Immunogenic versus tolerogenic mRNA Vaccines

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Inhoud

abstract

Vaccinology has seen unprecedented growth, especially in response to the COVID-19 pandemic, marked by the emergence of mRNA-based vaccines. This paper describes mRNA vaccine differences, focusing on the COVID-19 BNT162b2 vaccine from Pfizer/BioNTech and an mRNA-based Multiple Sclerosis (MS) vaccine in preclinical trials. Despite sharing the same mRNA platform and incorporating N1-methyl-pseudouridine (m1Ψ), both vaccines elicit opposite immune responses – the former inducing immunogenicity and the latter promoting tolerance. In this review, we will compare the vaccines in order to find an answer to the question of what causes their opposite immune reactions.

In the MS vaccine, tested in an autoimmune encephalomyelitis (EAE) mouse model representing MS, the vaccine is administered intravenously. The vaccine contains m1Ψ mRNA encoding myelin-derived antigens to induce peripheral tolerance by reducing effector T cells while promoting regulatory T cells. The formulation involves lipid nanoparticles (LPX) and a plasmid backbone with specific elements enhancing stability and translational efficiency.

In contrast, the COVID-19 vaccine, administered intramuscularly, stimulates an immunogenic response against the SARS-CoV-2 spike protein. This vaccine also contains m1Ψ mRNA and LPX, although its formulation differs in lipid composition. While both vaccines contain cholesterol they, contain different cationic lipids. Another difference is that whereas both vaccines use a T7 RNA polymerase for mRNA production, the COVID-19 vaccine presents a segmented poly(A) tail, while the MS vaccine does not. Some of these differences might explain the opposite immune reaction exerted by the two vaccines.

However, the main critical distinction seems to arise in mRNA purification methods. The MS vaccine rigorously removes double-stranded mRNA using HPLC or cellulose, confirmed by J2 dsRNA-specific antibody. In contrast, the COVID-19 vaccine seems to lack this extra purification step for double-stranded RNA removal, potentially influencing its immune response since double-stranded RNA is a well-known ligand for pattern recognition receptor signalling. Thus, the surprising finding of this thesis is that the immunogenic reaction of the COVID-19 vaccine might be at least partly attributable to a contamination with double-stranded RNA, whereas the tolerogenic reaction to the MS vaccine is explained the absence of this.

Introduction

Vaccinology is a booming field that has advanced due to the COVID-19 pandemic with the development of mRNA-based vaccines. Since the pandemic, there have been major developments in vaccine development for SARS-CoV-2, Cancer and Multiple sclerosis (MS). While the EMA and FDA have already admitted the mRNA-based vaccines from Pfizer/BioNTech and Moderna against SARS-CoV-2, various mRNA-based cancer vaccines are in phase 1 clinical trials (Rojas et al., 2023; Sahin et al., 2020). The COVID-19 BNT162b2 vaccine from Pfizer/BioNTech is the first vaccine that contains synthetic mRNA and is officially approved by several drug administration authorities worldwide (Nance & Meier, 2021).

Moreover, a tolerogenic MS vaccine is under investigation in an autoimmune encephalomyelitis (EAE) mouse model (Krienke et al., 2021). This MS vaccine by Krienke et al. is based on the same mRNA platform as the COVID-19 BNT162b2 vaccine, even though this vaccine should induce the opposite immune response. Thus while the COVID-19 BNT162b2 vaccine from Pfizer-BioNTech is based on the same technology platform as the MS vaccine from Krienke et al., they elicit a different response in the body. The COVID-19 vaccine elicits an immunogenic response in the body, and the MS vaccine elicits a tolerogenic response.

