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Imiquimod - A toll like receptor 7 agonist - Is an ideal option for management of COVID 19

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ABSTRACT

According to numerous recent publications, the COVID-19 patients have lymphopenia, higher infection-related biomarkers and several elevated inflammatory cytokines (i.e. tumor necrosis factor (TNF)-α, interleukin IL-2R and IL-6). The total number of B cells, T cells and NK cells are significantly decreased. RNA viruses, SARS-CoV-2 included, hit the innate immune system in order to cause infection, through TLRs 3, 7 and 8. Imiquimod is an immune-stimulator that activates TLR 7 and can be used to enhance the innate and adaptive immunity. Preclinical and clinical trials are proposed.

1. Introduction

Coronaviruses (CoVs) are classified to a large family of positive-sense single stranded RNA viruses ((+)ssRNA) in the genus of beta-coronaviruses that exploit their genetic material, after internalization in the host cells, to act as messenger RNA, so as to be translated to viral structural proteins, necessary for replication and transcription of the viruses (Li et al., 2020a, 2020b; Prompetchara et al., 2020; Yi et al., 2020). CoVs are greatly pathogenic in humans triggering respiratory infections ranging from the common cold to more severe diseases, like Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS). The virion of CoVs is of "core-shell" morphology illustrated as viral envelopes, with the viral CoVs' RNA genome being protected, in the core, inside nucleocapsids of protein origin and helical symmetry that are enveloped in an outer shell of lipidic membrane origin (lipid bilayer), typically deriving from the cellular membranes of the host cells. The viral envelope is of phospholipidic, proteinic and glycoproteinic origin and may serve as a kamuflaz to escape the immune system of the host, and may exploit its surface glycoproteins to identify and link to receptors on the cellular host membrane (Li et al., 2020a, 2020b; Prompetchara et al., 2020; Yi et al., 2020). CoVs belong to the RNA viruses with increased prospective of becoming pandemic worldwide (Cascella et al., 2020). The 2019 novel coronavirus, called 'SARS-CoV-2′ is a new strain that causes Corona Virus Disease 2019 (COVID-19), for which no effective treatment has been found until now. The outbreak of SARS-CoV-2, that first emerged in Wuhan in December 2019, has rapidly spread throughout the world (Huang et al., 2020; National Health Commission, 2020). Considering the ongoing outbreak in China and fast worldwide spread of COVID-19, infected by SARS-CoV-2, it has led to the declaration of Public Health Emergency of International Concern by the World Health Organization (WHO) on January 30, 2020 (World Health Organization, 2020). As of Apr 10, 2020, a total of more than 1,500,000 of laboratory-confirmed new cases of COVID-19 has been identified in the world with more than 89,000 deaths.

In order to deal with COVID-19 pandemic supportive and preventive therapeutic approaches have been followed with directives and guidelines of social distancing and isolation worldwide, while scientists, and researchers work in order to elucidate the clinical spectrum of COVID-19, the transmission mechanisms, the virus-host interactions, and seek new diagnostic, preventive (vaccines) and therapeutic approaches. In this short review, the basic molecular replication mechanism of SARS-CoV-2 and the consequences of the imminent cytokine storm on host cells are outlined on the base of CoVs immune elusion and host cells innate immunity, which represent key points for effective defense against COVID-19 infection. The drug Imiquimod a synthetic molecule able to enhance both the innate and acquired immune response is proposed as an effective therapeutic approach.

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2. Immunological data

2.1. Coronavirus replication in the host cell

Extensive studies have been researched in the field of etiology, epidemiology and pathophysiology of CoVs in relation to COVID-19 (Chan et al., 2020; Dong et al., 2020; Lei et al., 2018; Letko et al., 2020; Lu et al., 2020; Perlman et al., 2009; Raj et al., 2013; Sevajol et al., 2014; Song et al., 2018; Zhou et al., 2020a, 2020b; Zhu et al., 2019). In CoVs generally, the spike S glycoproteins (composed of S1 and S2 subunits) on the surface of the viral envelop guide the receptor mediated endocytosis by attachment to host cell receptors of Angiotensin-Converting Enzyme 2 (ACE2) for SARS-CoV and Dipeptidyl Peptidase 4 (DPP4) for MERS-CoV, respectively. By this binding process, CoVs manage to escape from immune surveillance, and thus host cellular membrane fusion is promoted, wherein the viral genome is released in the cytoplasm in order to be translated in the ribosomes of the host cell (Lei et al., 2018; Perlman et al., 2009; Raj et al., 2013; Sevajol et al., 2014). Then the released viral genome in the host ribosome, uses the open reading frames (ORFs), especially the ORF1a and 1 b, to be translated to long polyproteins 1a/1 ab (pp1a/pp1ab). Polyproteins are further processed by viral encoded proteases (such as, chymotrypsin-like protease 3CLpro, main Mpro protease and papain-like proteases) for the final product of 16 non-structural proteins (nsps 1–16) (Fehr and Perlman, 2015). The main role of nsps is to prepare a favorable cellular environment and enhance viral replication (nsps1), affect viral growth kinetics (nsps2), affect viral fitness, active-site mutants and conformational changes (nsps3-5), affect non-specific RNA-binding activity (nsps 7–10), contain the RNA replication and transcription enzymes (nsps12-16). Especially, in nsp 12 the coronavirus RNA-dependent RNA polymerase is contained (Knoops et al., 2008; Masters, 2006). According to Knoops et al., (2008), the transmembrane nsps are probably inserted into the endoplasmic reticulum (ER) membranes, where early viral RNA synthesis possibly occurs. At the time of increased replication expression being achieved, nsps promote membrane pairing and bending for membrane envelopes to be formed (Knoops et al., 2008).

