



# SARS-CoV-2 will constantly sweep its tracks: a vaccine containing CpG motifs in 'lasso' for the multi-faced virus

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Received: 13 May 2020 / Revised: 28 June 2020 / Accepted: 6 July 2020  
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## Abstract

During the current COVID-19 pandemic, the global ratio between the dead and the survivors is approximately 1 to 10, which has put humanity on high alert and provided strong motivation for the intensive search for vaccines and drugs. It is already clear that if we follow the most likely scenario, which is similar to that used to create seasonal influenza vaccines, then we will need to develop improved vaccine formulas every year to control the spread of the new, highly mutable coronavirus SARS-CoV-2. In this article, using well-known RNA viruses (HIV, influenza viruses, HCV) as examples, we consider the main successes and failures in creating primarily highly effective vaccines. The experience accumulated dealing with the biology of zoonotic RNA viruses suggests that the fight against COVID-19 will be difficult and lengthy. The most effective vaccines against SARS-CoV-2 will be those able to form highly effective memory cells for both humoral (memory B cells) and cellular (cross-reactive antiviral memory T cells) immunity. Unfortunately, RNA viruses constantly sweep their tracks and perhaps one of the most promising solutions in the fight against the COVID-19 pandemic is the creation of 'universal' vaccines based on conservative SARS-CoV-2 genome sequences (antigen-presenting) and unmethylated CpG dinucleotides (adjuvant) in the composition of the phosphorothioate backbone of single-stranded DNA oligonucleotides (ODN), which can be effective for long periods of use. Here, we propose a SARS-CoV-2 vaccine based on a lasso-like phosphorothioate oligonucleotide construction containing CpG motifs and the antigen-presenting unique ACG-containing genome sequence of SARS-CoV-2. We found that CpG dinucleotides are the least rare dinucleotides in the genomes of SARS-CoV-2 and other known human coronaviruses, and hypothesized that their higher frequency could be responsible for the unwanted increased lethality to the host, causing a 'cytokine storm' in people who overexpress cytokines through the activation of specific Toll-like receptors in a manner similar to TLR9-CpG ODN interactions. Interestingly, the virus strains sequenced in China (Wuhan) in February 2020 contained on average one CpG dinucleotide more in their genome than the later strains from the USA (New York) sequenced in May 2020. Obviously, during the first steps of the microevolution of SARS-CoV-2 in the human population, natural selection tends to select viral genomes containing fewer CpG motifs that do not trigger a strong innate immune response, so the infected person has moderate symptoms and spreads SARS-CoV-2 more readily. However, in our opinion, unmethylated CpG dinucleotides are also capable of preparing the host immune system for the coronavirus infection and should be present in SARS-CoV-2 vaccines as strong adjuvants.

**Keywords** SARS-CoV-2 · COVID-19 pandemic · Phosphorothioate oligonucleotides · CpG motif · Vaccine

Vaccines and drugs help mankind reduce the effects of natural selection when meeting and dealing with pathogens. On the one hand, this slows down the process of adaptation of

the human population to negative environmental factors; on the other hand, we save that which is most valuable—the life of each individual person. Nevertheless, it must be recognized that drugs and vaccines only delay the fatal meeting of the causative agent of the disease with those who are pathogen-sensitive or their genetic descendants. Natural selection is merciless—only the fittest survives. Since no one knows for sure which pathogen he or she will be sensitive to, everyone expects that at the nearest pharmacy a person will find, at the right time, a medicine that will save

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Responsible Editor: John Di Battista.

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his or her life. Obviously, this is not what happens. Today, during the sudden COVID-19 pandemic, we understand how fragile our habitual way of life has turned out to be. In fact, we are at the mercy of pure natural selection, and we are not able to oppose it with any effective vaccine or drug. Nevertheless, a sufficiently large number of research groups have already joined the race to create vaccines and drugs against the SARS-CoV-2 virus, which in the next few years will significantly slow down the effect of natural selection. In turn, weakened by vaccines and drugs, natural selection will continue to work and COVID-19 will morph into a disease with mild symptoms in the years to come. However, at this moment in time, during the exponential growth of the pandemic, the virus is aggressive and needs to be restrained as soon as possible.

SARS-CoV-2 is positive-sense, single-stranded RNA betacoronavirus from the Coronaviridae family. Genomic alignments suggest that the SARS-CoV-2 from the genus *Betacoronavirus* may be the result of a recombination of genetic material from two different viruses, one similar to the Chinese horseshoe bat virus and the other closer to the pangolin virus [1]. In fact, only two scenarios will occur simultaneously in the very near future: people who are genetically resistant to the virus will get sick, recover, and develop immunity, while people who are sensitive to the virus will need drugs and vaccines, which will have to be researched and developed if they are to recover. Since SARS-CoV-2 mutates constantly and more frequently than other RNA viruses (coronaviruses possess the longest genomes of all known RNA viruses, so more errors are made when they are copied; also, viral RNA-dependent RNA polymerases do not have a proofreading nuclease activity), it is likely that many new strains of the virus will appear and this will be a problem for creating vaccines. Still, vaccines are arguably the most powerful medical intervention in the fight against infectious diseases [2]. Generally, all vaccines work on the same basic principle. They present part or all of the pathogen to the human immune system, usually in the form of injections and in low doses, to induce the system to produce antibodies to the pathogen and activate induction of cross-reactive antiviral T cells, particularly the cytotoxic T lymphocytes that can kill virus-infected cells. Specific T lymphocytes and antibody-producing plasma cells (B cells) represent a kind of immune memory, which, once a virus is detected, can be quickly mobilized again if a person is exposed to the virus in its natural form. Traditionally, adjuvants (aluminum salts, squalene-in-water emulsions, imidazoquinoline derivatives, saponins, synthetic phosphorothioate-linked DNA oligonucleotides with optimized CpG motifs, etc.) have been used in vaccines to increase the magnitude of an adaptive response to a vaccine, based on antibody titer or ability to prevent infection. However, a second role for adjuvants has become increasingly important:

guiding the type of adaptive response to produce the most effective forms of immunity for each specific pathogen [3, 4]. Previous studies on several animal coronaviruses also suggest that not only humoral but also cellular immunity plays a role in protecting against virus infection [5, 6]. Classic methods involve taking a whole virus, weakening or killing it, then injecting its remains into a person's body.

For the human population, COVID-19 is the third significant coronavirus infection to occur in the twenty-first century, following severe acute respiratory syndrome (SARS) in 2002–2003 and Middle East respiratory syndrome (MERS) in 2012, which have not stopped yet. Since 2012 until 29 February 2020, the total number of laboratory-confirmed MERS-CoV infection cases reported globally to WHO is 2538 with 871 associated deaths. But while those outbreaks alerted the world to the virulent potential of the coronavirus family, SARS fizzled out faster than a vaccine could make its way through clinical trials, and MERS was not viewed seriously enough to generate sustained funding for vaccine developers. This despite the fact that the 2002 SARS and 2012 MERS outbreaks, both caused by close relatives of the new coronavirus, were warning shots that claimed approximately 774 and 871 lives, respectively.

One major hitch in developing a COVID-19 vaccine is that no medically proven predecessor exists for any type of human coronavirus. Only a small number of SARS-CoV vaccines made it to Phase I clinical trials before funding dried up because the virus was eradicated from the human population through non-pharmaceutical interventions when the case numbers were still small. The first vaccine tested in a clinical trial is made from the inactivated form of SARS-CoV. Several live attenuated, genetically engineered, or vector vaccines encoding the SARS-CoV spike (S) protein have been tested in pre-clinical studies. These vaccine candidates are effective in terms of eliciting protective immunity in the vaccinated animals. However, the use of whole virus or full-length S protein-based immunogens in humans should be approached with caution, because their use could induce harmful immune or inflammatory responses. Some authors have proposed the use of the receptor-binding domain (RBD) of SARS-CoV S protein (residues 318–510) in the development of a safe and effective subunit SARS vaccine. This functional domain, responsible for mediating virus-receptor binding, is also a major neutralization determinant of SARS-CoV [7]. These trials, performed using an inactivated virus vaccine and a spike-based DNA vaccine, have produced encouraging results; in addition to being safe, the vaccines induced neutralizing antibody titers [8, 9]. The structure of the S protein of SARS-CoV-2 was solved in record time at high resolution, contributing to our understanding of this vaccine target [10, 11]. It has been shown that some of the neutralizing monoclonal antibodies isolated against SARS-CoV, such as CR3022 [12, 13],

can cross-react with the receptor-binding domain of SARS-CoV-2. This suggests that SARS-CoV vaccines might cross-protect against SARS-CoV-2. However, because these vaccines have not been developed further than Phase I, they are currently not available for use. MERS-CoV vaccines are unlikely to induce strong cross-neutralizing antibodies to SARS-CoV-2 because of the phylogenetic distance between the two viruses. Nevertheless, we can still learn a lot from these vaccines about how to move forward with SARS-CoV-2 vaccine design [14].

Vaccines are usually developed on the basis of certain virus strains, as follows. The most well-known example is influenza vaccines. Influenza viruses belong to the Orthomyxoviridae family and are characterized by segmented, negative-strand RNA genomes. Infection with influenza viruses is a constant challenge, burdening people's health and economies worldwide every year. These infections cause epidemics that can become pandemics, affecting 5–30% of the global population annually, and are responsible for millions of hospitalizations and hundreds of thousands of deaths each year [15]. Natural reservoirs exist for influenza, infecting a wide range of animal hosts. These include wild aquatic birds, the natural reservoir for the influenza A virus, and pigs. Since pigs can become infected with influenza B virus, they may provide a natural reservoir for the virus [16]. While influenza, like COVID-19, causes morbidity among all ages, those most vulnerable to its serious complications, such as pneumonia, are the elderly [17, 18]. At the present time, the two available types of influenza vaccines are administered by different routes: parenteral inactivated influenza vaccines (IIV), delivered by injection, and intranasal live attenuated influenza vaccines (LAIV). LAIV mimic the course of natural infections and thus induce a stronger immune response than IIV [19]. Intranasal administration of LAIV produces a variety of adaptive immune responses, including serum antibodies and mucosal and cell-mediated immunity [20]. Based on data gathered by the Global Influenza Surveillance and Response System (GISRS), a partnership of 141 national influenza centers in 111 countries, six WHO collaborating centers, and four WHO essential regulatory laboratories, WHO updates the composition of influenza vaccines (both IIV and LAIV) annually. The WHO GISRS continually collects and analyses global influenza virus samples. By monitoring circulating influenza A and B viruses for how recently they have occurred and with what frequency, changes can be made to the components in the vaccines slated for the upcoming influenza season in both the northern and southern hemispheres [21]. Current influenza vaccination strategy induces cells to produce antibodies directed against the surface antigens of these viruses, with the main targets being the surface glycoprotein antigens hemagglutinin (HA) and neuraminidase (NA). The amino acid sequences encoding HA and NA are hypervariable, as a consequence of antigenic