While mRNA vaccine research has been ongoing for several decades, the COVID-19 pandemic propelled the development of mRNA vaccines. mRNA is an essential player in the cell since it is the code for the production of proteins by the ribosome (Franco & Koutmou, 2022). mRNA is the intermediate code between DNA and proteins (Franco & Koutmou, 2022). mRNAs can also be used to make proteins that have a therapeutic or prophylactic effect, such as in vaccines (Sahin et al., 2014). The vaccine platform is based on bringing mRNA coding for an antigen into an antigen-presenting cell (APC) and letting the cell bring it to expression to elicit an immune response (Miao et al., 2021). mRNA vaccines are a promising platform because protein expression is higher than in DNA vaccines, and RNA cannot be inserted in our genome sequence and thus cannot cause insertion mutations (Hargadon et al., 2018; Miao et al., 2021). Another benefit of mRNA vaccines is that they are rapidly produced, and their production is less expensive than other vaccines (Pardi et al., 2018). The most significant advantage of mRNA vaccines is that mRNA can encode almost any (auto) antigen as long as they are protein-based, making it suitable for every disease from which we know the antigen sequence (Krienke et al., 2021).

An immunogenic mRNA-based vaccine typically consists of three components: the synthetic mRNA molecules that code for the antigens, the carrier for mRNA delivery, and an adjuvant (Xie et al., 2023). Adjuvants are in the vaccine to activate the immune system, thereby increasing its efficacy and preventing tolerogenic responses (Xie et al., 2023). There are in principle three different categories of mRNA vaccine adjuvants in mRNA vaccines possible: 1) the RNA itself, 2) inflammatory components of the carrier, and 3) exogenous immunostimulatory molecules (Xie et al., 2023). mRNA with self-adjuvant characteristics encoded in its nucleotide sequence without modified uridine can activate inflammatory cells, for example via Toll-like receptor (TLR)-3/7 signalling (Karikó et al., 2005). Inflammatory components of the delivery system can also cause production of inflammatory signalling, which can also signal via TLR pathways (Xie et al., 2023). Different lipids or lipid-like materials can function as inflammatory components of the delivery system of mRNA (Han et al., 2023; Zhang et al., 2021). Examples of exogenous adjuvants are, for instance, bacterial products or Freund's adjuvants (Bolhassani et al., 2017).

The COVID-19 vaccine from Pfizer/BioNTech and Moderna does not contain an exogenous adjuvant. However, it has not become clear from the scientific literature why this vaccine triggers an immunogenic response, thus which component is responsible for the immunostimulatory activity.

Because of the speed at which the COVID-19 vaccine was developed and produced, there could be a possibility that there are things overlooked in the development of the vaccine. Therefore it is interesting to detect the critical difference between the widely-used COVID-19 vaccine and the newly developed tolerogenic MS vaccines as this could shed light on the mechanism of action of the COVID-19 vaccine.

This leads to the question of this thesis: What is the difference between the COVID-19 BNT162b2 vaccine from Pfizer-BioNTech and the MS vaccine from Krienke et al., which is currently under preclinical investigation in mouse models of MS since both vaccines elicit an opposite immune response?

This paper is focused on these vaccines because they are developed by the same principal investigators (Uğur Şahin and Özlem Türeci). These researchers contributed to the MS vaccine and are also the co-founders of the BioNTech company, which, together with Pfizer, developed the BNT162b2 COVID-19 vaccine.

Thus, this thesis will describe the MS vaccine from Krienke et al. and the Pfizer-BioNTech BNT162b2 COVID-19 vaccine and aims to provide an overview of both vaccines and their differences, which might be responsible for their opposite immune responses in the body.

Comparative analysis

MS vaccine

First, the vaccine against autoimmune encephalomyelitis (EAE) will be described. This vaccine is being investigated in mouse models with an established model for the autoimmune disease MS. MS is an inflammatory disease in which T and B cells are present in demyelinating plaques (Dobson & Giovannoni, 2019). These T and B cells induce the death of oligodendrocytes, causing demyelination of neurons. The vaccine aims to induce tolerance thereby resulting in reduced levels of effector T cells, and increased levels of regulatory T (Treg) cells that control the autoimmune disease (Krienke et al., 2021). These Treg cells control the antigen-specific immune response towards tolerance (Wildner & Selmaj, 2017). Thereby, this vaccine aims to induce a peripheral tolerance reaction against myelin-derived sequences to stop the disease or slow its progression (Krienke et al., 2021).