Even if CoVs RNA synthesis is incompletely understood, the viral genomic RNA following translation into polyproteins in accordance with structural (S (stripe protein, M (membrane protein), N (nucleocapsid protein), E (envelope protein) and non-structural proteins, serves as template for the synthesis of negative sense RNA species and smaller species of sub-genomic RNAs (sgRNAs) sequences of both polarities. The positive sense sgRNAs are received as messages for all ORFs expression. The viral genome is mainly acting as a loop template for replication and transcription. The replicated positive sense sgRNAs become the progeny genomes, where the viral N structural protein is directed to bind and form newly developed nucleocapsids for the progeny virions. Following the secretory pathway, the new nucleocapsid along with viral structural proteins S, E, and M penetrate into the ER or Golgi intermediate compartment, wherein through protein-protein interactions progeny virion assemblies with incorporated nucleocapsid are formed. Finally, the progeny viruses transported by Golgi vesicles penetrate into the ER lumen and after incorporating its lipid bilayer membrane, they are directed to the cellular membrane, in order to be exocytosed into the extracellular environment able to infect other host cells (Cascella et al., 2020; Fehr and Perlman, 2015; Knoops et al., 2008; Masters, 2006).

2.2. Cytokine storm in coronavirus infection

Unfortunately, a thorough research study on the virus-host interactions in case of SARS-CoV-2 during respiratory infection is yet limited, thus the available information arises from investigation on other RNA CoVs, such as SARS-CoV and MERS-CoV or for recent clinical date on COVID-19. According to numerous publications (Cameron et al., 2008; Channappanavar and Perlman, 2017; Fan et al., 2009; Huang et al., 2020; Qin et al., 2020; Shaw et al., 2013; Tang et al., 2011; Williams and Chambers, 2014; Wong et al., 2004; Xu et al., 2020; Zhang et al., 2020a, 2020b; Zhao et al., 2014; Zhou et al., 2020a, 2020b), the most well recognized hematologic abnormality for the patients is lymphopenia that in severe cases tend to be up to 85% (increasing rate with severity of the cases), higher infection-related biomarkers and elevated levels of several pro-inflammatory cytokines (i.e. tumor necrosis factor (TNF)-α, interleukin IL-2R and IL-6). In distinct report studies from China (Huang et al., 2020; Zhou et al., 2020a, 2020b; Qin et al., 2020; Xu et al., 2020) summarized in Table 1, increased levels of neutrophils, c-reactive protein and IL-6, accompanied with low levels of lymphocytes were observed, while increased levels of innate pro-inflammatory cytokines, such as TNF-α and chemokines such as IP-10 (interferon-γ-inducible protein), MCP-1 (monocyte chemoattractant protein), MIP-1α (macrophage inflammatory protein 1 alpha), were observed in patients needing intensive care unit (ICU) hospitalization. Higher serum levels of pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) and chemokines (IL-8) were also found in patients with severe COVID-19 compared to individuals with mild disease (Qin et al., 2020). The elevated plasma levels of neutrophils, pro-inflammatory cytokines and chemokines and the decrease on lymphocyte levels have been correlated with disease severity and mortality (Qin et al., 2020; Xu et al., 2020) (Table 1).

The intense and uncontrolled release of pro-inflammatory cytokines has been associated to cytokine storm (CS), a syndrome that can be promoted by infectious diseases, such as COVID-19, resulting in systemic inflammation, acute lung injury, acute respiratory distress syndrome (ARDS), multiple organ failure (especially acute kidney injury, cardiac injury, spleen atrophy, lymph node atrophy) (Chan et al., 2020; Zhang, W., et al., 2020) and death (Qin et al., 2020; Xu et al., 2020; Zhang, D., et al., 2020). One of the foremost and most severe consequences of SARS-CoV-2 is ARDS, an immunopathologic syndrome in patients. In Huang et al., 2020 report, based on patients hospitalized in Wuhan with early stage symptoms of COVID-19, 26 out of 41 (63%) patients developed lymphopenia and 12 of 41 (29%) suffered of ARDS, while 13 (32%) patients were admitted to an ICU and 6 (15% of 41 patients) died. All patients hospitalized in ICU presented cytokine storm syndrome with elevated levels on plasma serum of pro-inflammatory cytokines and chemokines, such as IL-7, IL-10, GSCF, IP-10, MCP-1, MIP-1α, and TNFα. Even if the pathophysiology of COVID-19 is not fully understood yet, the presented cytokine storm has been related to disease severity (Cameron et al., 2008; Channappanavar and Perlman, 2017; Fan et al., 2009; Qin et al., 2020; Shaw et al., 2013; Tang et al., 2011; Williams and Chambers, 2014; Wong et al., 2004; Xu et al., 2020; Zhang D. et al., 2020; Zhang W. et al., 2020). The elevated secretion levels of pro-inflammatory cytokines in CoV infections, such as IL-6, IL-12, IFNγ, IP-10, MCP-1 has thought to possibly promote the activation of T-helper-1 (Th1) cell responses, while the GCSF, IP-10, MCP-1, MIP-1α, and TNFα pro-inflammatory chemokine storm has been associated to a possible promotion of T-helper-2 (Th2) cell responses (Huang et al., 2020; Liu et al., 2020). In the 2019 inflection of SARS-CoV-2 elevated secretion levels of cytokines related with Th1 responses and forthcoming pulmonary inflammation, and cytokines of Th2 responses associated with suppression of inflammation, have been reported according to the stage of the disease (Guo et al., 2020).