drift or shift, respectively. This characteristic makes them responsible in large part for both epidemic and pandemic influenza outbreaks [15]. In addition, these frequent changes to the surface antigens of influenza viruses allow them to avoid antibody-mediated immunity. On the other hand, it has been shown that cytotoxic T-lymphocyte populations, when directed against the internal antigens of influenza A virus, demonstrate a broad cross-reactivity with the influenza virus subtypes [22]. Both IIV and LAIV vaccines have a history of safe use in children and adults. However, both can cause adverse events and/or rare adverse events, some more prevalent in children, others more likely to occur in adults [23]. Each year, necessary changes are made to the formulation of influenza vaccines and when they are administered. Factors that affect those being vaccinated, both genetic and environmental, are difficult, if not impossible, to control. For these reasons, even though vaccines undergo strict monitoring before authorization, the possible occurrence of adverse effects, such as narcolepsy [24], cannot be completely eliminated. Consequently, annual post-licensure vaccine safety surveillance is fundamental [25]. For all of these reasons, no universal influenza vaccine is yet available against all influenza virus subtypes and seasonal influenza vaccines require annual updates. The lack of preventive vaccines for clinical use in humans against such viruses makes emerging influenza and coronaviruses a serious global threat [26]. Currently, available vaccines keep deaths from influenza relatively under control. However, when any influenza virus lineage emerges in a novel mammalian host population, the threat of a direct or indirect pandemic is a real risk [27].

The creation of vaccines for seasonal influenza viruses is justified because they work, but for some other RNA viruses, this is not so. The human immunodeficiency virus, types 1 and 2 (HIV-1 and HIV-2), is an example of a zoonotic infection that has spread all over the world without being stopped. At the moment, approximately 38 million people have been infected with HIV, but there is not a single successful vaccine able to effectively contain the HIV pandemic [28]. Both types of HIV are the result of multiple cross-species transmissions that occurred when simian immunodeficiency viruses (SIV) naturally infected African primates [29]. The human immunodeficiency virus (HIV), which consists of two identical negative-sense single-stranded RNA molecules, belongs to the genus *Lentivirus* in the *Retroviridae* family, subfamily *Orthoretrovirinae* [28]. Attempts to develop a safe and effective HIV vaccine have been hindered by the difficulty in clearly defining the specific immune responses responsible for preventing infection and limiting disease progression. Since early attempts to use vaccines to induce neutralizing antibodies ended in disappointment, recent research efforts have focused on vaccines that induce T-cell immunity. The belief that the specific antiviral CD8 T lymphocyte response is crucial for immune

control of viral replication has provided an important facet for ongoing efforts to develop effective vaccines against HIV. Unfortunately, while the actions of T cells are known to limit HIV and other viral infections after the infection has already occurred, their role, if any, in protecting against initial infection is much less clear [2]. HIV-1 is highly mutable. This, combined with its complex structure and life cycle, has placed further obstacles in the path to developing an effective vaccine. The virus is a master of obfuscation. For instance, it uses variable glycosylated residues to camouflage the conserved receptor- and coreceptor-binding sites on the viral envelope (Env) glycoproteins engaged in the virus's attachment and fusion to host membrane receptors [30]. In addition, the conserved sites are thermostably concealed [31]. These are just two of the features that allow the virus to nimbly evade detection by cross-reactive antibody (Ab)-producing B cells that recognize conserved sites. As a part of its life cycle, HIV-1 is able to integrate into its host's genome, forming a latent provirus that escapes immune recognition owing to the absence of significant protein expression [32]. Neutralizing antibodies (NAb) typically play a key role in controlling viral infections and contribute to the protective effect of many successful vaccines. Compelling data gathered using experimental animal models have demonstrated that NAb can prevent HIV-1 acquisition; however, no similar data exists for humans and the role of NAb in controlling established infection in humans is also limited. It is clear that HIV-specific NAb drives the evolution of the HIV-1 envelope glycoprotein within an infected individual. The ability of the virus to evade immune selection may be the main reason HIV-1 NAb exert limited control during infection. Attempts to define NAb capable of providing a broad spectrum of protection against diverse circulating HIV-1 strains have encountered the difficulties posed by the extraordinarily antigenic diversity of HIV-1 [33]. Reports that the breadth and potency of humoral responses are higher in subtypes C and A than in subtype B infections provides evidence that viral genetic subtype may also be important [34, 35]. In addition to its talents for concealment, the virus can also make mistakes. The high error rate of the reverse transcriptase [36], combined with the rapid turnover of plasma virions [37], further hinders the development of an effective vaccine by providing a dizzying range of variants from which to select and escape from both cellular and humoral immune responses [38, 39]. At present, at least 12 recognized HIV-1 genetic subtypes are known to exist in humans. Each of these subtypes has rapidly diversified to yield intersubtype recombinants [40, 41] that present an even greater challenge in the development of a universal HIV vaccine.

Another 'multi-headed beast' sweeping its tracks is the hepatitis C virus (HCV). HCV is a positive-sense single-stranded RNA virus of the Flaviviridae family [42].