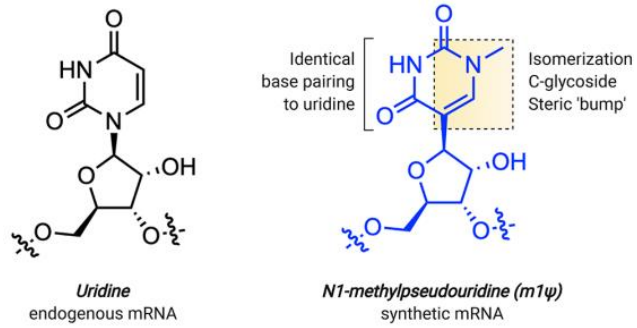

Figure 1 shows Uridine and N1-methyl pseudouridine structures taken and adapted from (Nance & Meier, 2021, p. 2).

Krienke et al. made a nanoparticle-formulated mRNA-lipoplex formulation (mRNA-LPX). The different papers described in this paper use different acronyms (mainly LPN and LPX) to indicate lipid nanoparticles, but in this thesis, we use the term LPX for consistency.

In the RNA, they replaced uridine with non-immunogenic 1-Methylpseudouridine (m1Ψ) (Krienke et al., 2021). The difference in structure between normal uridine and m1Ψ is shown in Figure 1. For comparison, they also made another nanoparticle-formulated mRNA-LPX consisting of immunogenic unmodified (U) RNA (Krienke et al., 2021). This thesis focuses on the formulation with m1Ψ mRNA since this is the non-immunogenic formulation, and this m1Ψ was also used in the COVID-19 vaccine.

The vaccine contains m1Ψ-modified mRNA that encodes MOG_{35-55} (Krienke et al., 2021). MOG_{35-55} is the myelin oligodendrocyte glycoprotein, the immunogenic epitope in the EAE mouse model representing MS (Bittner et al., 2014). Mice treated with the m1Ψ mRNA showed substantially reduced secretion of inflammatory (IFN-α) mediators and less activation of different immune cells, including antigen-presenting cells (APCs), natural killer cells (NK) and B cells, compared to mice injected with unmodified U mRNA (Krienke et al., 2021). This indicates that the m1Ψ mRNA is not immunogenic in these experiments.

In the experiments, the vaccines containing 20 µg MOG35-55 m1Ψ-modified, 20 µg irrelevant m1Ψ-modified mRNA or saline were administered intravenously (IV) (Krienke et al., 2021). The mice were treated with this on days 7 and 10 or at an EAE score of 1-2 after disease induction, and were injected twice per week after this (Krienke et al., 2021). The EAE score is a measure of the severity of disease severity (Shahi et al., 2019). In addition to the MOG_{35-55} epitopes, experiments were also conducted with a multiepitope vaccine containing 40 µg multiple myelin-derived sequences (Krienke et al., 2021). The multiepitope vaccine consisted of different myelin-derived sequences: MOG_{35-55}, $PLP_{139-151}$, $PLP_{178-191}$ and MBP_{84-104}, $OVA_{25-7-264}$ (Krienke et al., 2021). Mice were injected with 200 µl mRNA-LPX

containing 20 µg MOG_{35-55} m1Ψ mRNA (Krienke et al., 2021). Mice were injected IV into the retrobulbar venous plexus or tail vein (Krienke et al., 2021).

The authors studied the treatment of MOG_{35-55} m1Ψ mRNA in EAE mice, and the clinical signs of EAE were stopped after this treatment, which indicates that mice were cured (Krienke et al., 2021). After administration with MOG_{35-55} m1Ψ mRNA, there was a high cell count of MOG_{35-55} specific Treg cells in the spleen (Krienke et al., 2021). These findings suggest that the vaccine can induce tolerance against MOG_{35-55}, since Treg cells are important for tolerance reaction and thereby, can control the disease (Sage & Sharpe, 2016).

