Review

SARS Immunity and Vaccination

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Severe acute respiratory syndrome (SARS) is a serious and fatal infectious disease caused by SARS coronavirus (SARS-Cov), a novel human coronavirus. SARS-Cov infection stimulates cytokines (e.g., IL-10, IFN-γ, IL-1, etc.) expression dramatically, and T lymphocytes and their subsets CD4$^+$ and CD8$^+$ T cells are decreased after onset of the disease. SARS-specific IgG antibody is generated in the second week and persists for a long time, whereas IgM is expressed transiently. The spike protein and neucleocapsid protein are most abundant in SARS-Cov and contribute dominantly to the antibody production during the course of disease. Spike protein, especially the ACE-2 binding region (318-510aa) is capable of producing neutralizing antibody to SARS-Cov. Neucleocapsid protein induces protective specific CTL to SARS-Cov. Therefore, applications with spike subunit, neucleocapsid subunit as well as inactivated SARS-Cov are three prospective vaccination strategies for SARS. *Cellular & Molecular Immunology*. 2004;1(3):193-198.

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The causative agent of severe acute respiratory syndrome (SARS) was identified as a new type of coronavirus, the SARS coronavirus (SARS-Cov). SARS-Cov genome contains five major open reading frames (ORFs) encoding the replicase polyprotein, the spike (S), the envelope (E), membrane (M) glycoproteins and the nucleocapsid protein (N) in the same order and of approximately the same size as those of other coronaviruses (1-3). Additionally, SARS-Cov also has several small non-structural ORFs that are found between the S and E genes and between the M and N genes. So far, the SARS virus seems remarkably invariant: the genome sequences of 14 isolates from SARS patients in Singapore, Toronto, China and Hong Kong have not revealed any changes. This feature is beneficial for SARS vaccination.

SARS-Cov brought about strong immunological responses contributed to viral protection and pathogenesis. Elucidating such immunity of SARS is important for understanding SARS pathogenesis and developing SARS vaccines. In this review, we will focus on SARS immunity and potential vaccination.

SARS immunity

As a typical virus infection of a cell, SARS-Cov binds to host cells *via* a specific SARS receptor, angiotensin converting enzyme 2 (ACE-2) (4-7). Following entry of the virus uncoats, nucleic acid is released, and transcription occurs followed by the production of viral proteins. During this course, host defense system involving B and T cells is stimulated. As we know, cytotoxic T cells (CD8$^+$) and T helper cells (CD4$^+$), have distinct effector functions. CD8$^+$ cytotoxic T lymphocytes (CTLs) play a pivotal role in the clearance of intracellular pathogens through the recognition and elimination of infected cells. When CD4$^+$ T helper cells recognize antigenic peptides presented by professional antigen presenting cells (APC), they produce cytokines that promote cell-mediated and/or humoral immunity. During SARS-Cov infection, the immunity was characterized as lymphopenia, specific antibody production, cytokine profile and specific responses to individual viral proteins.

Cytokines profile

The pattern of cytokines elicited by a particular pathogen plays a critical role in determining disease outcome by influencing the types of immune effectors that develop against the infectious agent (8-15). During SARS-Cov infections, IFN-γ, a Th1 cytokine which is associated with potent cell-mediated immunity and resistance to intracellular pathogens, was increased dramatically. IL-4, the dominant Th2 cytokine, which promotes humoral immunity that protects against extracellular microbial infections, was decreased after onset of SARS-Cov infection. It indicates a Th1 dominated-responses caused by SARS-Cov infection whereby eliminated the viral pathogen from the body. However, another Th2 cytokine,

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Abbreviations: SARS, severe acute respiratory syndrome; CTL, cytotoxic T lymphocyte; APC, antigen presenting cell; IBV, infectious bronchitis virus; ACE, angiotensin converting enzyme.

Table 1. *Cytokines profile after SARS onset.*

Cytokines	Producing cells	Event	References
IL-1	macrophages	+	10, 12
IL-2	Th1 cells	-/+	8-11, 14
IL-4	Th2 cells	-	10
IL-6	Th2 cells, fibroblasts, macrophages, endothelial cells	+	13
IL-8	activated T cells, fibroblasts, macrophages	+	10, 12
IL-10	Th2 cells	+	14
IL-12	macrophages, monocytes, dendritic cells, B cells	+	10, 14
IL-13	Th2 cells	-	13
IL-16	$CD8^+$ T cells, epithelial cells, mast cells, acidophils	+	13
IL-18	activated T cells, macrophages	-	13
IFN-γ	Th1 cells	+	9, 13
TNF-α	macrophages, T cells	+/-	10, 12, 13
TGF-β	T cells, macrophages, platelets	+	13

All cytokines are detected from serum or plasma. +, represents elevation after SARS infection; -, represents reduction after SARS infection.

