SC2 structural proteins, HEK-293T and Vero E6 cells were

16) transfected with vectors expressing S, M, E or N,
respectively.

➡■ Protein M was able to easily release supernatant independent of other structural proteins into the medium, indicating that M was an essential driver of VLP formation.

17) Furthermore, S and N were less detectable in the culture supernatant, in the absence of other structural constituents during detection with the Flag antibody.

➡■To learn more about how S, M, E and N can be mutually regulated in terms of protein production, we

18) then co-transfected HEK-293T and Vero E6 cells with 2 out of 4 adenovectors.

➡■Interestingly, the presence of M resulted in cleavage of the membrane fusion subunit of the spike protein (Sc).

Collectively, this data establishes for the first time that M is the

19) most critical protein driving SC2 VLP output.

➡■ Although the output power of the protein is stronger in HEK-293T cells than in Vero E6 systems, the morphology of VLP SARS-CoV-2 derived from Vero E6 cells was more stable and unified in relative terms than that of HEK-293T.

20) SC2 VLP from Vero E6 cells, the particles show a typical crown-like structure which is the signature of the optimal trimer spike formation on the SC2 envelope.

➡■The VLPs formed by HEK-293T were more variable in both shape and size. In particular, the average

21) diameter of the SC2 VLPs of HEK-293T cells drops to approximately 90 nm, while those assembled in Vero E6 cells were smaller, showing approximately 71 nm.

➡■Hence, Vero E6 cells are more efficient at optimally forming trimeric tip.

22) https://t.co/DNaPm0WUWu

https://t.co/IdDpVwkw5F

➡■ Considering that viral infectivity strongly depends on S / Spike residing in the external appendages of the trimers, emerging at the M membrane of SC2, it is therefore important to underline how S / Spike hosts a

23) sequencing motif (PRRARS), identified between S1and S2,not present in any other coronavirus (which also highlights the "SIGNATURE of the manufacturer").

➡■This sequencing motif was found to be very similar to the Staphylococcal Enterotoxin of Bacterial Superantigen B(SEB).

24) The co-existence of TGEV (gastroenteritis), the E. Coli bacterium, and HIV was also found in S.

https://t.co/Y7K4MlkJl7

➡■To understand the formation of the VLP particles of an SC2 vaccine, made with mammalian cells, it is necessary to explain its compositional elements:

25) ■HEK 293 cells

Human embryonic kidney 293 cells, often also referred to as HEK 293, HEK-293, 293 cells or less precisely as HEK cells, are a specific cell line originally derived from human embryonic kidney cells grown in tissue cultures taken from a female fetus.

26) HEK 293 cells have been used extensively in cell biology research for many years, due to their reliable growth and propensity for transfection. They are also used by the biotech industry to produce therapeutic proteins and viruses for gene therapy.

27) HEK 293 cells were generated in 1973 by transfecting cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA from a healthy female fetus, these cells were then cultured, by transduction, from adenovirus.

28) They are called HEK since they originated in human embryonic kidney cultures.

Having probably adapted to tissue culture, the cells of this clone developed in the relatively stable HEK 293 line.

Subsequent analyzes showed that the transformation was

29) incorporated into the human chromosome.

However, the original transformation of the adenovirus was inefficient, suggesting that the cell that eventually produced the HEK 293 line may have been unusual in some way.

HEK 293 cells, generated by adenovirus transformation of

30) human embryonic kidney cells, have many properties of immature neurons, suggesting that adenovirus preferentially transformed a cell of the neuronal lineage in the original kidney culture.

A comprehensive study of the genomes and transcriptomes of

31) HEK 293 and five derived cell lines compared the transcriptome of HEK 293 with that of human renal, adrenal, pituitary and central nervous tissue and found that the HEK 293 model resembled more from close to that of the adrenal cells, which however have many

32) neuronal properties.

Given the location of the adrenal gland (adrenal means "close to the kidney"), some adrenal cells could conceivably have appeared in an embryonic kidney-derived culture and could be preferentially transformed by adenovirus.

33) Adenoviruses transform neuronal lineage cells much more efficiently than typical human kidney epithelial cells.

The HEK 293 pattern most closely resembled that of adrenal cells, which have many neuronal properties.

34) HEK 293 cells have a complex karyotype, showing two or more copies of each chromosome and with a modal chromosome number of 64. They are described as hypotriploid, containing less than three times the chromosome number of a haploid human gamete.

35) (Chromosomal abnormalities include a total of three copies of the X chromosomes and four copies of chromosome 17 and chromosome 22.)

The presence of multiple X chromosomes and the lack of traces of sequences derived from the Y chromosome suggest that

36) the fetus of origin was a female.

This is one of the reasons why the AZ vaccine particularly affects women more than men, favoring the formation of neural micro thrombi, due to the adenoviral vector used.