We will now go into further detail about the formulation of the MS vaccine to be able to determine if there are critical differences with the COVID-19 vaccine.

The mRNA for the vaccine was cloned into the multiple-cloning site (MCS) of the pST1-hAg-MCS-FI-A30LA70 plasmid (Krienke et al., 2021). As specified in the paper from Krienke et al. they made a plasmid backbone that consists of the following elements, which are shown in Figure 2:

- Human alpha globin 5'-untranslated region (UTR) (hAg).
- A 3'-UTR consisting of FI element and a poly(A) tail of 100 nucleotides, which was interrupted by a short linker after 30 nucleotides, placed before the poly(A) tail. This FI element is a patented amino-terminal enhancer sequence, which leads to increased stability and translational efficiency (Orlandini Von Niessen et al., 2017).
- Coding sequences of antigen-encoding templates (MOG35-55, PLP139-151, PLP178-191 and MBP84-104, OVA257-264 (SIINFEKL) constructs). The coding sequences were flanked upstream by mmsec and downstream by mmMITD sequences (pST1-hAg-mmsec(opt)-MCS-mmMITD(opt)-A30LA70). These are a leader peptide and a MHC trafficking signal (MITD). An MHC trafficking signal guides the antigen on MHC class II to present it to T cells (Kreiter et al., 2008). These were added because a leader peptide and MHC trafficking signal improve antigen presentation by APCs ((Kreiter et al., 2008).

In addition, myelin epitopes were extended with seven amino acids on the C-terminus and the N-terminus to ensure proper antigen processing (Krienke et al., 2021). For example the peptide sequence of MOG flanked by seven amino acids on both sides thus was MOG27-63: SPG KNA TGM EVG WYR SPFS RVV HLY RNG KDQ DAE AQP (Krienke et al., 2021).

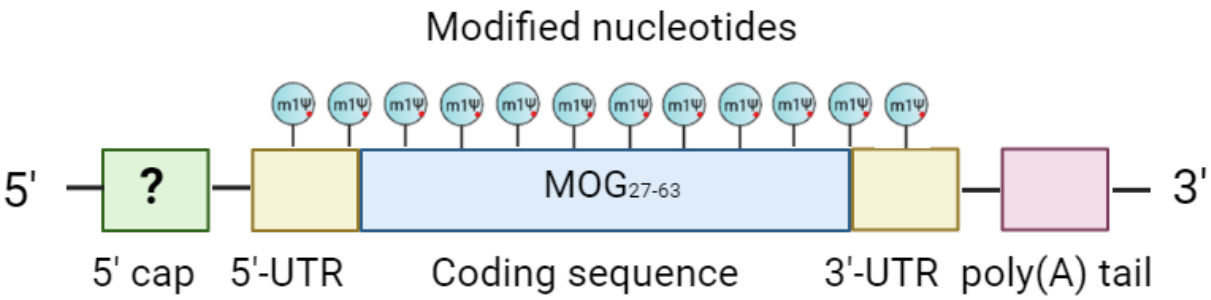

Figure 2, the different elements in the MS vaccine from Krienke et al., Created with BioRender.com

The researchers linearised the pST1-hAg-MCS-FI-A30LA70 plasmids with EciI, SapI, or BpiI restriction enzymes (Kreiter et al., 2008). this was done to produce mRNA transcripts of a defined length. The researchers have already used this method for linearisation as described in an earlier publication from Kreiter et al. (Kreiter et al., 2008). After linearisation of the plasmid, in vitro transcription was performed after purification with phenol-chloroform extraction and sodium acetate precipitation (Kreiter et al., 2008). A commercially available kit (the mMESSAGE mMachine Ultra T7 kit) from Ambion was used for in vitro transcription (Kreiter et al., 2008). After the transcription process, the

RNA concentration and quality were assessed by spectrophotometry and agarose/formaldehyde gel electrophoresis (Kreiter et al., 2008).