2.3. Immune responses and innate immunity

SARS-CoV-2 infection has been related to lymphopenia with decreased levels of lymphocytes (T cells, B cells and natural killer cells, NK) being correlated with disease severity in most patients. Previous studies on MERS-CoV and SARS-CoV infections have identified that T cells especially $CD4^+$ and $CD8^+$ T cells, play a significant antiviral role (Cameron et al., 2008; Liu et al., 2020). In recent reports on the clinical features, the total number of B cells, T cells and NK cells significantly decreased in patients with COVID-19 and more evident in the severe cases, compared to the non-severe group (Guo et al., 2020; Liu et al.,

Table 1
Characteristic examples of clinical studies in patients with COVID-19 and rate of pro-inflammatory cytokines.

Study	Patients	Middle age (years)	Symptoms	Virus-Related Complications	ICU	Died	Plasma Levels	Ref
COVID-19: pneumonia cases in Wuhan, China	41	49	Common symptoms: Fever, Cough, myalgia or fatigue, Less Common symptoms: headache, haemoptysis, sputum production, diarrhea	Dyspnea, lymphopenia Pneumonia, ARDS, RNAaemia, acute cardiac injury, secondary infection	13	6	IL2, IL7, IL10, GSCF, IP10, MCP1, MIP1A, and TNFα	Huang et al. (2020)
COVID-19: pneumonia cases in Wuhan, China	3	adults	Common symptoms: cough, chest discomfort, fever,	Pneumonia		1		Zhu et al. (2019)
COVID-19: pneumonia cases in Wuhan, China	1	50	Common symptoms: fever, chills, cough, fatigue and shortness of breath	lymphopenia Pneumonia, ARDS,		1	CCR6+ Th17 in CD4 T cells	Xu et al. (2020)
COVID-19: pneumonia cases at Tongji Hospital	452	58	Common symptoms: Fever, expectoration, dyspnea, fatigue, dry cough, myalgia	Short of breath, fatigue			lymphopenia, higher infection-related biomarkers (serum ferritin, C-reactive protein), inflammatory cytokines/ chemokines (TNF-α, IL-2, IL-6, IL-8)	Qin et al. (2020)

2020; Qin et al., 2020; Zhang W. et al., 2020). Especially, T cells were shown to be more affected by SARS-CoV-2, as T cell count was nearly half the lower reference limit. The function of $CD4^+$, $CD8^+$ T cells, and NK cells was within normal range and no significant difference was found between severe cases and non-severe ones (Qin et al., 2020). In viral infections, the response of the host immune system after virus invasion is to recognize and control the infectious DNA or RNA genome released in the host cells cytoplasm. CoV infections can cause uncontrolled immune responses, through the cytokine storm syndrome and result in immunopathogenesis in the host (Cameron et al., 2008; Channappanavar and Perlman, 2017; Li et al., 2020a, 2020b; Prompetchara et al., 2020; Tang et al., 2011; Williams and Chambers, 2014; Yi et al., 2020; Zhao et al., 2014). In particular, SARS-CoV infections the S protein on the virion corona is linked to the ACE2 receptor of the host cell and after fusion via the cytoplasmic membrane, the viral RNA genome is released into the cytoplasm. Then, normally the innate immune response signaling cascade starts with the recognition of the viral genome, representing a pathogen-associated molecular pattern (PAMP), by pattern recognition receptors (PRRs), which in general are proteins responsible for detecting pathogenic stimuli (Bussey and Brinkmann, 2018; Li et al., 2020a, 2020b; Prompetchara et al., 2020; Yi et al., 2020). It is well known that the innate immune system is important in early life, when the adaptive functions are underdeveloped. At present, the response of the innate immune through the PRRs is the first line of defense against viral infections, thus PRRs, such as toll-like (TLR), RIG-I-like (retinoic acid-inducible Gene-I-like receptors, RLR), NOD-like (nucleotide-binding oligomerization domain-like receptors, NLR), C-type lectin-like (CLRs), should provide robust and efficiently synchronized effect (Bussey and Brinkmann, 2018; Li et al., 2020a, 2020b; Prompetchara et al., 2020; Yi et al., 2020).