Although the zoonotic origins of HCV in humans remain unclear, recent and direct cross-species transmission from horses to dogs appears plausible [43]. Highly diverse HCV-related viruses have also been detected in bats and rodents [44], in cattle [45], and in black-and-white colobus monkeys [46]. There is good news in the form of direct-acting antivirals (DAA) against the hepatitis C virus (HCV) infection, which have been shown to achieve a complete cure in > 95% of cases [47, 48]. It has been suggested that DAA have the potential to eliminate an infection that affects more than 71 million individuals worldwide [49]. This is particularly important because many individuals do not seek treatment for constant hepatitis C because they are unaware that they are infected. Sadly, it is often a long time after the initial infection, when hepatitis C has precipitated serious health issues, that these individuals look for help. It is estimated that only 5% of HCV cases worldwide are diagnosed [50, 51]. This is one reason that the development of vaccines that protect against persistent HCV infection remain a public health priority. Without access to vaccines that can limit viral transmission, even the broad use of highly effective DAA is unlikely to achieve HCV elimination. In addition, DAA treatment does not protect against reinfection, further underscoring the need for an effective vaccine [52]. Current preclinical and clinical trials are being carried out for two vaccines that target either the antibody or the T cell response. The next generation of vaccines is likely to involve a combination of these two strategies. The first vaccine contains a recombinant form of the virus envelope glycoproteins gpE1 and gpE2, aimed at inducing neutralizing antibodies and CD4 helper T cells [53, 54]. The second vaccine is vector-based and encodes nonstructural (NS) proteins of the virus (NS3-NS5) using chimpanzee adenovirus priming and a modified vaccinia Ankara (MVA) boost. These represent two of the most promising strategies: production of broadly neutralizing antibodies (bNAb) to neutralize the infectivity of the virus and generation of potent virus-specific CD4 and CD8 T cells to eliminate infected hepatocytes [55]. Multiple studies of HCV in humans and chimpanzees have provided strong evidence for the kinetic association between spontaneous viral clearance and the induction of a broad, sustained group of HCV-specific CD4 and CD8 T cells [56]. As it turns out, these T cells are polyfunctional, producing multiple cytokines and effector functions [57]. For instance, as the infection is being cleared, virus-specific T cells develop a memory T cell phenotype that upregulates cell surface expression of the IL-7 receptor CD127 [58–60]. If the virus persists, CD8 T cells that recognize intact epitopes (i.e., epitopes that have not mutated) become exhausted and express exhaustion markers such as programmed death 1 (PD1), T-cell immunoglobulin and mucin domain-containing-3 (Tim-3), cytotoxic T-lymphocyte protein 4 (CTLA4), 2B4, CD160, KLRG1, T-cell immunoreceptor with Ig



and ITIM domains (TIGIT), and CD39 [56, 61]. There is an increasing amount of evidence that the specificity and potency of the early antibody response can influence the acute infection outcome and that neutralizing antibodies seem to play a part in controlling HCV during chronic infection [62]. In particular, it is worth noting the data from studies showing that bNAb isolated from chronically infected and spontaneously resolved individuals have the capacity to block infectivity in mouse models of HCV infection, further emphasizing the important protective effect of the antibody response [63–65].

Thus, in one way or another, all vaccines aimed at preventing zoonotic RNA viruses are focused on producing specific antibodies and activating cytotoxic T lymphocytes that can cause the death of virus-infected cells. In general, the lack of effective vaccines against some RNA viruses, such as coronaviruses, HIV, and HCV, can be explained by the large number of mutations that occur during replication, as well as weak antibody activity. To some extent, the slow progress in the development of HCV and SARS coronavirus vaccines is also due to the lack of sufficient funding and the advent of highly effective DAA in the case of HCV. Nevertheless, a large number of RNA viruses of zoonotic origin will always carry with them the danger of new pandemics; the creation of vaccines can help humans be better prepared to meet them. Ideal vaccines are those that will be effective for the vast majority of people and against most of the strains of the target circulating RNA virus. One promising strategy is to create vaccines based on conservative sequences of viral genomes using oligonucleotides. Despite the impressive success of currently approved adjuvants for generating immunity to viral and bacterial infections, there remains a need for improved adjuvants that enhance protective antibody responses, especially in populations that respond poorly to current vaccines. Today, the larger challenge is to develop vaccines that generate strong T cell immunity with purified or recombinant vaccine antigens. Oligonucleotides can be used as both antigens and adjuvants.

Several studies have shown that oligonucleotides can improve the efficacy of vaccines, either by inducing antigen modification (i.e., by enhancing expression of immunogenic molecules) or by targeting certain components of the host immune system to achieve the desired immune response. However, despite their extensive use, problems such as insufficient stability and low cellular delivery have not been sufficiently resolved to achieve effective and safe oligonucleotide-based vaccines [66]. For many years, nucleic acids and short nucleotide molecules have been used as vaccine components. Their use has ranged from DNA [67, 68] or RNA vaccines [69, 70], to oligonucleotide sequences containing unmethylated cytidine phosphate guanosine (CpG) motifs with significant immunostimulatory (adjuvant) properties [71, 72]. More recently, strategies that manipulate the

expression of genes controlling the immune response or the expression of antigens of interest are being used to improve immunogenicity and vaccine efficacy. This line of research has a history of more than 30 years; in one of the first experiments, when spleen cells from BALB/c mice were incubated with the nucleic acid fraction from different bacteria, the natural killer (NK) activity of the cells was remarkably elevated and the cells produced factors that activated macrophages and inhibited viral growth. The factor that activated the macrophages was interferon (IFN)-gamma and the other factor, which inhibited viral growth, was IFNalpha/beta. However, the nucleic acid fraction from neither of the vertebrate cells showed any such activity. This report provided evidence for the first time that, while DNA from microorganisms and invertebrates could induce IFN, the DNA from vertebrates, and probably from plants as well, could not [73].