To make the RNA non-immunogenic, the researchers used m1Ψ instead of the normal uridine during in vitro transcription (Krienke et al., 2021). After the transcription, the mRNA was purified using high-performance liquid chromatography (HPLC) or cellulose (Krienke et al., 2021). The single-stranded mRNA was purified because double-stranded mRNA binds with higher affinity to cellulose in an ethanol-containing buffer (Baiersdörfer et al., 2019). This method has been proven to remove 90% of the double-stranded mRNA (Baiersdörfer et al., 2019). This was confirmed via a dot blot analysis using a J2 dsRNA-specific antibody (Baiersdörfer et al., 2019). The literature describes that both methods are equally effective in removing double-stranded mRNA because both methods showed similar J2 antibody signal intensities (Baiersdörfer et al., 2019). To prove that the mRNA was successfully purified and double-stranded mRNA was removed, Krienke et al., used the antibody mAb J2 (Krienke et al., 2021). mAb J2 is a monoclonal antibody that can recognise double-stranded mRNA in the presence of nucleoside modifications such as m1Ψ (Karikó et al., 2011).

mRNA-LPX particles were made, and this was done in sterile and RNAse-free conditions (Krienke et al., 2021). The mRNA-LPX consisted of mRNA together with the cationic lipids: 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) from Merck & Cie or 1,2 Dioleoyl 3 Trimethylammoniopropane (DOTAP) from Merck Eprova, and also helper lipid 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) from Avanti Polar Lipids or Corden Pharma and cholesterol from Sigma-Aldrich (Kranz et al., 2016). mRNA-LPX particles are lipid carriers that protect the mRNA from extracellular ribonucleases, which can degrade the mRNA (Kranz et al., 2016). The mRNA-LPX is also beneficial for efficient uptake and expression of the mRNA by antigen-presenting cells, which is proven by a luciferase assay (Kranz et al., 2016).

Covid vaccine

The vaccine against SARS-CoV-2 BNT162b2 will be discussed in this paragraph. In contrast to the MS vaccine, the BNT162b2 vaccine from Pfizer/BioNTech has already been approved by the EMA and FDA. Like the MS vaccine, the COVID-19 vaccine is also a mRNA-based vaccine, and the COVID-19 vaccine is the first synthetic mRNA vaccine that has been admitted by the FDA (Nance & Meier, 2021). The COVID-19 vaccine codes for the SARS-CoV-2 spike protein that plays an essential role in receptor recognition of the virus and membrane fusion processes (Huang et al., 2020). In the spike protein in the vaccine, there are two mutations introduced (K986P and V987P) which ensure that the spike protein is in the prefusion conformation (Hsieh et al., 2020; Huang et al., 2020; Nance & Meier, 2021; Vogel et al., 2021). This means that the spike protein stays in the folded conformation prior to binding to the ACE2 receptor on host cells, so that antibodies will be produced that recognize the viral particle prior to infection (Lan et al., 2020). The effect of the vaccine is immunogenic, thus APCs present the spike protein to T cells after vaccination, which causes the production of antibodies and T cells against this spike protein (Nance & Meier, 2021). In this way, infection can be prevented by neutralizing antibodies, and severe diseases can be prevented by cellular immune responses.

The COVID-19 vaccine is based on a mRNA platform similar to the MS vaccine (Nance & Meier, 2021). The other components of the vaccine are water, salt, sugar and lipids (Nance & Meier, 2021). The lipid component of the COVID-19 vaccine is mRNA-LPX composed of optimised ionisable cationic lipids (Kulkarni et al., 2018; Nance & Meier, 2021). The exact lipid composition is composed of 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC); ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) (ALC-0315); 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159) and cholesterol (Comirnaty | European Medicines Agency). As in the MS vaccine, they thus also used LPX particles in the COVID-19 vaccine, but they are composed of different lipids.