However, when immune response is dysregulated it will result in an excessive inflammation, even cause death (Qin et al., 2020). Qin et al., 2020 demonstrated pronounced lymphopenia and low counts of $CD3^+$ and $CD4^+$ cells in COVID-19 cases. Similar clinical features are observed in SARS-CoV with patients of severe groups after convalescent indicating multifunctional $CD4^+$ T cells producing IFN-γ, TNF-α, and IL-2, and $CD8^+$ T cells producing IFN-γ, TNF-α, and CD107a and elevated immune responses of Th2 cytokines (IL-4, IL-5, IL-10), while in MERS-CoV patients of increased severity elevated $CD8^+$ T cell levels were observed with $CD4^+$ T cell responses being minimal. In the latter case after convalescence severity-dependent antibody responses and antigen-reactive cells were observed (Qin et al., 2020). From the clinical findings, it is considered that T cell responses are clearly related to CoVs severity. In the lungs usually, PAMPs in the form of genomic RNA viruses or dsRNA intermediates are recognized, during replication in the host, by the Toll-like receptors (TLRs) 3, 7, 8, and endosomal RNA receptors or cytoplasmic RNA PRRs, such as RIG-I (retinoic-acid inducible gene I, also known as DDX58) and MDA5 (melanoma differentiation-associated 5) representing highly important receptors, since they provide first line defense against infections (Bussey and Brinkmann, 2018; Guo et al., 2020; Kikkert, 2020; Li et al., 2020a, 2020b; Nazmi et al., 2014). The attachment of viral genome in the receptors give rise to innate immune response signaling pathways. Especially, for TLRs expressed on the membranes of leukocytes (i.e. immune cells, such as dendritic cells, macrophages, natural killer cells, cells of the adaptive immunity T cells, and B cells) the attachment of viral RNA triggers molecular cascades of innate immune responses and development of acquired antigen-specific immunity. The activation of TLRs and the involvement of T cell antigen receptors (TCR) promotes critical signal transduction cascades through the activation of signal transducing adapter proteins (STAPs), such as MYD88, to transfer the antigen-induced signal transduction pathway. A series of molecular events is involved in signal transduction, usually protein phosphorylation catalyzed by protein kinases (IKKi, IRAK1, IRAK4, and TBK1) resulting in cytokine production and innate immune responses (Bussey and Brinkmann, 2018; Guo et al., 2020; Kikkert, 2020; Li et al., 2020a, 2020b; Nazmi et al., 2014). Moreover, in viral infections the innate immune system senses foreign material that is possibly pathogenic, and this triggers downstream signaling to ultimately induce transcription factors in the nucleus, which in turn stimulate expression of types I and III IFNs and other pro-inflammatory cytokines (Bussey and Brinkmann, 2018; Guo et al., 2020; Kikkert, 2020; Li et al., 2020a, 2020b; Nazmi et al., 2014). Once released, type I IFNs bind to IFN-α/β receptor (IFNAR) on viral cells resulting in the activation of JAK-STAT signaling pathway. In a sequence of molecular events induced by IFNs action, encountering enzyme production (protein kinase R, RNAse L), and protein phosphorylation, the viral and host RNA genome within the cells are destroyed, in order to reduce virus and infected cells production and replication (Bussey and Brinkmann, 2018; Guo et al., 2020; Kikkert, 2020; Li et al., 2020a, 2020b; Nazmi et al., 2014). Although, CoVs are highly sensitive to IFNARs the SARS-CoV and MERS-CoV viruses remain extremely pathogenic, possibly since the N structural proteins of CoVs employ tactics of immune escape proteins in order to serve antagonistically against host IFN response (Kikkert, 2020; Nazmi et al., 2014).

Generally, IFN induce the production and transcription of IFN-stimulated genes (ISGs) under the control of IFN-stimulated response element (ISRE), with the action of prevention and suppression of viral replication, particularly at an early stage of the infection (Prompetchara

et al., 2020). Moreover, autocrine signaling induces the secretion of hormones or autocrine messenger agents, such as IL-1, IL-2, IL-6, in the host cell, which attach to autocrine receptors leading to stimulation of genes inside the host cell itself. Alternatively, paracrine signaling promotes secretion of paracrine factors, which diffuse extracellularly, in the relatively close environment, to diffuse to nearby cells and bind to paracrine receptors, where signal transduction cascades are initiated. Mainly, there are four families of paracrine receptors and consequent signaling pathways the fibroblast growth factor family (FGF with receptor tyrosine kinase pathway and JAK-STAT pathway), Hedgehog family, Wnt family, and TGF-β superfamily. Subsequently, a second round of autocrine and paracrine signaling ensures that infected, and the surrounding uninfected cells, express a myriad of IFN-stimulated genes that establish a so-called antiviral protection state (Kikkert, 2020; Lei et al., 2018; Nazmi et al., 2014; Perlman et al., 2009; Raj et al., 2013; Sevajol et al., 2014). The strict distinction between innate and adaptive (T cells, $CD4^+$ T cells, and $CD8^+$ T cells, B cells) immune responses is probably not accurate. In the respiratory tract, several cell types and mechanisms that integrate aspects from both branches of human immunity are thought to be very important for the defence against respiratory infections. NKs, T cells, mucosal-associated invariant T cells, and neutrophils, form a bridge between the innate and adaptive machineries and play very important roles during the clearance of respiratory viruses (Kikkert, 2020).

In overall, the role of type I IFN is vital in respiratory CoV infections, especially in SARS-CoV and MERS-CoV cases with increased mortality. Both type of CoVs promote an associated disease severity by utilizing their structural proteins and ORFs of the non-structural proteins to meddle in the host cells' signaling pathways and at the same time downregulate signaling receptors, such as IFN receptors. This type of interfering leads to pulmonary and systemic inflammatory responses with elevated levels of leukocytes, including lymphocytes, neutrophils and monocytes/macrophages (Cameron et al., 2008; Channappanavar and Perlman, 2017; Kikkert, 2020; Liu et al., 2020; Qin et al., 2020; Shaw et al., 2013; Williams and Chambers, 2014; Zhang et al., 2020a; Zhang et al., 2020b, Zhao et al., 2014). SARS-CoV-2 genomic sequence presents similarities with SARS-CoV and MERS-CoV genomes, thus it is considered that upon COVID-19 infection the low levels of pro-inflammatory cytokines, leukocytes and type I IFN is related to early disease stage, however as infection severity increases cytokine storm and elevated levels of type I IFN leads to pulmonary immunophathologic syndromes, such as pneumonia and ARDS in accordance to clinical features presented in Table 1 (Li et al., 2020a, 2020b; Prompetchara et al., 2020; Yi et al., 2020). Unfortunately, a protective vaccine against CoVs that would provide essential protection against these infections has not been developed yet, even if is highly researched. Thus, effective therapeutic measures need to be researched to eliminate the virus, taking advantage of the insight on the innate immune system, at an early stage for valuable immunological responses, wherein CoVs infections can be controlled efficiently with least pulmonary immunological consequences.

