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Infection, Genetics and Evolution 85 (2020) 104489

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Immunoinformatic construction of an adenovirus-based modular vaccine platform and its application in the design of a SARS-CoV-2 vaccine

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ARTICLE INFO

Keywords:
Immunoinformatics
SARS-CoV-2 vaccine
Epitope display
HAdV vectors
Immunomodulatory prediction
Vaccine strategy

ABSTRACT

The current SARS-CoV-2 pandemic has imposed new challenges and demands for health systems, especially in the development of new vaccine strategies. Vaccines for many pathogens were developed based on the display of foreign epitopes in the variable regions of the human adenovirus (HAdV) major capsid proteins (hexon, penton and fiber). The humoral immune response against the HAdV major capsid proteins was demonstrated to play a role in the development of an immune response against the epitopes in display. Through the immunoinformatic profiling of the major capsid proteins of HAdVs from different species, we developed a modular concept that can be used in the development of vaccines based on HAdV vectors. Our data suggests that different immunomodulatory potentials can be observed in the conserved regions, present in the hexon and penton proteins, from different species. Using this modular approach, we developed a HAdV-5 based vaccine strategy for SARS-CoV-2, constructed through the display of SARS-CoV-2 epitopes indicated by our prediction analysis as immunologically relevant. The sequences of the HAdV vector major capsid proteins were also edited to enhance the IFN-gamma induction and antigen presenting cells activation. This is the first study proposing a modular HAdV platform developed to aid the design of new vaccines by inducing an immune response more suited for the epitopes in display.

1. Introduction

In the midst of the new SARS-CoV-2 pandemic (WHO, 2020; Wu et al., 2020), studies focusing on the design of rapid and efficient vaccine strategies are fundamental as a response to control the disease. The SARS-CoV-2 is an enveloped single-stranded RNA virus that recently emerged and rapidly spread across the world, raising a pandemic scenario (Layne et al., 2020; Yang et al., 2020). Severe consequences to public health can already be observed and strategies to contain the pandemic are currently of great urgency (Chen et al., 2020). At the moment, there is no licensed vaccine against SARS-CoV-2. The HAdVs have been considered promising vectors to be used in delivery strategies (Crystal, 2014; Emmer and Ertl, 2020; Neukirch et al., 2020). Vaccine strategies based on HAdV vectors can be developed using different methods, such as the display of foreign antigenic sequences in the proteins present in the HAdV capsid (Sharma and Worgall, 2016; van Winkel et al., 2018). It has been shown that as a consequence of the SARS-CoV-2 infection, there is a cytokine storm with high plasma production of IL-1beta, IL-2, IL-6, IL-7, IL-8, IFN-gamma, TNF-alpha, MIP-1alpha, MIP-1beta, IP-10 among others (Pettersen et al., 2004) (Huang et al., 2020; Qin et al., 2020), which has been associated with the disease severity (Huang et al., 2020; Mehta et al., 2020). In general, a protective immune response to a virus involves adaptive responses mediated by CD4+, CD8+ T cells and B cells. Indeed, during SARS-CoV infection, another coronavirus, the T cell response and neutralizing antibody production were detected in survivors for longer time and the induction of a Th1 profile was considerate desirable for a vaccine (Liu et al., 2017). However, the protective immune response to SARS-CoV-2 is not yet known. Furthermore, the epitopes of SARS-CoV-2 that are recognized by human immune cells are still poorly characterized. Using bioinformatic tools to predict B and T cell epitopes that might be recognized by these human cells may allow the selection of regions of the virus that can be potential targets for the immune system and vaccine development (Baruah and Bose, 2020; Grifoni et al., 2020).