Another feature of the COVID-19 vaccine is the replacement of natural uridine with m1Ψ (Nance & Meier, 2021). Replacement with m1Ψ in the COVID-19 vaccine is used to increase the production of proteins and for immune evasion (Nance & Meier, 2021). In addition, m1Ψ increases translation and half-life by altered interaction with the ribosome (Svitkin et al., 2017). The usage of m1Ψ is another commonality of the COVID-19 vaccine with the MS vaccine. Both vaccines try to enhance protein expression via the usage of m1Ψ-modified mRNA. While the use of m1Ψ mRNA is common between both vaccines, there is also a difference in the usage of this m1Ψ mRNA, since Krienke et al. indicate this as an essential part for inducing tolerogenic responses. However in the COVID-19 vaccine, the same m1Ψ mRNA is used, but it still elicits an immunogenic response in this vaccine.

The mRNA of the COVID-19 vaccine is a linear sequence of 4284 nucleotides in length (Nance & Meier, 2021). As described in the paper of Nance & Meier. the vaccine consists of 5 different elements with different functions, which are shown in Figure 3:

- A 5'-cap (m7(3'OMeG)(5')ppp(5')(2'OMeA)pG, commonly referred to as trinucleotide Cap 1 (Nance & Meier, 2021). This part has two functions: the protection of RNA and the recruitment of the ribosome for translation to proteins (Henderson et al., 2021). In the paper from Krienke et al. the 5'-cap is not described and it is not stated if the MS vaccine contains a Cap1.
- A 5'-UTR derived from the human α-globin mRNA with an optimised Kozak sequence (Nance & Meier, 2021). A Kozak sequence is a sequence that is the initiation site for protein translation (Kozak, 1989). Like the MS vaccine, the COVID-19 vaccine also has a 5'-UTR derived from the human α-globin.
- A codon-optimized coding sequence that specifies production of the transmembrane-anchored immunogenic SARS-CoV-2 spike glycoprotein (Nance & Meier, 2021). It is not stated for which codon optimisation is used, but according to the literature, it can increase protein expression (Mauro & Chappell, 2014).
- A 3'-UTR consisting of two sequences derived from the amino-terminal enhancer of split mRNA and the mitochondrial encoded 12S rRNA (Nance & Meier, 2021). This part of the sequence plays an essential role in stabilising the mRNA and, therefore, is beneficial for protein expression (Orlandini von Niessen et al., 2019). While the COVID-19 vaccine thus contains two sequences that stabilise the mRNA, the MS vaccine consists only of a 3'-UTR of FI element.
- An unusual 3'-terminus consisting of two segmented poly(A) tracts (Nance & Meier, 2021). A segmented poly (A) tail consists of at least two A-containing elements, each defined as a nucleotide sequence consisting of 40–60 adenosines, separated by a spacer element of different length (Trepotec et al., 2019). This part takes care of a reduction in recombination of DNA during the production of the plasmid, while it does not affect mRNA translation (Trepotec et al., 2019). Researchers made plasmids with different segmented poly (A) tails (Trepotec et al., 2019). They performed a capillary gel electrophoresis to measure the size of the poly (A) tails (fragments), and they observed less recombination with fragmented poly (A) tails (Trepotec et al., 2019). The COVID-19 vaccine and the MS vaccine both contain a poly (A) tail, but in case of the COVID-19 vaccine this poly (A) tail is segmented.

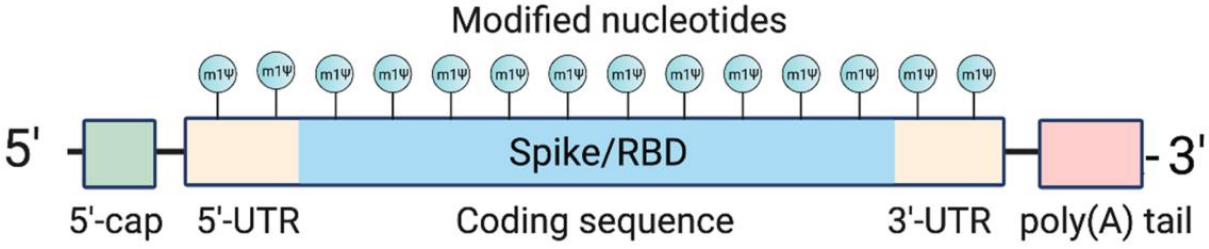

Figure 3 shows the different elements in the COVID-19 vaccine from Pfizer/BioNTech, taken from (Nance & Meier, 2021, p. 3).