3. Imiquimod as an immunostimulator

3.1. Imiquimod effect in immune system

Imiquimod ((IMQ) is a non-nucleoside heterocyclic amine which belongs to the class of 1H-imidazo-[4,5-c] quinolones (Garland, 2003; Gupta et al., 2002; Sauder, 2003; Suzuki et al., 2000). The precise mechanism of action of this synthetic molecule is unknown. However, preclinical studies revealed that imiquimod modifies the immune response by enhancing both the innate and adaptive immune system, in particular the cell-mediated pathways (Stanley, 2002). Generally, imiquimod acts as an immune response modifier, in an indirect manner, as it induces immune reactions and the secretion of many cytokines, which in turn stimulate T cells (Dahl, 2002; Gupta et al., 2002; Reiter et al., 1994). Moreover, it has been used as a potent antiviral and antitumor agent in different animal models (Dahl, 2002; Gupta et al., 2002; Reiter et al., 1994).

Innate immune system is based on the recognition of pathogens from the organism and activation of many cell types, which eliminate them. Imiquimod exerts its action in innate immune system by binding to cell surface receptors, such as toll like receptors (TLRs). There are 10 types of TLRs that recognize microorganisms or specific components derived from pathogens. Imiquimod exerts its biological action through TLR-7 and TLR-8 (Garland, 2003; Stanley, 2002; Schön and Schön, 2007; Vidal, 2006; Yoon et al., 2019). This interaction leads to activation of a signaling cascade, which promotes translocation of nuclear factor-kappa B (NF-κB). NF-κB binds to DNA and induces the expression of many pro-inflammatory cytokines from the peripheral blood mononuclear cells, such as interferon-α (IFN-α), tumor necrosis factor α (TNF-α), interleukin IL-1, IL-2, IL-6, IL-8, IL-12, granulocyte colony stimulating factor (GM-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), as well as chemokines, such as CCL4 and CCL2 (Chosidow and Dummer, 2003; Dahl, 2002; Gupta et al., 2002; Reiter et al., 1994; Stanley, 2002; Schön and Schön, 2007; Vidal, 2006). The acute antiviral and antitumor effects of imiquimod are largely originating from its ability to induce innate immune responses, especially its ability to induce secretion of IFN-α and other cytokines, such as IL-6, IL-12 and TNF-α, which has been observed in many studies (Dahl, 2002; Reiter et al., 1994; Vidal, 2006; Yoon et al., 2019). Increased expression of IL-1 and IL-6 leads to the activation of T lymphocytes and IL-12, which is produced by macrophages, induces the production of IFN-γ by natural killer (NK) and T cells. Furthermore, imiquimod activates antigen presenting cells, such as dendritic cells, macrophages activated to secrete both cytokines and nitric oxide and B lymphocytes activated to proliferate and differentiate, which in turn activate the adaptive system (Chosidow and Dummer, 2003; Dahl, 2002; Gupta et al., 2002).

Another cell type that is activated and migrates towards the side of the infection, in response to IFN-α, IFN-γ and IL-12 are Langerhans cells, which in turn induce immune responses. These cells show increased mobility in the presence of imiquimod migrating to the regional lymph node and function as major antigen presenting cells (Chosidow and Dummer, 2003; Dahl, 2002; Gupta et al., 2002). Moreover, cytokines that are produced from innate immune responses, induce indirectly the production of IFN-γ from T helper cell type 1 (Th1), which are part of the adaptive immune system. IFN-α enhances the expression of IL-12 receptor B2 subunit on Th1 cells, which in turn respond to IL-2 and become the main source of IFN-γ. Moreover, IFN-γ in combination with IL-2 produced from Th1 cells, activates CD8 cells that are converted to cytotoxic T cells, in order to eliminate cells infected from virus and to provide the immune memory, which is necessary for a future contamination (Chosidow and Dummer, 2003; Gupta et al., 2002; Stanley, 2002; Vidal, 2006; Schön and Schön, 2007; Vidal 2006, 2006; Yoon et al., 2019). Furthermore, IFN-γ and IFN-α inhibit the production of cytokines IL-4 and IL-5 from Th2 cells (Vidal, 2006; Vidal, 2006). Overall, imiquimod has a unique mode of action as it does not directly kill the infected cells, but its strong antiviral and antitumor activity is due to its ability to enhance the production of many pro-inflammatory cytokines and to trigger an immune response (Chosidow and Dummer, 2003; Dahl, 2002; Gupta et al., 2002). The role of Imiquimod to stimulate innate immunity indicates its potential to treat viral infections, such as SARS-CoV-2 in early stages of the disease, where activation of innate immunity by a TLR-7 agonist is of vital importance. However, in late stage infected patients by SARS-CoV-2 Imiquimod could provoke further up-regulation of the "cytokine storm", thus an alternative therapeutic intervention should be chosen for late stage infection, as described by Zhao et al., 2019.