In 1995, Krieg et al. reported that unmethylated CpG dinucleotides (CpG ODN) within bacterial DNA activated host defense mechanisms that led in turn to innate and adaptive immune responses [74]. CpG ODN is a ligand of Toll-like receptor 9 (TLR-9) in antigen-presenting cells. Toll-like receptors (TLR) are a family of non-clonal pattern-recognition receptors that recognize conserved pathogen-associated molecular patterns (PAMP). Under homeostatic conditions, TLR discriminate 'non-self' from 'self' in that they do not sense host-derived 'self-molecules' but become activated by PAMP during infection [75]. Ligand binding by TLR3, TLR7, TLR8, and TLR9 occurs in the acidic environment of the endosome [76] and causes cellular activation, presumably by stabilizing preformed TLR dimers or inducing TLR-dimer formation. CpG ODN/TLR-9 interaction induces an innate immune response that promotes the subsequent development of adaptive immunity [71, 77]. Their utility as vaccine adjuvants has been evaluated in different clinical trials and the results indicate that CpG ODN augments the induction of vaccine-specific cellular and humoral responses [72]. In 2017, the FDA approved HEPLISAV-B, the first vaccine with a CpG ODN as an adjuvant, for hepatitis B vaccines [78]. However, it has also been reported that CpG ODN can induce high levels of pro-inflammatory cytokines, with the potential risk of developing or worsening autoimmune diseases and systemic inflammatory response syndrome (SIRS) [79, 80].

TLR9 agonists have demonstrated a substantial potential as vaccine adjuvants, and as mono- or combination therapies for the treatment of cancer and infectious and allergic diseases. Commonly referred to as CpG oligodeoxynucleotides (ODN), TLR9 agonists directly induce the activation and maturation of plasmacytoid dendritic cells and enhance differentiation of B cells into antibody-secreting plasma cells. Preclinical and early clinical data support the use of TLR9 agonists as vaccine adjuvants, where they can enhance both the humoral and cellular responses to diverse

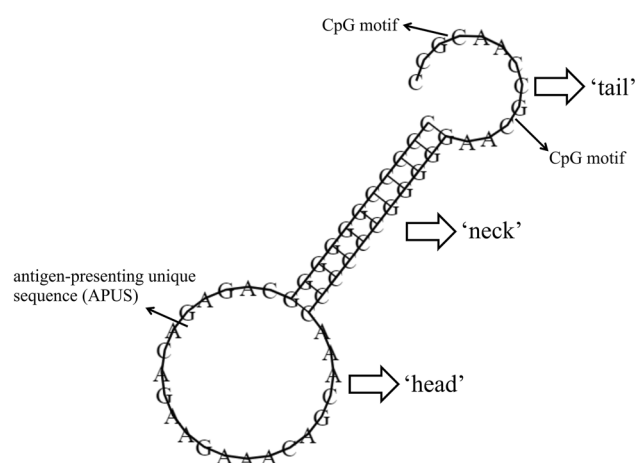
antigens [77]. When used as vaccine adjuvants, CpG ODN improve the function of professional antigen-presenting cells and boost the generation of humoral and cellular vaccine-specific immune responses. These effects are optimized by maintaining the ODN and the vaccine in close proximity. The adjuvant properties of CpG ODN are observed when administered either systemically or mucosally, and persist in immunocompromised hosts. Preclinical studies indicate that CpG ODN improve the activity of vaccines targeting infectious diseases and cancer. Clinical trials demonstrate that CpG ODN have a good safety profile and increase the immunogenicity of co-administered vaccines [71].

The majority of studies carried out previously in SARS-CoV-infected humans and animals pointed to a dysregulated/exuberant innate response as the leading contributor to SARS-CoV-mediated pathology. Almost two decades after the 2002–2003 SARS epidemic, we still do not have any approved preventive or therapeutic agents available with which to respond to the re-emergence of SARS-CoV or other related viruses. A strong neutralizing antibody response generated against the spike (S) glycoprotein of SARS-CoV is completely protected in the susceptible host. However, neutralizing antibody titers and the memory B cell response are short lived in SARS-recovered patients and the antibody will only target the primary homologous strain. Follow-up studies from patients who recovered from SARS suggest that the SARS-CoV-specific antibody response is fleeting. In these patients, the SARS-CoV-specific IgM and IgA response lasted less than 6 months, while the virus-specific IgG titer peaked 4 months post-infection and declined markedly after 1 year. Despite the lack of virus-specific memory B cell response, SARS-CoV-specific memory T cells persist in SARS-recovered patients for up to 6 years post-infection. Consistent with the results from these human studies, results from animal studies also suggest that strong virus-specific T cell responses are required to protect mice from lethal SARS-CoV-MA15 infection. Future vaccine interventions should, therefore, consider strategies that enhance the T cell response to provide a robust long-term memory. Since tissue-resident memory T cells provide better protection, boosting both local and systemic memory T cell responses would be a more useful strategy than either of these interventions alone. Although the amount of data available on virus-specific memory T cell responses to emerging coronaviruses is limited, existing studies indicate that lung resident virus-specific memory CD8 T cells provide substantial protection following SARS-CoV challenge. These studies suggest a potential role for virus-specific memory T cells in broad and long-term protection against SARS-CoV infection. This is important as the response of neutralizing Abs and memory B cells to SARS-CoV declines significantly 1–2 years post-infection; in addition, these responses are strain specific [81]. Taken together, these data clearly