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https://doi.org/10.1016/j.meegid.2020.104489
Received 22 April 2020; Received in revised form 8 July 2020; Accepted 29 July 2020
Available online 03 August 2020

Human adenovirus (HAdV) are classified in the *Adenoviridae* family, genus *Mastadenovirus*, and, to date, 103 genotypes have been described (Brister et al., 2019). The HAdVs are non-enveloped viruses with icosahedral symmetry and their major capsid proteins, hexon, penton and fiber, stand out as the main targets of anti-HAdV immune responses (Lopez-Gordo et al., 2014; Onion et al., 2007; Tischer et al., 2016). The hexon protein is the most abundant protein in the HAdV capsid, with about 940 amino acids (aa). In its aa sequence, this protein presents, across species, four conserved regions (hCRs), that are alternated by three variable regions (VRs). These VRs are further classified in nine hypervariable regions (HVRs) (Onion et al., 2007; Rux et al., 2003). The penton protein has about 540 aa and is involved in the capsid stability and flexibility. This viral protein also aids cell entry through the Arginine-Glycine-Aspartate (RGD) motif (Cuzange et al., 1994; Shayakhmetov et al., 2005). Three conserved regions are present in its aa sequence (pCRs). The fiber protein is the third major protein, with its aa sequence ranging from 300 to 500 aa, and is responsible for interacting with cellular receptors (Zhang and Bergelson, 2005).

Construction of vaccines based on the display of foreign epitopes in the VRs of the HAdV capsid proteins has been demonstrated to be a strategy that has the potential of eliciting a robust and protective immune response against epitopes of a wide variety of pathogens (Farrow et al., 2014; Tang et al., 2017; Wu et al., 2015b). Different HAdV genotypes have been used as prototypes to display foreign epitopes, and genotype HAdV-5 from species C is the most studied in the context of epitope display and anti-HAdV immune responses (Vujadinovic and Vellinga, 2018). Studies have shown that the insertion of the same foreign epitope in the hexon, or in the fiber sequences, results in different profiles of immune responses anti-foreign epitope (Lanzi et al., 2011).

Production of cytokines, upon immune cells recognition of viral components, has a decisive role in anti-HAdV successful responses. Previous studies have identified HLA class I and II promiscuous epitopes present in the HAdV capsid proteins that are recognized by different CD4 and CD8 T cells of healthy individuals (Hutnick et al., 2010; Onion et al., 2007). Neutralizing antibodies are also important for protection against HAdVs, since they act extremely efficiently in neutralizing HAdV particles (Sumida et al., 2005; Sumida et al., 2004).

In the recent years, immunoinformatics tools have become more accurate and techniques of epitope prediction have greatly evolved, enabling the construction of computational models that can reliably reveal peptides characteristics (Backert and Kohlbacher, 2015; Bahrami et al., 2019). Therefore, the use of such tools for the analysis of the immunogenic potential of protein sequences has played a role in the consolidation of vaccine development methods, allowing the study of proteins' immunological profile (Bahrami et al., 2019; Bazmara et al., 2019). Thus, making feasible the study of antigenic determinants of the HAdV major capsid proteins and proteins present in other pathogens.

Starting from this perspective, the present study aimed at the design of a modular HAdV-based platform that proposes to reconstruct the conserved aa sequences of the major capsid proteins of the HAdV-5 based on the analysis of representative genotypes from all HAdV species. The modular approach was also designed for the selection of SARS-CoV-2 aa sequences to be displayed at the HAdV capsid. In the context of epitope display in HAdV vectors, this analysis contributes with the optimization of aspects inherent to the vector, with the use of modules designed from immunomodulatory aa sequences found in HAdV from other species.

2. Materials and methods

2.1. Sequence alignment and amino acid diversity analysis

Initially, four sets of sequences were created. The set one contained the complete aa sequences of the hexon, penton and fiber proteins, from the 101 available HAdV genotypes (Supplementary table 1). The set two contained the aa sequences of the major capsid proteins from seven genotypes, each one representing one of the seven HAdV species (A-G) (Table 1). The set three contained only the aa sequences of the hCR1–4 and pCR1–3 from the genotypes present in set two, and also the complete aa sequence of the fiber protein from HAdV-5C (Table 1). The set four contained the hCR1–4 and pCR1–3 of the hexon and penton proteins from all 101 published genotype sequences (Supplementary table 1).

Table 1
Reference sequences used in the construction of sets 2 and 3.