Synthetic DNA fragments containing the COVID-19 sequence were used to produce the plasmids (Nance & Meier, 2021). In the plasmid, the COVID-19 sequence is placed behind a T7 RNA polymerase promotor (Nance & Meier, 2021). A T7 RNA polymerase is incubated with the plasmids containing the COVID-19 sequence and nucleotide triphosphates (NTPs) to produce mRNA (Nance & Meier, 2021). This is another similarity between both vaccines since in both cases a T7 RNA polymerase is used to produce mRNA. While it is not described in detail how the mRNA is linearised like in the MS vaccine, it is stated that the COVID-19 vaccine contains an enzymatically made linear sequence (Nance & Meier, 2021). T7 RNA polymerase is used because this polymerase is error-free and is able to transcribe long RNAs (CHAMBERLIN et al., 1970; Chamberlin et al., 1983). An even more important characteristic of the T7 RNA polymerase is that it is able to incorporate modified nucleotides such as m1Ψ (Goldberg & Rabinowitz, 1961).

The researchers state that every natural uridine (also in the UTRs) are replaced with m1Ψ in the COVID-19 vaccine, but it is not stated how this is confirmed (Nance & Meier, 2021). Although the researchers only added m1Ψ, there should be no normal uridine in the mRNA, but it could be tested if the mRNA only contains m1Ψ via mass spectrometry.

The vaccine was injected into mouse muscle for preclinical trials of the COVID-19 vaccine (Nance & Meier, 2021). In these preclinical trials, mice were injected intramuscular (IM) once with 0.2, 1 or 5 µg of the COVID-19 vaccine (Khehra et al., 2021). This is a much lower (10-100 fold) dose compared to the mouse trials with the tolerogenic MS vaccine. Later on, clinical trials in humans older than 16 were performed (Frenck et al., 2021; Polack et al., 2020). In these clinical trials, participants received an IM injection of 30 µg BNT162b2 vaccine (Frenck et al., 2021; Polack et al., 2020). The administration of the vaccine in mouse models thus differs from the administration of the MS vaccine, which was IV administered. It is impossible to compare the vaccines in humans since the MS vaccine has not been tested in humans yet.

It is not stated whether double-stranded regions were removed during the production of the COVID-19 vaccine, while this was the case with the MS vaccine. What is clear from the literature is that using modified nucleotides such as m1Ψ causes a substantial reduction in double-stranded secondary structures in the mRNA (Nance & Meier, 2021). in addition the segmented poly-A tail increases stability of the mRNA and thereby possibly reducing doubled stranded secondary structures in the mRNA.

Discussion

Because of the COVID-19 pandemic, the field of research about mRNA vaccines has become very popular. Besides the immunogenic mRNA vaccines such as the BNT162b2 COVID-19 vaccine from Pfizer/BioNTech, mRNA has other potential applications, such as the tolerogenic MS vaccine.

This paper aimed to describe both vaccines in detail so that it is possible to compare the vaccines. Although the vaccines have many similarities, some differences could explain the opposite response. The adjuvant activity in the BTN162b2 vaccine is not well understood. However, somewhere in the vaccines, there must be a critical difference, either in the nanoparticles, the RNA-folding, or the UTRs, that underly the opposite immune responses.

Both vaccines are based on an mRNA platform and contain m1Ψ mRNA in which N1-methyl-pseudouridine replaces all the uridine nucleotides. The research groups from both papers describe that m1Ψ is non-immunogenic, so it is notable that there is still an immunogenic reaction in the COVID-19 vaccine, although they also incorporated this m1Ψ (Krienke et al., 2021; Nance & Meier, 2021). For the production of both vaccines, essentially the same method to construct their plasmids for the production of mRNA was used. For both vaccines, a T7 RNA polymerase, which is suitable for incorporating m1Ψ modified RNA, was used.