suggest that the CpG ODN/TLR-9 interaction induces a strong innate immune response that promotes the subsequent development of an adaptive immunity triggered by the production of T helper cells one and pro-inflammatory cytokines.

With advancing age, both humans and animals become highly susceptible to SARS-CoV and other respiratory virus infections. Such an age-dependent increase in susceptibility is associated with a significant reduction in the magnitude of the virus-specific T cell response [82–84]. Young C57BL/6 mice (6 weeks of age) generate a SARS-CoV-specific CD8 T cell response that is approximately eightfold greater than that observed in 12-month-old mice [85]. Virus-specific CD8 T cells play a critical role in viral clearance; CD4 T cells provide the necessary support for the antiviral function of the CD8 T cells [86]. Generally, results suggest that the role of virus-specific primary CD4 and CD8 T cell responses to respiratory or other coronavirus infections are both virus and mouse strain dependent [81]; in other words, they depend on the genetic component of the host-virus relationship. Eventually, in the absence of vaccines and drugs, natural selection will rapidly lead to the survival of only genetically resistant individuals in a host population.

At this juncture, we propose a SARS-CoV-2 aerosol-based vaccine based on a lasso-like phosphorothioate oligonucleotide construction containing CpG motifs and antigen-presenting unique sequence (APUS) (Fig. 1). Aerosol immunization seems to be a promising method of vaccination [87, 88], including as it does aerosolized delivery of nucleic acids optimized for lung uptake [89] and nasal spray [90]. Several independent studies have now demonstrated that the dependence on CpG motifs for TLR9 activation is



**Fig. 1** Structure of a SARS-CoV-2 vaccine based on a lasso-like phosphorothioate oligonucleotide construction containing CpG motifs and antigen-presenting unique ACG-containing genome sequence of SARS-CoV-2 (performed by RNAfold WebServer; <https://rna.tbi.univie.ac.at>)

restricted to synthetic phosphorothioate oligodeoxynucleotides (PS-ODN), and that natural phosphodiester oligodeoxynucleotides (PD-ODN) bind and activate TLR9 via the 2' deoxyribose backbone in a sequence-independent manner [91–95]. Indeed, further control can be achieved through recognition of cytosine methylation, which acts as a negative determinant, i.e., its presence in mammalian DNA prevents the triggering of TLR9.

In the proposed construction, the main role of the antigen-presenting part of the vaccine will be played by the phosphorothioate oligonucleotides found on the APUS region (Fig. 1). Phosphorothioate (PS) oligonucleotides, which contain a non-bridging sulfur, are the most widely studied oligonucleotides; compared to unmodified oligonucleotides, they possess higher solubility and nuclease stability, with improved membrane penetration. Nuclease stability is important because human lung fluid possesses DNase activity. This activity can be inhibited by the use of direct nuclease inhibitors, which enhance the uptake of oligonucleotide-based vaccines by cells [96] or through oligonucleotide modification. Therapeutic use of oligonucleotides is highly dependent upon their efficient uptake by cells. The literature contains many reports of experiments both in tissue culture and in vivo using a phosphorothioate backbone to generate antisense effects mediated by an RNase H-dependent mechanism. These data have led to the investigation of phosphorothioate oligonucleotides in therapeutic clinical trials [97]; it has also been demonstrated that CpG motifs within phosphorothioate (PS)-modified DNA effectively drive Toll-like receptor 9 (TLR9) activation [98].

We found that CpG dinucleotides are the least rare dinucleotides in the genomes of SARS-CoV-2 and other known human coronaviruses (Table 1), and hypothesized that their higher frequency could cause the unwanted increased lethal effect on the host, resulting in a 'cytokine storm' in people prone to overexpress cytokines through the activation of specific Toll-like receptor interactions with single-stranded RNA in a manner similar to those of TLR9-CpG ODN interactions. Most RNA viruses that infect mammals and other vertebrates profoundly suppress CpG dinucleotide frequencies [99]. The functional nature of this apparent selection

against CpG dinucleotides operating on both host and viral cytoplasmic RNA sequences remains unexplained.