Protein	Specie	Acession number*
Hexon	A	AEK79922.1
	B	AAW33354.1
	C	AAW65514.1
	D	AGT76808.1
	E	AAW33307.1
	F	NP_040862.1
	G	ABK35044.2
Penton	A	AEK79908.1
	B	AAW33349.1
	C	AAW65509.1
	D	AGT76803.1
	E	AAW33302.1
	F	NP_040857.1
	G	ABK35039.1
Fiber	A	AEK79935.1
	B	AAW33370.1
	C	AAW65529.1
	D	AGT76825.1
	E	AAW33322.1
	F long	NP_040876.1
	F short	NP_040875.1
	G long	ABK35059.1
	G short	ABK35058.1

* The accession numbers of the 101 HAdV genotypes are available at the supplementary material and are available at the GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Sequences in set one were aligned using the Clustal Omega platform at the EMBL-EBI server (https://www.ebi.ac.uk/Tools/msa/clustalo) (Madeira et al., 2019). The alignments were then analyzed at the Protein Variability server (Garcia-Boronat et al., 2008), using the Wu-Kabat variability method, returning graphics showing the aa variability along the hexon and penton proteins.

2.2. In silico prediction of HLA class I and II epitopes in the major capsid proteins

Set two was submitted for the prediction of HLA class I and II epitopes in the major capsid proteins. The first step of this analysis was performed using the NetMHCpan 4.0 and NetMHCIIpan 3.2 softwares (Jensen et al., 2018; Jurtz et al., 2017) with the default thresholds, predicting epitopes for 126 and 27 HLA class I and II alleles, respectively (Supplementary table 2). The lengths of the predicted sequences were of 9 and 15 aa for the HLA class I and II epitopes, respectively.

The epitopes predicted for the hexon, penton and fiber proteins were filtered based on their promiscuity. For the epitopes predicted from the hexon and fiber, the adopted cutoffs were of 40 HLA class I and 15 class II alleles. For the epitopes from the penton protein the adopted cutoffs were of 30 HLA class I and 15 HLA class II alleles. These cutoffs were adopted in order to provide a stringent number of predicted epitopes. All the filtered epitopes had their conservation evaluated at the Epitope Conservancy Analysis toll (http://tools.iedb.org/conservancy/) (Bui et al., 2007), using the sequences in set one as reference.

2.3. In silico prediction of B-cell epitopes in the major capsid proteins

The prediction of B cell epitopes was performed using set two, in order to map regions containing B-cell epitopes that are present in genotypes representing different species. This set was submitted at the ABCpred server (Saha and Raghava, 2006) with a 0.90 threshold, a window length of 16 aa for the epitopes and the overlapping filter was ON. The conservation of the epitopes was evaluated with the Epitope Conservancy Analysis tool (http://tools.iedb.org/conservancy/) (Bui et al., 2007). A filtering process was applied to the predicted epitopes, selecting only the epitopes present in the hCRs and pCRs present in set three. All predicted B-cell epitopes present in the HAdV-5 fiber protein were selected.

2.4. Prediction of immunomodulatory sequences

The predictions of sequences capable of activating antigen presenting cells (APCs), inducing IFN gamma, IL-4, IL-17, IL-10 and proinflammatory sequences were performed with set two (Table 1). The prediction of epitopes capable of activating APCs was performed with the VaxinPAD platform (Nagpal et al., 2018) at the GPRSdocker, with the dipeptide composition as the prediction method and a 0.5 threshold. The prediction of proinflammatory sequences was performed at the ProInflam server (http://metabiosys.iiserb.ac.in/proinflam/prot.php) (Gupta et al., 2016) with a 0.5 threshold. The prediction of IL10 inducing epitopes was performed with the IL10pred platform (Nagpal et al., 2017) at the GPSRdocker with a 0.5 threshold. The prediction of IL4 inducing epitopes was performed at the IL4pred platform (http://crdd.osdd.net/raghava/il4pred/) (Dhanda et al., 2013a), with a 0.5 threshold and the support vector machine (SVM) based prediction method were selected. The prediction of IFN gamma inducing epitopes was performed at the IFNepitope server (http://crdd.osdd.net/raghava/ifnepitope) (Dhanda et al., 2013b) and the SVM based approach, a 0.5 threshold and the IFN gamma vs non IFN gamma model for prediction were selected. The prediction of sequences capable of inducing IL-17 was performed at the IL17eScan (http://metabiosys.iiserb.ac.in/IL17eScan) (Gupta et al., 2017) with the DPC-based model of prediction and a 0.5 threshold. All predictions were performed with 15 aa overlapping epitopes generated from set two and the SVM scores obtained by the predictions for each of these epitopes were plotted (Supplementary figs. 6–13).