HEK 293 cells are used in the production of the

37)■ Oxford-AstraZeneca C19 vaccine (AKA AZD1222).

HEK 293 cells were adapted to grow in suspension culture, which allowed for the growth of large amounts of recombinant adenovirus vectors.

A more specific use of HEK 293 cells is in the propagation of adenoviral vectors.

38) Viruses offer an efficient means of carrying genes into cells, which they evolved to do, and are therefore of great use as experimental tools. However, as pathogens, they also present a risk to the investigator.

This danger can be avoided by using viruses that lack key genes

39) and are therefore unable to replicate after entering a cell.

To propagate such viral vectors, a cell line is required that expresses the missing genes. Since HEK 293 cells express a number of adenoviral genes, they can be used to propagate adenoviral

40) vectors in which these genes (typically E1 and E3) are eliminated.

An important variant of this cell line is the 293T cell line.

Contains SV40 large T antigen which enables episomal replication of transfected plasmids containing the SV40 origin of replication.

41) This allows for amplification of transfected plasmids and extended temporal expression of desired gene products.

HEK 293 cells, and in particular HEK 293T, are commonly used for the production of various retroviral vectors, such as HIV.

42) Various retroviral packaging cell lines also rely on these cells.

Depending on various conditions, the gene expression of HEK 293 cells can vary.

The following proteins of interest (among many others) are commonly found in untreated HEK 293 cells:

43) corticotropin type 1, EDG1, EDG3 and EDG5, acetylcholine M3, TRPC1, TRPC3, TRPC4, TRPC6.

Despite uncertainty about the origin of the fetus used to obtain the cell line, circumstantial evidence strongly suggests that it came from an elective abortion.

44) this presents ethical difficulties for the use of HEK 293 and derivative products, such as vaccines.

Currently, vaccines are being developed using all types of technology platforms available, including:

- nucleic acid (DNA and RNA),

- virus-like particle (VLP),

-peptide

45) - viral vector (replicating and non-replicating),

-recombinant protein,

-live attenuated virus,

- approaches with inactivated viruses.

Much of the research and development on C19 / SARS-CoV-2 involves the introduction of DNA vectors into mammalian

cells, particularly

46) HEK293 cells, on a large scale.

-For example, DNA transfection of the adenovirus vector containing the SARS-CoV-2 S protein gene into HEK293 cells to produce C19 vaccines;

-transfection of the SARS-CoV-2 structure protein genes into HEK293 cells to produce vaccines

47) with virus-like particles (VLP);

-transfection of the SARS-CoV-2 S protein gene into HEK293 cells to produce the S protein trimer vaccine.

- The average VLP particle size is approximately 60 nm, similar in size to wild type TGEV.

48) Max Planck Institute, has created a dedicated spin-off laboratory, called ContiVir, for the production of VLP particles and the purification of inactivated SARS-CoV-2 particles:

50) https://t.co/wt9vzR0QBg

https://t.co/jNXn5rX8J6

➡■ HEK293 cells presented problems, as if the high reproducibility of the results is an advantage of HEK293 cells, however if they are cultured for an extended period of time their health deteriorates.

51) One of the major disadvantages of using any human cell line is that there is a risk of contamination from human specific viruses.

Of course, the key to any successful cell culture is excellent aseptic technique to avoid infection.

52) However, a mycoplasma infection cannot be avoided. Mycoplasma can wreak havoc on your culture by affecting cell health, gene expression, and downstream experimental results.

https://t.co/S3R1HDm8K4

53) The use of human embryonic kidney (HEK) cell line 293T to produce vectors for in vivo applications raises safety concerns due to the presence of coding sequences for the SV40 T antigen.

This explains the cause of laboratory accidents, such as that

54) sadly happened at Whuan's BSL4, in which the use of Adenoviral vectors in laboratory animals in vivo, determines their high volatility and therefore evasion also in Lab BSL4 with negative pressure rooms.

Normally, but not always, CRISPR-Cas9 genome editing is

55) used to remove sequences encoding the SV40 T antigen from HEK293T cells by transfecting them with a recombinant Cas9-expressing plasmid and two distinct single guide RNAs (sgRNAs) corresponding to the beginning and at the end of the coding region of the T antigen.

56) Cell clones lacking sequences encoding the T antigen were identified by PCR.

Genome-wide sequencing of the parents' HEK293T cell line revealed multiple SV40 T antigen encoding sequences that replaced cell sequences on chromosome 3.

57) Western blot analysis of cell extracts prepared from null clones of the T antigen confirmed that the SV40 large and small T antigen proteins were absent.

Lentiviral vectors produced using the null clones of the T antigen showed very low titers of

58) transduction units, while the titers obtained from the parent HEK293T cell line were much higher.

HEK293T cells are easily transfected and the transfection rate can be around 80%.

59) We now move on to analyze VLP SC2 cells expressed in an insect cell culture.