IL-10 was also elevated in the SARS patients. Mainly, IL-10 is produced by Th2 and it has a dual effect on T lymphocytes in terms of inhibiting Th1 cells to produce IL-2 and interferons as well as tumor necrosis factor (TNF), and promoting the proliferation and cytolysis activity of CD8 and NK cells. Therefore, it is possible that elevation of IL-10 expression is associated with the susceptibility to the disease. As for IL-2 expression, Li et al (14) and Duan et al (11) claimed a high expression level after SARS onset whereas others did not (8, 9). Expectedly, inflammatory cytokines elevated dramatically. The cytokine profile is summarized in Table 1.

Similar to the H5N1 "avian flu" influenza infection, of which the influenza virus has been shown to be a potent inducer of proinflammatory cytokines (16, 17) particularly, there is substantial upregulation in tumor necrosis factor-α production. SARS infection induced a similar inflammatory cytokine pattern and might contribute to unusual severity of human disease. Consistently, this point was supported by the clinical evidence of SARS treatment with corticosteroid or its analogue that the levels of TNF, IL-1β and other inflammatory cytokines were reduced after administration and such reductions were associated with clinical severity. Thus, inhibition of inflammatory cytokines may be a beneficial strategy for SARS therapy. However, Jones (9) found that SARS infection developed a weaker ability of periphery monocytes to produce cytokines (IFN, IL-2, IL-10, IL-12, etc.) after mitogen stimulation and they suggested that increased cytokine level might be beneficial for SARS treatment. Indeed, administration of cytokine IFN was demonstrated efficacious for SARS therapy (18-20).

Antibody profile

Similar to common acute viral infection, such as hepatitis A, the profile of antibodies against SARS virus has a typical pattern for IgG and IgM production. All patients with SARS infection had antibody responses to SARS virus during the convalescent phase. As shown in Figure 1. the SARS-specific IgG antibody persisted for a long time, but SARS-specific IgM antibody remained measurable for a much shorter period (within 13 weeks), suggesting that IgG antibody against SARS virus represents primary humoral immune response to protect patient against SARS. It is believed that the SARS-specific IgG antibody is dominantly contributed by production of N-specific and S-specific antibody (21-25). Similar to other coronavirus, such as murine coronavirus, turkey coronavirus and porcine reproductive and respiratory syndrome virus, SARS N protein is a most abundant protein and a strong immunogen, and the resultant antibody may be a good marker for SARS infection. Presently, N protein-based immunological methods for SARS serological diagnosis have been developed and the sensitivity and specificity are up to 97-100% (26, 27). Spike protein is another abundant protein of SARS virus. Western blot assay indicated it also dominantly contributed to SARS-specific antibody production. Importantly, the S-specific antibody was confirmed to exert the activity by neutralizing SARS virus as discussed in later section.

Lymphopenia

Lymphopenia is a very common feature for SARS infection (29-33). According to Wong's report (31), 153 (98%) of the 157 patients had lymphopenia (absolute lymphocyte count $<1000/mm^3$) during their course of illness. Most patients had normal lymphocyte count at the onset of disease. Progressive lymphopenia occurred in the early course of illness and reached its lowest point in the second week in most cases. The lymphocyte count commonly recovered in the third week. Several reports supported such expression pattern of lymphocytes. Analysis for lymphocyte subset showed that $CD4^+$ and $CD8^+$ T cells were decreased significantly which was associated with adverse outcomes. Thus, in SARS infection, lymphopenia reflects the severity of infection and may be a good marker of disease activity.

Why and how did SARS infection induce lymphopenia? Some investigators proposed that depletion of lymphocytes was due to apoptosis (34-38). In severe parmyxovirus infections in humans such as measles, lymphopenia is commonly presented and associated with more severe disease, and the apoptosis is believed to be the mechanism

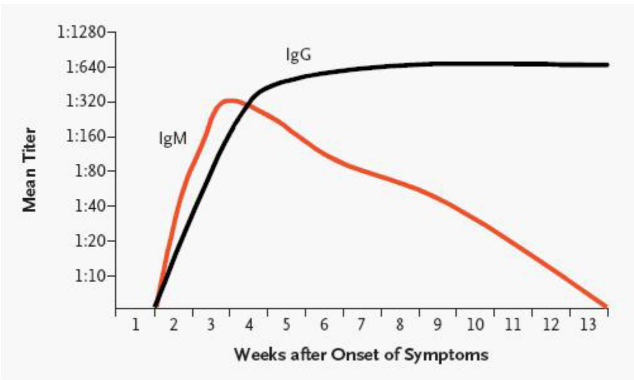

Figure 1. Production of SARS-Cov-specific antibodies after infection onset. This figure is adopted from reference (25).