The results of these predictions were filtered and only the sequences present in hCRs and pCRs from set three were selected. All epitopes present in the HAdV-5C fiber protein were selected.

2.5. Structural analysis of the HAdV-5C hexon and penton homomers

In order to evaluate the conservancy of aa residues involved in the hydrogen bonds and salt bridges present in the interfaces of the hexon and penton homomers. The structures of the hexon trimmer (PDBid: 3TG7) and the penton pentamer (PDBid: 6B1T) of the HAdV-5C were extracted from the PDB databank and analyzed at the PDBePISA platform (https://www.ebi.ac.uk/pdbe/pisa/) (Krissinel and Henrick, 2007). The positions of the amino acids responsible for the hydrogen bonds and salt bridges, present in these interfaces were obtained. The conservation of these residues was evaluated using the alignments of the hexon and penton proteins from set two.

2.6. Detection of predicted sequences in the HAdV major capsid proteins

The T and B cell epitopes predicted from the HAdV-5 hexon, penton and fiber proteins, scoring above the cutoffs in the previous steps, were aligned with the prototype sequences from the major capsid proteins of HAdV-5C. All regions containing T and B-cell epitopes were highlighted (supplementary fig. 3). The T and B cell predicted epitopes from all HAdV species are available at supplementary tables 3–11. The SVM scores obtained by the prediction of immunomodulatory sequences present in set three were plotted (supplementary fig. 6–13) and the predicted induction potential of the hCRs and pCRs from each HAdV species were compared.

2.7. Applying the modular design in the development of a SARS-CoV-2 HAdV-based vaccine

The aa sequences of the spike glycoprotein, membrane and envelope proteins from the SARS-CoV-2 (acession number MN908947.3) were submitted to the same prediction protocol described in 2.2–4. For the HLA class I and II epitopes prediction, the promiscuity cutoffs adopted for the spike glycoprotein and membrane protein was of 40 and 15 alleles. For the envelope protein the cutoffs were of 30 and 10 alleles for the HLA class I and II epitopes, respectively. In the B-cell epitope prediction, the score threshold adopted for the spike glycoprotein was 0.90. For the membrane and envelope proteins the score threshold was 0.80. For the immunomodulatory predictions the same protocol described in 2.4 was used and only the top ten sequences scoring above the thresholds were selected. The results of the prediction were aligned to the sequences of the proteins. Regions of these proteins containing T and B-cell epitopes and immunomodulatory sequences were defined as target and induction modules, respectively, and selected to be displayed in the VRs of the HAdV major capsid proteins. The position of the RGD motif was investigated in the structure of the spike glycoprotein (pdb id: 6VSB) (Wrapp et al., 2020a). Induction and target modules designed based on SARS-CoV-2 proteins were inserted in the HAdV vector using the linker GPGPG.

3. Results

3.1. Sequence alignment and amino acid variation analysis

The results of the conservation analysis for the HAdV major capsid proteins, along with the conservation analysis of the hCRs and pCRs, are displayed in Table 2. The number of amino acids present in the hCR1, 2, 3 and 4 are approximately 136, 28, 100 and 494 respectively. The locations of the sequences from all the hCRs and pCRs are better depicted in Fig. 1 and Table 2, which includes the amino acid variation analysis. Variations in the identity percentages of the analysis may be found when compared to previous studies that had also considered the major capsid proteins due to the adoption of these positions and lengths for the hCRs (Ebner et al., 2006).

3.2. HLA class I and II prediction

For the hexon protein, the HLA binding prediction indicated the existence of 69 HLA class I epitopes (scoring above the cutoff). Some of these epitopes are conserved only intra-species, but epitopes conserved in two or more species were also found. From all the predicted epitopes,

Table 2
Minimum identity and conservation analysis of the HAdV major capsid proteins.

Protein	Regions	aa position (reference genome: AY601635.1)	Minimum identity (%)	Global conservation (%)
Hexon	hCR-1	1–131	87.79	73.09
	hCR-2	220–247	57.14	
	hCR-3	317–417	90.1	
	hCR-4	549–952	77.89	
Penton	pCR-1	41–149	81.65	72.03
	pCR-2	163–292	83.08	
	pCR-3	389–571	79.67	
Fiber	–	–	–	29.21

Minimum identities obtained from the aligment performed at the Clustal Omega Server (https://www.ebi.ac.uk/Tools/msa/clustalo).