3.2. Imiquimod in clinical therapy

Imiquimod (IMQ), an immune response modifier, has shown to have

antiviral and antitumor attributes. Specifically, imiquimod induces (2′–5′)-oligoadenylate synthetase, which confers an antiviral state and upregulates natural killer cells activity *in vivo* and *in vitro*. IMQ also enhances cell-mediated immunity (Miller et al., 1999). Lately Nerurkar et al., 2017, presented upregulated levels of chemokines in imiquimod treated mice presented three to five days after treatment. Fuertes et al., 2019 observed a higher IMQ efficacy for the treatment of anal condyloma compared to anal HSIL in HIV-infected individuals.

In *in vitro* studies against human peripheral blood mononuclear cells (PBMCs), imiquimod at 1–5 μg/ml induces the production of several cytokines including several subtypes of IFN-α, TNF-α, IL-1, IL-1RA, IL-6, IL-8, IL-10, IL-12 p40, granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF) and macrophage inflammatory protein 1-α (MIP-1), MIP-1β and macrophage chemotactic protein (MCP-1) (Fuertes et al., 2019; Gibson et al., 1995) and at a low drug concentration about 0,5 μg/ml is found that IFN-a and IL-1RA are the only cytokines increased (Papadavid et al., 2007). Moreover, in *in vivo* studies that IMQ was administrated orally to mice (p.o.), stimulated a dose-dependent increase in serum levels of IFN-α, being active with doses as low as 3 mg/kg (Dahl, 2002). IFN-α levels were detectable 1 h after treatment, with peak levels occurring at 2 h after treatment. Optimal dosage of imiquimod ranged between 10 and 100 mg/kg, with higher dosages (150–250 mg/kg) inducing similar secretion levels. Multi-dose regimens were also tested, separated by 2 h, showing enhanced levels of IFN-α. Multiple doses of imiquimod on the same day caused augmented IFN-α levels, however high daily doses of IMQ to mice resulted in a hypo responsive state characterized by reduced cytokine induction, while separation of the doses by four or more days caused normal levels of cytokine induction. In rats, oral administration of imiquimod, in doses 2 mg/kg or more, induced increased serum levels of IFN-α and TNF-α and the kinetics of induction were similar to those seen in mice induction (Dahl, 2002).

Very recently, a thought-provoking analysis was put forward, demonstrating the effect of IMQ against influenza A virus infection (To et al., 2019). The authors provided with proof that delivery of imiquimod in mice, directly to the lungs via intranasal administration resulted in decreased expression in viral replication, airway inflammation, leukocytes levels (i.e. inflammatory cells such as macrophages, neutrophil, eosinophil), and pro-inflammatory cytokines/chemokines (IL-6, IFN-γ, IL-1β, GCSF, CCL3, CXCL2, TNF-α)) following influenza A virus infection. The effect was actually stronger in intranasal administration compared to an epicutaneous one. The therapy resulted in a near 5-fold elevation in Type I IFN-β in the lung tissue of mice after 3 days of treatment. Moreover, imiquimod significantly suppressed the mRNA levels of IL-6, CCL3, CXCL2 and IL-1β, otherwise increased in non-IMQ treated mice, whereas yielded a significantly higher antibody response in IgG1, IgG2a, IgE, IgM and total levels of IgG during infection. Additionally, TRL7 activation by IMQ was observed through a pronounced Type I IFN prompted response. Finally, triggered response of adaptive immune system was observed by efficient activation on the response of T lymphocytes ($CD8^+$) and T helper cells ($CD4^+$). In overall, IMQ effective treatment of influenza A virus was presented by suppression of inflammatory cells, cytokines/chemokines, activation of important type I IFN, and triggered adaptive system responses, while no significant lung dysfunction was observed as presented by tissue damping of naïve in comparison to influenza infected animals (To et al., 2019). Imiquimod, a potent TLR7 agonist, could be a strong primer of the immune response to infectious pulmonary viruses, such as CoVs (Table 2).

Numerous clinical trials present imiquimod as a potent and versatile compound that can enhance cellular activity and innate immunity (Grimm et al., 2012; Kaspari et al., 2002; Kjaer et al., 1996; Kreuter et al., 2006; Mao et al., 2006; McCuaig et al., 2009; Schiffman et al., 1993). Especially, clinical trials in humans either with topical treatment or with suppositories revealed prevention of recurrences in anal canal condyloma, whereas in infants with infantile hemangioma the moderation in surface erythema was notably faster than in prior experience (Kaspari et al., 2002; McCuaig et al., 2009). Kreuter et al., 2006, further stressed the fact that application of imiquimod suppositories in intra-anal HPV Types 6 and 11 in HIV-Infected men after the surgical removal of intra-anal *Condylomata Acuminata* may yield better recurrences under the absence of sexual intercourse, while HIV-associated immunosuppression probably leads to a new increase in HPV-11 DNA load. Both Kaspari et al., 2002 and Kreuter et al., 2006 utilized 5% imiquimod (in cream Aldara) in suppositories anally. IMQ due to its antiviral and anti-inflammatory action has also been researched in its role for the treatment of HPV in the development of cervical intraepithelial neoplasia (CIN) (Kjaer et al., 1996; Mao et al., 2006; Schiffman et al., 1993). Specifically, in fifty-nine patients suffering from CIN an incremental administration was followed, in which administered dose was increased every two weeks until the maximum dosage of three vaginal suppositories (6.25 mg IMQ). CIN patients were divided in two groups one of placebo administration and the other of IMQ suppositories.66 The IMQ group presented higher histologic regression and remission in correlation to the placebo one. A 60% clearance of CIN was observed in the IMQ treated patients proving a well-established antiviral effect of imiquimod against HPV (Grimm et al., 2012).