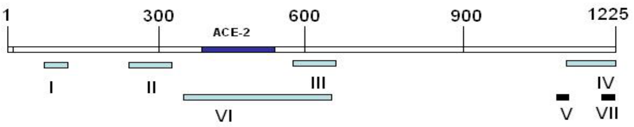

Figure 2. Immunological epitopes in spike protein of SARS. Peptides in region I to IV have reactivities with antiserum of SARS patients. Region V (RLNEVAKNL) exerts CTL induction activity by binding to HLA-A2 for antigen presentation. VII is a immunogenic T-cell epitope and elicits an overt specific T-cell response in HLA-A2(+) SARS-CoV-infected patients. The number indicates amino acids of the protein.

of lymphopenia. The inhibitors of apoptosis ameliorate illness and prevent death. However, there is no convincing evidence to support such hypothesis so far. Another explanation for the lymphopenia is that lymphopenia occurs when the body's mechanisms for down-regulation of lymphocyte differentiation, particularly mediated by IL-10 from the cytokine cascade, swing into action. It may also be hastened by down-regulation following infection and activation of T lymphocytes (39, 40).

Immunity of spike proteins

Spike protein is located at outside of virus and is a biggest protein with 1225 amino acids and 24 glycosylation sites. By using SARS-specific antibody (antiserum) and peptide synthesis, several antigenic motifs were identified in S protein (41-43). Four regions with highly immune reactivity are located at 67-119aa (I), 265-345aa (II), 588-645aa (III) and 1130-1234aa (IV), respectively. In region IV, there are two regions (V and VII) responsible for CTL induction, as shown in Figure 2. Based on antigenic prediction, ACE-2 binding region is a weak antigenic determinant. However, when the mice or monkeys were injected with DNA vaccine or adenoviral-delivery vaccine containing expressible full length of spike gene, anti-ACE-2 binding region antibody was generated and exerted neutralizing activity by blocking binding of S to ACE-2. Our recent unpublished data showed the capacity of ACE-2 recombinant protein to generate neutralization antibody effectively. Thus, ACE-2 binding region of spike protein is an antigenic determinant and it may be used as an antigen for SARS vaccination. Additionally, epitopes of region V and VII of spike protein bind to HLA-2A and induce production of protective CTL by antigen presentation.

Roles of SARS nucleocapsid protein in immunity

Nucleocapsid protein is a most abundant protein of coronavirus. During virion assembly, N protein binds to viral RNA and leads to formation of the helical nucleocapsid. It is predicted that N protein is a highly charged, basic protein of 422 amino acids with a short lysine rich region suggestive of a nuclear localization signal (1). Interestingly, N protein contains no cysteine and exerts high polar property by dominant hydrophilicity. The recombinant N proteins with different sizes are expressed in soluble form in *E.coli* despite expression rate as high as 40-50% (recombinant protein over total proteins) (44). The abundance and high hydrophilicity of N protein are supposed to contribute to potent immunity after SARS infection.

About a week after SARS onset, N protein-specific antibody may be detected and sustains for long time. The corresponding epitopes in N protein were summarized as Figure 3. N371-390 and N385-407 have a potent ability to react with the serum of 94-97% patients, suggesting the epitope site at the C-terminus of the N protein is likely to be located at codons 371-407 (24).

As other coronavirus, N protein of SARS virus is able to induce specific CTL by use of DNA vaccine. The epitopes for CTL induction remain unknown.

SARS vaccination

For vaccine development, it is critical to generate protective immune responses including neutralization antibody and CTL generation. The SARS-Cov is a novel coronavirus, but vaccines for other human coronaviruses have not been successfully developed. Lots of experiences in developing vaccine for veterinary coronaviruses have been obtained. Vaccines against infectious bronchitis virus (IBV) of chickens, for example, have been the most successful of vaccines for diseases caused by coronaviruses (45), the others being against bovine, canine, feline and porcine coronaviruses. Attenuated IBV strains (by passage in chicken embryonated eggs) were introduced as vaccines in the 1950s, followed a couple of decades later by inactivated vaccines for boosting protection in egg- laying birds. All of chickens may be protected, but the protection was transient, the decline being apparent 9 weeks after vaccination. The recombinant spike glycoprotein S1 subunit induced virus neutralization antibody while the protection percentage was less than 50%. When fowl adenovirus was used for vaccine vector, the protection percentage went up to 90-100%. The poor cross-protection induced by S1 was found and it suggested a very limited epitopes for neutralization antibody production. Recombinant N protein of IBV could not induce protective response, while its DNA vaccine induces protective immunity. Although the basis of IBV vaccine immunity is not well understood, it provided us lots of instructive clues for SARS vaccine development. Currently, three kinds of SARS vaccines, inactivated virus-based vaccine, S-based vaccine and N-based vaccine, are under extensive studies.