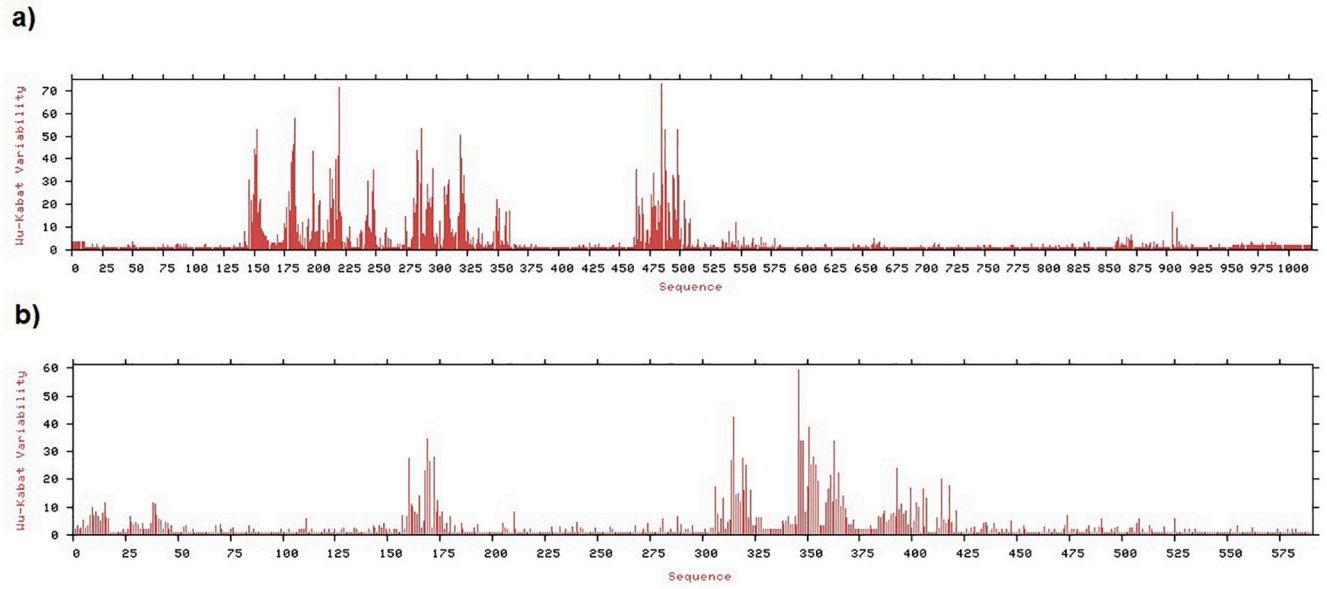

Fig. 1. AA variability analysis of the hexon and penton proteins. The sequences of the hexon and penton protein from 101 available genotypes were aligned and submitted for analysis at the Protein Variability Server. A) Four conserved regions are present in the hexon protein. B) Three conserved regions are present in the penton protein.

five epitopes are conserved among all 101 HAdV genotypes included in this analysis. One of these epitopes is located in the hCR1 and the other four are located in the hCR4. The most promiscuous HLA class I epitope found in this analysis is located at the sequence SM-PQW-YM. In species A, C, D, F and G this epitope and was found to be promiscuous for 76 HLA alleles. In species B and E, the variant of this epitope (SMLPQW-AYM) was predicted as being promiscuous to 41 alleles (Supplementary table 3).

The prediction of HLA class II epitopes indicated the presence of 73 epitopes. The best scoring epitope is present in the region LW–FLY-N—LYLP. In species E this region was predicted as promiscuous to 23 alleles. All the variations of this sequence present in other species also scored above the promiscuity cutoff. The region DRMYSFFRNFQPMS-RQV was indicated to contain three HLA class II epitopes overlapped that are promiscuous to 16, 17 and 18 HLA alleles and conserved in all 101 genotypes (Supplementary table 6).