In other trials the topical application of IMQ was researched (de Berker et al., 2017; Marks et al., 1988; Schwartz, 1996; Torres et al., 2007). Actinic keratosis, a common cutaneous, pre-cancerous neoplasm appearing as rough, dry, scaly lesions that occur primarily on chronic

Table 2
Characteristic examples of *in vivo* animal trials and clinical trials of Imiquimod administration with virus disease, such as influenza A, H1N1, H3N2 and influenza B virus.

Disease	Nr of patients	Middle age of patients (years)	Administration	Period of treatment	Outcome	Side effects/adverse events	Ref.
In vivo animal tests against influenza A virus and H1N1							
Influenza A virus	–	–	directly to the lungs via intranasal	–	decreased expression in viral replication, airway inflammation, leukocytes levels	–	To et al. (2019)
H1N1 Influenza Virus	–	–	intraperitoneal	–	stronger B cell responses to proliferate and differentiate into antigen specific IgM and IgG secreting antibodies with viral neutralizing activity	–	Li et al. (2018)
Clinical phase 2 b/3 trial with Vaccines against Influenza H1N1, H3N2 virus							
H1N1	216	18–30	topical treatment with imiquimod immediately before intradermal influenza vaccination	Single dose	Seroconversion, seroprotection, increased geometric mean titre (GMT)	Fever, headache, malaise, myalgia, arthralgia, and severe adverse events and local symptoms included redness, swelling, induration, ecchymosis, and pain	Hung et al. (2016)

ultraviolet (UV) light exposure on skin of middle-aged and elderly people is a field that researchers took interest in (de Berker et al., 2017; Marks et al., 1988; Schwartz, 1996). Torres et al., 2007 through a double-blind, placebo-controlled, randomized study in 17 patients presented an increased expression of TLR3, TLR7, and TLR8, consistent with increased expression observed in human peripheral blood mononuclear cells upon treatment with imiquimod. The induction of several members of the cytoplasmic helicase innate immune pathway, as well as several TLRs, indicates that in addition to activation of the TLR7 pathway treatment with IMQ also results in priming of other innate pathways, which may augment other aspects of the innate immune response. Moreover, it is also stated the fact that the increase of CD8β, SELL, NT5E (CD73), LGALS2 (galectin 2), and LAIR1 (leukocyte-associated immunoglobulin-like receptor 1) receptors, as well as T-cell receptor (TCR) subunits TRD and TRG, TCR-signaling pathway genes (such as Fyn, Fyb and LCP2), genes associated with T-cell activation (such as HCK, CD69, PTPRC (CD45) SELL (CD62L, L-Selectin)), ITGA4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor), and LAG3 are indicative of the stimulation of the adaptive immune system.70 Observations regarding the increase of T-cell activation were also provided in a fifty-two patient study with topical treatment and the same administration by van Seters et al., 2008.

The local anti-tumor effect of topical TLR7 agonist imiquimod 5% cream in breast cancer patients with skin metastases was investigated in a ten-patient study (de Berker et al., 2017). In this study, the variability of preexisting lymphocytic infiltrates within the cutaneous metastases and the lack of consistent quantitative changes of the infiltrate in biopsies were in contrast to the induction of a T-cell inflammatory infiltrate. The authors speculated that the effect of imiquimod may depend on the tumor microenvironment. Nevertheless, it is stated that IMQ can promote a pro-immunogenic tumor microenvironment with histological tumor regression based on the evidence of an immune-mediated response (Adams et al., 2012). Concerning Vulvar Paget Disease (VPG), there have also been attempts of imiquimod treatment (Cowan et al., 2016; Feldmeyer et al., 2011; Marchitelli et al., 2014; Sendagorta et al., 2010; van der Linden et al., 2012). van der Linden et al., 2012, have already performed a twenty patient study following treatment with 5% IMQ, topically, three times per week over a 16-week period. Other researchers and physicians have presented either case reports, where IMQ 5% was administered topically in 3 patients daily for 3 weeks and every other day for 3 following weeks (Sendagorta et al., 2010) or one patient 3 times per week (Feldmeyer et al., 2011) or greater in groups for every other day in 10 patients (Marchitelli et al., 2014) or 3 times per week in 8 patients (Cowan et al., 2016). A high clinical and histologic remission of the disease was achieved in high occurrence (75% in Cowan et al., 2016, 90% in Marchiteli et al., 2014), whereas Feldmeyer et al., 2011 confirmed the findings in his study as well. The above mentioned studies summarize the potency of IMQ in the handling of VPG.