Inactivated virus-based vaccine

Live vaccines have the great advantage of providing an increased antigenic challenge that lasts days or weeks, and inducing it in the right site. They are likely to contain the

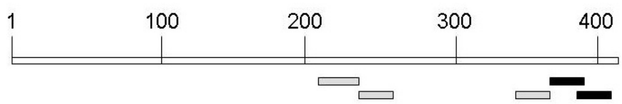

Figure 3. Antigenic motifs of SARS nucleocapsid protein. Blank bar indicates stronger ability to induce antibody production. The number indicates amino acids of nucleocapsid protein.

greatest number of viral antigens. Generally, live vaccines are more effective than killed ones. However, in the case of SARS-Cov, live vaccine is dangerous both to vaccine producer and vaccine receivers. Such difficulties lead us to think about an alternative strategy, such as using killed vaccine. The killed vaccine of SARS-Cov suffers from three extra disadvantages: T-cell independence, major histocompatibility complex restriction and, since SARS is a highly infectious disease, the serotype alternation caused by vaccination may affect immunological analysis of epidemic monitoring. Even so, application of killed SARS vaccine is acceptable to develop before we get a better vaccination method. In China, killed SARS vaccine is testing in clinical trails.

S-based vaccine

As mentioned above, DNA vaccine of spike may induce neutralization and specific CTL. Thus it is considered as a prospective vaccine candidate for SARS-Cov.

Consistent with several authors' reports that immunization with recombinant S1 protein or plasmid encoding the S1 subunit of IBV could induce protective immune response (46-48), the first report on immunizing masques with structure genes of SARS-Cov including S1, N and M could elicit a high titer of neutralizing antibody and T-cell response (49). Yang et al. immunized animals with DNA vaccine containing S gene (S1+S2) alone and obtained high titer of neutralization antibody and cellular immunity (50). The neutralizing antibody is capable of blocking viral infection, but the adoptive cellular immunity lacked protective effect on SARS-Cov infection. Bisht et al. constructed recombinant forms of the highly attenuated modified vaccinia virus Ankara (MVA) containing the gene encoding full-length SARS-CoV-S (51). The resultant MVA/S administered by intranasal or intramuscular inoculations elicited protective immunity, as shown by reduced titers of SARS-CoV in the upper and lower respiratory tracts of mice after challenge. Thus, S gene is thought to contribute to neutralizing antibody production and is prospective target for vaccination.

Interestingly, Zeng et al. used S1 (18-495aa) and S2 gene (52), rather than combined with other genes, as DNA vaccine and claimed that S1 and S2 induced high titer of neutralizing antibody also, but the neutralizing antibody was contributed by cooperation of anti-S1 and anti-S2 antibodies. Recent data demonstrated that ACE-2 is SARS-Cov receptor and its ligand is located at junction region of S1/S2 (318-510aa). Our recent data indicated that this region contributed to neutralizing antibody production in animals (Zhu MS et al., unpublished data). Therefore, we proposed that ACE-2 binding region of SARS-Cov spike fragment might contain two antigenic epitopes for neutralizing antibody production and these epitopes might be useful for SARS vaccination.

N-based vaccine

Several reports demonstrated that protective responses elicited by antigens of some viruses that were not present on the surface of the virion, such as the N protein, were more likely to be due to CTL. For example, nucleoproteins of Ebola virus (53, 54), measles virus (55), lymphocytic choriomeningitis virus (56) and influenza virus (57, 58) may induce protective CTL. In porcine coronavirus, transmissible gastroenteritis virus (TGEV), N protein is a representative antigen for the T cell response and may induce cellular and humoral immune responses (59). As expected, in the case of SARS coronavirus, intramuscular injection with expression plasmid containing full length of SARS N gene induces potent protective CTL also (44, 60). Interestingly, if the N gene was fused with calretinin, CTL induction by the DNA vaccine was improved significantly. It is claimed that calretinin may help peptides of N protein to be presented. Current data of N protein-based DNA vaccine were obtained by use of full neucleocapsid gene including a nuclear location signal with possible pathological risk, and the CTL induction activity of N protein fragments remains unknown.

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