There are some differences between both vaccines. For instance, the MS vaccine is administered IV, while the COVID-19 vaccine is administered IM. Moreover the amount of vaccine given in mouse models is very different: 10-100 fold higher for the MS vaccine. However these differences are unlikely to cause the differences in immune response. In anything, the systemic IV administration of a far higher doses of vaccine would be expected to increase the immunogenicity and not induce tolerance as observed with the MS vaccine. This is supported by other studies with immunogenic mRNA-based vaccines, for example other groups are developing anti-cancer vaccines that are administered IV and generate an immunogenic response (Baharom et al., 2022).

Also, both vaccines consist of LPX particles. Although both vaccines contain a lipid part, different lipids form the lipid nanoparticle. Both vaccines contain cholesterol but the other lipids are different between the vaccines. As lipids are well-known substrates of pattern recognition receptors, especially TLRs and scavenger receptors these might contribute to the different immunogenicity of the vaccines.

Whereas both vaccines have a 5’-UTR that consists of hAg, there could be differences in the 5’ end. The paper from Nance et al. states that there is a 5’-cap with a Cap1 that protects the RNA and recruits the ribosome for translation. The paper from Krienke et al. does not describe the 5’-cap. However, it is very likely that they also used a 5’-cap as its absence would lower the mRNA stability, decrease antigen production, and increase its immunogenicity, whereas this vaccine is aimed at inducing high levels of antigen production and inducing tolerance.

At the other end of the mRNA, both vaccines contain a poly (A) tail, but in the COVID-19 vaccine, this tail is segmented, while this is not the case in the MS vaccine. It does not directly become obvious from literature but this could be the critical difference causing an opposite immune response.

Both vaccines are linear. Both vaccines are linearly made in an enzymatic way. However, while it is clearly stated that the EciI, SapI, or BpiI restriction enzymes are used to linearise the plasmid in the MS study, this is not stated in the COVID-19 paper. However, the specific enzymes used are unlikely to cause the opposite immune response of the vaccines.

However, there is one major difference between both vaccines that stands out. The MS study from Krienke et al. specifically described that they purified the mRNA by HPLC or cellulose. This was done to remove double-stranded mRNA from their vaccine. The researchers even described that they confirmed with an antibody that no double-stranded mRNA was present. For the COVID-19 vaccine, the researchers do not state that they screened for double-stranded mRNA. The researchers only described that the use of m1Ψ is beneficial to reduce double-stranded mRNA substantially, but this is not a guarantee that there is no double-stranded RNA present, especially since the MS vaccine also contains this modification. As dsRNA is a ligand to pattern recognition receptors like TLR3 and TLR7, it is thus possible that the immunogenicity of the COVID-19 vaccine is at least partly caused by the presence of double-stranded RNA.

Thus, although it cannot be excluded that differences in the lipid composition, the 5'-cap and the 3'-UTR are responsible for the opposite immune response induced by the MS and COVID-19 vaccines, it might also be that this is caused by a contamination with double-stranded RNA in the COVID-19 vaccine. To address this the COVID-19 vaccine should be purified with HPLC and confirmed that double-stranded mRNA is not present with J2 dsRNA-specific antibody. In addition, it would be interesting to perform mouse experiments with the MS vaccine where this purification step is omitted, and with making the composition of the lipid composition, the 5'-cap and the 3'-UTR identical to that used in the COVID-19 vaccine.

If a contamination with double-stranded RNA is responsible for the immunogenicity of the COVID-19 vaccine, this would have far-reaching consequences, as it means that a key functional component of the COVID-19 vaccine (i.e. the adjuvant) is in fact an impurity that is neither defined nor registered at the FDA/EMA. However, this conclusion should be considered with caution, as, based on the published information, there is too little evidence to conclude if this is really the cause for the different immune responses.

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