The prediction of HLA class I epitopes in the penton protein indicated 32 epitopes. The most promiscuous epitope is present in the region RSW-L-N-Y, and in species D it was predicted to 64 alleles. All the variations of this sequence, present in other species, also scored above the cutoff. The most conserved epitope found by the prediction is present in all species (99 genotypes) except for species F, and it was predicted to 34 alleles (Supplementary table 4).

The prediction of HLA class II epitopes in the penton protein revealed 23 epitopes that scored above the cutoff. The majority of predicted epitopes in this protein are conserved only intra-species. The most promiscuous epitope was predicted to 18 alleles and is conserved for two genotypes from species A (Supplementary table 7).

For the fiber protein, the HLA class I and II epitopes prediction indicated 64 and 87 epitopes, respectively, that are mostly genotype-specific. The most promiscuous HLA class I epitope (SAYSITFEF) was predicted to 70 alleles and is conserved in two genotypes of species D. The most promiscuous HLA class II epitope was predicted to 25 alleles and is present in the only genotype of species G (HAdV-52). All the predicted T cell epitopes from the HAdV major capsid proteins are available in supplementary tables 3–8.

3.3. B-cell predicted epitopes

The B cell epitope prediction in the HAdV major capsid proteins indicated the presence of intra-species conserved epitopes. In the hexon protein, B-cell epitopes were found in all four hCRs and 45 epitopes (epitopes in the VRs included) were predicted in the seven species. The analysis of the penton protein also pointed to epitopes present in all three pCRs, and 30 B-cell epitopes (epitopes in VRs included) were found. In the fiber protein, due to its low conservation, most of the predicted epitopes are genotype-specific and 34 epitopes were predicted. It is important to reinforce that prediction analysis was performed using one genotype representing each species (set two). All the predicted B cell epitopes are available in supplementary table 9–11.

3.4. Immunomodulatory sequences prediction

The summary of the immunomodulatory profile of the CRs of the hexon protein is indicated in Fig. 2. Each of the induction potentials obtained by the hexon and penton proteins of all species are detailed considering the aa positions along the CRs in the graphs available at the supplementary figs. 6–12. Graphs are also available for the fiber protein of HAdV-5 (supplementary fig. 13).

The prediction of sequences capable of activating APCs in the penton protein sequences indicated regions of the pCR3 of species A, C, F and G. All the pCRs of all species were predicted to contain sequences capable of inducing IL-10. Considering IFN-gamma induction, the pCR2 was indicated to contain such sequences in species B, D, E, F and G. The pCR1 of species C and the pCR3 of species A and B were indicated to contain sequences capable of inducing IFN-gamma. For the prediction of IL-4 inducing sequences the pCR2 of all species and the pCR3 of species A, B, C, F and G were indicated as IL-4 inducers. The pCR1 of species B was the only not indicated as containing sequences capable of inducing IL-4. In the penton protein, all pCRs of all species were indicated as containing proinflammatory sequences.

In the fiber protein, the prediction of sequences capable of activating APCs returned only four sequences that are present in two genotypes of species D and two genotypes of species F. Sequences capable of inducing IFN-gamma, IL-10, IL-4 and proinflammatory