However, it has been frequently hypothesized that recognition of CpG motifs is part of an as yet uncharacterized 'self-non-self' recognition system coupled to an innate immunity [100]. This may be functionally and perhaps evolutionarily related to the Toll-like receptor 9 that recognizes non-methylated CpG dinucleotides in DNA sequences [101, 102]. Atkinson et al. decided to investigate the roles played by the various components of the innate immune system in recognizing dinucleotides and recruiting antiviral defenses. Their interest was caught when they observed that elevated frequencies of both UpA and CpG attenuated viral replication, whereas frequencies lower than those of native viral sequences enhanced replication [99]. However, our thinking goes in the other direction: natural selection aims to strengthen the genotypes of RNA viruses with fewer CpG dinucleotides, because otherwise, a strong 'cytokine storm' is unleashed in people prone to overexpress cytokines, causing the death of the host, which for the virus presents a developmental impasse. Any virus that completely eliminates its host population finds itself in an evolutionary dead end since viruses are obligate intracellular parasites. A certain balance of power exists in the virus-host relationship; to an extent, all of its participants remain 'gentlemen' and provide their opponents with the opportunity to survive and reproduce. The system sacrifices units, but the community is saved. Dinucleotides appearing and disappearing during the microevolution of the coronavirus CpG apparently help to adjust (more or less) the optimal level at which the parasite disseminates when the virus replicates, resulting in a moderate number of deaths of individuals in the host population. Since SARS-CoV-2 appeared only recently in the human population, in the near future we will see just such an adjustment as the number of CpG oligonucleotides changes in the direction optimal for the virus to replicate. Interestingly, the genome of virus strains sequenced in China (Wuhan) on February 11, 2020 contained on average 1 CpG dinucleotide more than was observed in later strains from the USA sequenced on May 6, 2020 (Table 2). Obviously, at the beginning of the microevolution of SARS-CoV-2 in

**Table 1** Occurrence of various dinucleotides in the genomes of known human coronaviruses

	CG	CC	GG	GC	UC	GA	AG	GU	AC	CU	CA	AA	AU	UA	UU	UG
SARS-CoV-2 NC_045512.2	439	784	973	1168	1413	1612	1742	1990	2023	2081	2084	2169	2308	2377	2454	2589
SARS-CoV NC_004718.3	568	850	1072	1433	1553	1680	1752	1871	1979	2191	2206	1922	2236	2081	2207	2664
MERS-CoV NC_019843.3	711	973	1084	1490	1695	1472	1654	2180	1766	2309	1931	1727	2284	2302	2407	2704
HCoV-NL63 NC_005831.2	332	535	907	884	1147	1185	1280	2436	1363	1717	1345	1716	2380	2493	3171	2893
HCoV-HKU1 NC_006577.2	340	527	877	860	1248	1429	1531	2437	1194	1777	1184	1898	3093	3205	3428	2854
HCoV-OC43 NC_006213.1	485	701	1150	1327	1186	1626	1823	2411	1331	1758	1600	1925	2867	2794	2892	3056
HCoV-229E NC_002645.1	488	615	984	1165	1131	1399	1417	2241	1559	1727	1640	1782	2097	2034	2522	2900

**Table 2** Occurrence of CpG dinucleotides in the SARS-CoV-2 isolates from China ( $n=7$ ) and USA ( $n=36$ ) found in GenBank

Virus origin	Occurrence of CpG dinucleotide mean $\pm$ SE
SARS-CoV-2 China, Wuhan, February 11, 2020	439.14 $\pm$ 0.09
SARS-CoV-2 USA, New York, May 6, 2020	438.44 $\pm$ 0.14*

\* $p < 0.01$

the human population, natural selection tends to favor viral genomes containing fewer CpG motifs so as not to trigger a strong innate immune response. Apparently, people who become infected with the virus have moderate symptoms and spread SARS-CoV-2 more readily. It is worth noting that the influenza A virus, which originated from an avian reservoir, has undergone a significant reduction in the number of CpG nucleotides since its introduction into humans [102]. This hypothesis is further reinforced by the fact that the CpG motif in an AU-rich oligonucleotide can significantly stimulate the immune response of plasmacytoid dendritic cells [100, 103].

In our 'lasso' construct, we placed the CpG motifs in the 'tail' region in the to 5'-purine-purine-unmethylated deoxycytosine-deoxyguanosine-pyrimidine-pyrimidine-3' position [104]. The 'neck' region of the construct features a double-stranded DNA region stabilized by 30 hydrogen bonds between 10 cytosines and 10 guanines. The double-stranded DNA 'neck' makes it possible to form a loop in the region of the antigen-presenting 'head'. The presence of such a loop allows us to take advantage of the frequent formation of RNA loops in cells and their possible interaction with the corresponding Toll-like receptor interacting with single-stranded RNA. Only adenines, cytosines, and guanines are present in the construct, which makes it possible to activate pattern recognition receptors interacting with both 'non-self' DNA and 'non-self' RNA. Since the PS backbone is the basis of the construct, the presence of deoxyribose will be of little importance and everything will depend on the nucleotide sequence of the construct [98]. CpG motifs in nuclease-resistant PS backbones have been found to dramatically enhance B cell stimulatory properties [105, 106]. In almost all vertebrates, the optimal PS CpG ODN are extraordinarily strong mitogens for B cells [107–110]. While the inhibitory effect observed for poly G motifs of CpG ODN reduces the level of B cell activation [111], in our construct, this motif is found in the double-stranded 'neck' and may have a positive inhibitory effect during possible acute inflammation that occurs after vaccination. As the body temperature rises, the double-stranded DNA 'neck' melts to produce a single-stranded oligonucleotide sequence containing two poly G motifs. Of note,

in vertebrate genomes, CpG dinucleotides occur only ~ 25% as frequently as would be predicted if base utilization were random [112]. Furthermore, the bases flanking CpGs in vertebrate genomes are not random: the most common base preceding a CpG is a C and the most common base following a CpG is a G [113]. These types of CpG motifs do not support strong immune stimulation. In addition to these differences in CpG content, methylation of CpG dinucleotides differs between virus and host. These dinucleotides, which are not methylated in the pathogen DNA, are routinely methylated in vertebrate DNA at the 5th position of ~ 70% of the cytosines [112]. If immune recognition of CpG DNA has evolved as an effective defense, then it seems likely that pathogens would evolve counter strategies to block or evade the defense. One potential strategy would be for a pathogen to reduce the level of CpG in its genome to decrease its immune-stimulatory effects. In principle, this should be much easier for pathogens having small genomes and many of them were shown to have very low CpG content [114, 115].