There have also been interesting attempts to utilize IMQ as a vaccine adjuvant. Immunization of guinea-pigs with Herpes Simple Virus glycoprotein and imiquimod, reduced effectively virus recurrence in comparison with unimmunized controls (Gibson et al., 2002). In particular, even though the mechanism of action was not identified, the results indicated that the use of IMQ as an enhancer significantly diminished recurrent lesion days by 53–69% (Gibson et al., 2002). The effect of IMQ has also been examined against Influenza Virus, where Li et al., 2018 focused on the combination of imiquimod with H1N1/415742Md influenza virus particle. Specifically, a compound of IMQ with the virus (50 μg/10 μg, respectively) were intra-peritoneal administered in mice, whereas other treatment schemes were utilized as controls (such as, IMQ only group and H1N1 influenza only group). An upregulation of cytokines expressions was noted in both Th-1, Th-2 cytokines with elevated IL-10 secretion levels induced in the IMQ only group, while in the virus only group cytokine storm with increased levels of pro-inflammatory cytokines (IL-6, IFN-γ, IL-2, IL-4 and IL-5) was observed. The outcome revealed that the combination (IMQ and virus group) induced much stronger B cell responses to proliferate and differentiate into antigen specific IgM and IgG secreting antibodies with viral neutralizing activity. The main result was that imiquimod integrated with vaccine antigen can advance potent B cell activation and differentiation leading to accelerated viral specific antibody production, which contribute to the protection against imminent incoming pathogen (Li et al., 2018). In an another novel research, imiquimod has been administrated systemically in order to be tested as an adjuvant that improves immunogenicity of a tumor-lysate vaccine, inducing the rejection of a highly aggressive T-cell lymphoma (Papakostas, 2015). The results indicate that Tumor cell lysate vaccination using imiquimod as an adjuvant, enhanced the protection from tumor growth and induced a Th1-type as well as humoral immune responses against LBC cells. The research concluded that imiquimod administered alone significantly enhanced immune response to a tumor lysate vaccine and produced an elevated number of $CD4^+$ T-cells and an IFN-γ Th1-type response along with specific antibodies (Papakostas, 2015).

Imiquimod has been evaluated in controlled phase 2 b/3 clinical trial as a topical pre-treatment agent in combination with trivalent influenza vaccination against influenza B, H1N1 and H3N2 viruses (Hung et al., 2016). By this study, improved protection against circulating strains of influenza viruses was provided with effective immunogenicity of influenza vaccination due to the topical imiquimod pretreatment before intradermal vaccination.

3.3. Imiquimod oral availability

Imiquimod, a TLR7/8 agonist that induces a potent anti-viral response, is characterized by the production of type I interferons (IFN), pro-inflammatory cytokines and chemokines (Gibson et al., 2002). A growing literature clearly points a systemic response following topical imiquimod treatment. The tissue response to Aldara treatment across a detailed time-course model determined the most likely mechanism driving systemic inflammation (Nerurkar et al., 2017a, 2017b). It has been shown that topical Aldara treatment induced a potent chemokine and cytokine response throughout the peripheral tissues and brain, with these responses being temporally distinct (Nerurkar et al., 2017a,b). IMQ binded to TLR7 on inflammatory cells, such as the Langerhans cells of the epidermis, dendritic cells and monocytes and induced cytokines secretion (IFN-α and TNF-α) (Papakostas, 2015). Specifically, the results also presented that IMQ was present in both plasma and brain as early as 4 h after treatment, suggesting that the primary mechanism of immune activation of topical Aldara treatment was through the direct ligation of IMQ with TLR7 receptors throughout the body (Nerurkar et al., 2017a,b). IMQ treatment could induce unintended medium grade systemic side effects that ranged in severity and frequency, including fever, fatigue, headaches, erythema, myalgia, application site inconvenience and raised erythrocyte sedimentation rate (Del Rosso et al., 2009; Gollnick et al., 2020; Kumar and Narang, 2011; Schwartz, 1996).

According to a clinical pharmacokinetic report conducted in order to assess the effect of food on the oral IMQ absorption, to characterize its pharmacokinetics, and to estimate its oral bioavailability on individuals that received a 100 mg oral dose of IMQ, the oral bioavailability was near 47%, with an absorption half-life of close to 1 h and independent of food consumption (Soria et al., 2000). The study suggested that food provided no effect on the rate, extent of absorption or bioavailability of oral imiquimod, and proved the suitability of IMQ for oral administration (Soria et al., 2000). In a phase I clinical study, tolerability, toxicity and biological effects of daily oral imiquimod administration were investigated in 21 patients with refractory cancer.88 Patients were treated with doses of 25 mg, 50 mg, 100 mg or 200 mg on a projected 112 day course, in which only three patients completed the course, all at the 50 mg dose. Treatment toxicities were dose related and mainly comprised flu-like symptoms, nausea and lymphopenia. Interferon production was not demonstrated within the first 24 h of the initial dose

but, following repeated doses, ten of the patients developed detectable serum interferon concentrations with a maximum value of 5600 IU ml recorded. Daily oral administration of imiquimod presented dose-depented activation of the interferon production system but at higher doses resulted in flu-like side effect (Savage et al., 1996).

4. Conclusions

In coronavirus influenzas the ability of the viruses to escape immune surveillance may lead to increased pathogenicity, as an outcome of lymphocytopenia and cytokine storm syndrome. The severity of all types of CoVs is highly related to elevated levels of leukocytes, pro-inflammatory cytokines and desregulation of type I IFN. TLR-7 and 8 play a significant role and in cooperation with PRRs represent the first line of defence against viruse infections. The RNA viruses after infection of host cells trigger signaling cascades resulting in the upregulated secretion of pro-iflammatory mediatora and Type I IFN, which render cells' resistance to CoVs infections. Imiquimod (IMQ) is an immune response modifier that induces immune reactions and the secretion of cytokines reulting in antiviral and antiinflammatory responses. Imiquimod exerts its action in innate immune system by binding to cell surface receptors, especially TLR-7 and TLR-8. The action of Imiquimod to stimulate innate immunity indicates its potential to treat viral infections. We have clear evidence that Imiquimod is able to offer satisfactory stimulation of innate and acquired immunity, helping the elimination of SARS-CoV-2, at least during the early phases of infection.

Author contribution

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Declaration of competing interest

All the authors declare no conflicts of interest.

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