Currently the VLP particles are composed of S, M, E or S, M, N, E, in mammalian cells, but there are also variants of VLP paerticelle created with the insect cell expression system (baculovirus).

60) In fact, the creation of the VLP vaccine can use bacterial scaffolding, yeast, mammalian cells, or ... insect ...

➡■ Since yeast is not perfect for the expression of animal proteins, the compromise is to use insect cells.

(The first VLP vaccine that

61) addresses malaria, Mosquirix, (RTS, S) was expressed in yeast. RTS, S is a portion of the Plasmodium falciparum circumsporozoite protein fused with the hepatitis B surface antigen (RTS), combined with hepatitis B surface antigen (S) and adjuvanted with AS01,

62) consisting of (MPL) and saponin.)

➡■ Let's try to analyze the components of the malarial VLP and we will notice the presence of many pathogens, common in SC2:

Plasmodium falciparum has the ability to evade the immune system while in the bloodstream,

63) evading immune cells, producing over 2,000 cell membrane antigens, and penetrating hepatocytes.

-HBsAg is the surface antigen of the hepatitis B virus (HBV) (diameter 42 nm.) These antigenic proteins can be genetically produced (eg Transgene E. coli) to

64) produce material for a simple antigen test , which detects the presence of HBV.

-Recently, the HBV (Hepatitis B) genome has been cloned into E.coli. The determination of its primary structure allowed the localization of the gene (called the S gene) coding for HBsAg and the

65) synthesis of the core antigen in E. coli was reported. They constructed a derivative of the lambda bacteriophage that carries a fusion between the beta-galactosidase (lacZ) gene and the HBsAg (lambdalacHBs-1) coding sequence. Infection of E. coli with lambdalacHBs-1 leads to

66) the biosynthesis of a polypeptide carrying antigenic determinants of the HBV surface antigen.

➡■ In summary, Baculovirus expressing plasmodium falciparum virus in mosquitoes, Hepatitis B virus core antigen gene (HBcAg) has been deleted at some unique restriction enzyme

67) sites and inserted into E. coli expression plasmids which had the tryptophan promoter.

-MPL myeloproliferative leukemia virus, which is a thrombopoietin antagonist is the main regulator of megakaryocytopoiesis and platelet formation.

https://t.co/4c7pDP2wNF

68) ➡■ SARS-CoV-2 VLP-A / SARS-CoV-2 VLP-B, are 2 types VLP, expressed for SC2, developed in insect cell cultures.

➡■SARS-CoV-2 virus-like particles (VLPs) were expressed in Sf21 cells, assembled with SARS-CoV-2 spike (S, Flag-labeled), envelope (E) and membrane (M) proteins.

69) But what exactly is the Sf21 Production Host?

■Basically Sf21 = insect cells.

70) https://t.co/3SFxgYbo85

71) To confirm that protein S was incorporated within the VLP particles, once purified, they were analyzed by Western blot using the SARS-CoV-2 S protein polyclonal antibody which was shown to have been incorporated.

The morphology of the SARS-CoV-2 VLPs was studied by

73) and are structurally similar to native virions.

74) The insect cell system has been shown to be a powerful system for the quick and easy production of VLP,

➡■The main limitation of the insect cell system is the possible contamination of the enveloped baculovirus particles.

To solve this problem, they

75) developed a non-replicative baculovirus that minimizes pollution ... this for the development of a VLP SC2 vaccine, but what would happen if a highly infectious virus (such as SC2 wild)?

➡■Simple, this technique of culturing insect cells with a wild

76) baculus would be adopted, in order to favor contamination in the in vitro culture.

(The Trichoplusia-derived insect cell line was used for the production of the Papillomavirus HPV L1-VLP vaccine.)

77)
➡■ Conclusion:

The use of VLP particles developed in mosquito cell cultures, in vitro, has allowed the construction, in the laboratory, of the wild virus SC2 which has, in its N nucleus, highly pathogenic agents such as the BaT CoV virus, and Plasmodium Yoelii /

78) Plasmodium Falciparum (Malaria); this last condition explains the strongly antiviral effect of INVERMECTIN, known to be an anti-parasitic and anti-bacterial with other benefits.

➡■Then the parasite, which generated the baculovirus in the mosquito, allowing the spread of

79) the malarial agent, is completely neutralized by the invermectin drug.

➡■Here it is demonstrated how the excessive use of the gain-of-function (GOF) has allowed an experiment, not controlled, with dangerous pathogenic viruses, developed in the laboratory, generating the

80) double virus/vaccine link, which then strongly characterized the construction of the chimeric virus VLP / SC2, developed with ■Sf21 cells, and its subsequent laboratory escape, due to the unregulated use of ■Adenoviral

81) vectors, on animals in vivo, which cannot be controlled even in a ■BSL4.

END