Thus, in the proposed construct, the CpG 'tail' will trigger cells that express Toll-like receptor 9 (including human plasmacytoid dendritic cells and B cells) to mount an innate immune response characterized by the production of T helper cells 1 and pro-inflammatory cytokines, which will improve the function of the professional antigen-presenting cells and boost the generation of humoral and cellular vaccine-specific immune responses. Also CpG 'tail' is supposed to trigger Toll-like receptors that interact with the single-stranded RNA containing CpG motif in a manner similar to interactions with TLR9-CpG ODN. Generally, the unmodified 'tail' will serve as an adjuvant and will prepare the immune system for the meeting with the antigen found on the 'head'. The sequence of the 'head' (5'-GCAGAGACA GAAGAAACAGCAAAC-3') is well conserved. It is found in every SARS-CoV-2 genome located in GenBank at this moment and belongs to a nucleocapsid phosphoprotein gene [protein\_id="QJC2104QJC21049.1"] that has 87% query cover with Homo sapiens protein kinase C eta (PRKCH) (NG\_011514.1). 9.1] that has 87% query cover with Homo sapiens protein kinase C eta (PRKCH) (NG\_011514.1).

Summing up, the proposed design contains CpG dinucleotides, important for in the activation of innate immunity, and a unique genomic sequence of the virus, the antigen-presenting component that activates adaptive immunity. Similar vaccine constructs based on phosphorothioate oligonucleotides can have a wide variety of unique sequences based on the circulating SARS-CoV-2 strains, and each vaccine may contain a dozen of these lasso-like constructs. In the very near future, we plan to test such vaccines on the murine coronavirus, taking care not to miss any possible negative effects, and remaining aware that the PS backbone dramatically increases the non-specific ODN binding to a wide variety of proteins [116, 117].



The mechanisms of the 'cytokine storm' and how they contribute to fatalities reported for COVID-19 are still under investigation. Currently, it is believed that SARS-CoV-2 dampens antiviral interferon responses, which results in uncontrolled viral replication. For the other coronaviruses, SARS-CoV and MERS-CoV, the response to viral infection by type I IFN is suppressed. The influx of neutrophils and monocytes/macrophages results in hyperproduction of pro-inflammatory cytokines [118]. In a study of 41 patients hospitalized with COVID-19, high levels of pro-inflammatory cytokines were found, including IL-2, IL-7, G-CSF, IP-10, MCP-1, MIP-1A, and TNF- $\alpha$ , in the most severe cases [119]. This overproduction of a large number of pro-inflammatory cytokines dubbed a 'cytokine storm,' begins a cascade of events that is hard to stop. For instance, the cytokine storm can initiate viral sepsis and inflammatory-induced lung injury, which leads to other complications including pneumonitis. While 'cytokine storms' can overwhelm people of any age, some scientists believe that this phenomenon may explain why otherwise healthy young people died during the 1918 Spanish flu pandemic and more recently during the SARS, MERS, and H1N1 epidemics. One of the most frequently studied organisms known to cause cytokine storms is influenza a virus, particularly the pandemic subtypes. It has been shown that the H1N1 strain responsible for the 1918 pandemic induced higher levels of pro-inflammatory immune cells and cytokines in the lungs than the seasonal influenza viruses. This contributed to its high virulence and may account for the unusually high mortality rate seen among young adults during the outbreak [120]. Severe influenza infections caused by highly virulent subtypes such H1N1 and H5N1 are characterized by over induction of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) [121, 122], which eventually results in multiple organ dysfunction and failure and increased vascular hyperpermeability [122].

With respect to the tendency of the body to overexpress cytokines and let loose a 'cytokine storm' during coronavirus infection in some humans, we are proposing vaccine construct having the CpG motifs which will help people's immune systems train for this inflammation reaction in the absence of a real pathogen and real danger. In a broad sense, the immune system copes with the virus much more effectively if it has already encountered it. The unmethylated CpG motif is certainly that portion of the virus genome for which the immune system of sensitive people needs to be prepared. Generally, CpG ODN boosted the humoral immune response induced by vaccines against a large number of pathogens, including influenza virus, measles virus, lymphocytic choriomeningitis virus, orthopoxviruses, and other viruses [71]. At the height of the epidemic, when more than 500,000 people have already died, apparently it is time to try this approach when creating a vaccine against

SARS-CoV-2. Even though SARS-CoV-2 will constantly sweep its tracks, an effective vaccine should contain a CpG motif as a conservative fingerprint of the pathogen to train the human immune response. The fight against COVID-19 will be difficult and lengthy. Nevertheless, the current situation will help to gain experience and the ability to prevent future coronavirus pandemics. Life goes on.

**Acknowledgements** This work was supported by state program No FZEG-2020-0060 of the Ministry of Science and Higher Education of Russian Federation.

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