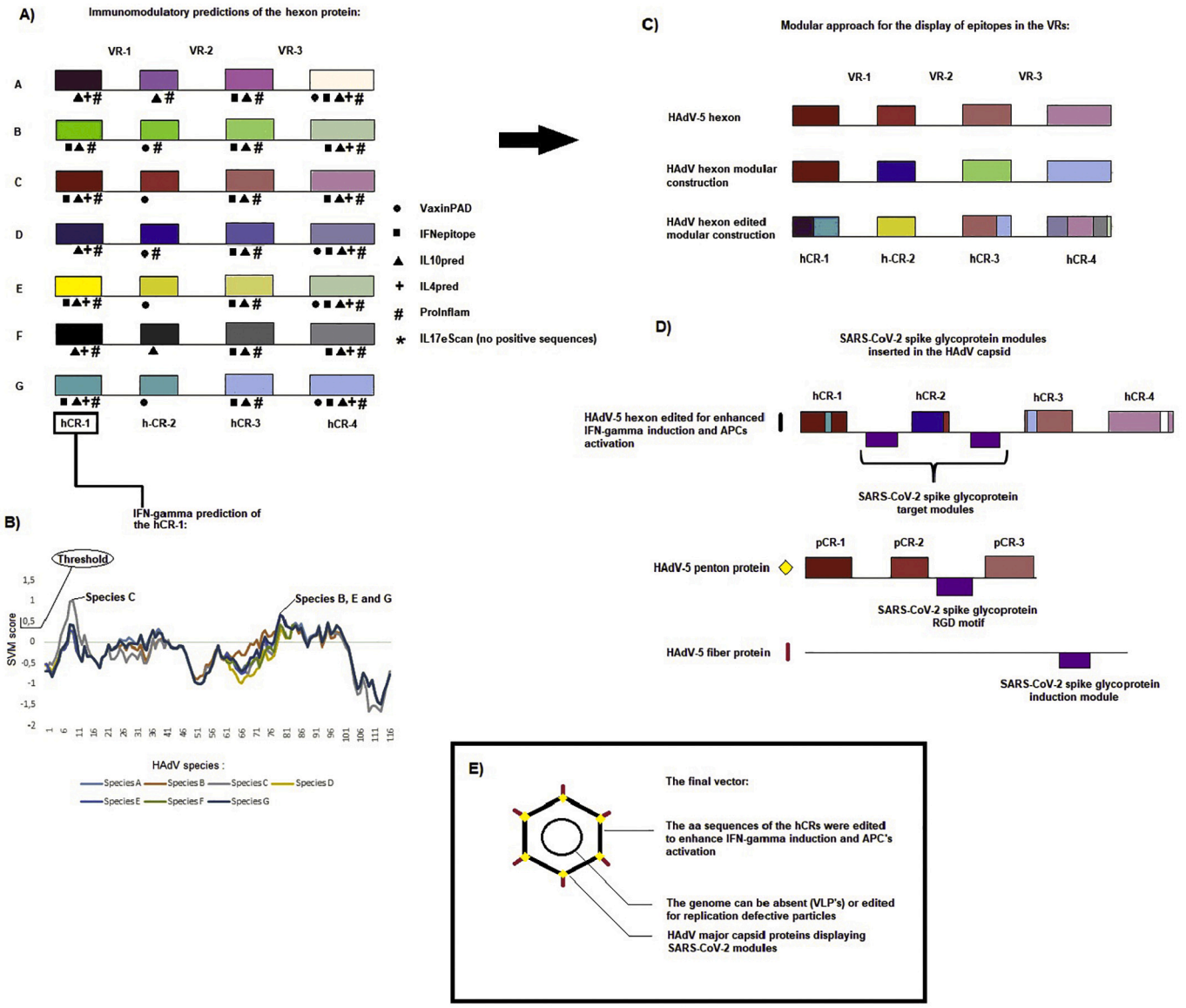

Fig. 2. Strategy for modular construction of HAdV vectors for epitope display. A) The vector modules were designed from the predictions performed with the sequences of the conserved regions present in the hexon (hCR1–4) and penton (pCR1–3) proteins of the seven analyzed genotypes representing the seven HAdV species. B) By consulting the scores obtained by the predictions, it is possible to edit or merge conserved regions (CRs) from different species of HAdV, in order to modulate the immune response against the vector. C) The modular approach can be obtained by using CRs from different species or by merging sequences from different species in the same CR. D) The concept of the modular HAdV vector displaying the SARS-CoV-2 spike glycoprotein modules in the variable regions (VR1–3) (the aa sequences of the vector are depicted in Fig. 3) E) Final vector structure. The hexon, penton and fiber proteins are depicted in black, yellow and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequences were predicted in the fiber protein of all seven species. Sequences capable of inducing IL-17 were not indicated in any HAdV major capsid protein of any species.

3.5. Structural analysis of the HAdV-5C hexon and penton homomers

The conservation of the amino acids involved in the hydrogen bonds and salt bridges, formed in the interfaces of the monomers composing the hexon trimmer and penton pentamer, is demonstrated in the alignment available at supplementary figs. 1 and 2. The majority of amino acids involved in these interactions are conserved across all seven HAdV species. This information is particularly useful if editing of CRs is needed.

3.6. Modular design of the HAdV hexon and penton proteins

All the epitopes and immunomodulatory sequences predicted from the HAdV-5 hexon and penton proteins, scoring above the cutoffs in the previous steps, were aligned with the sequences from the major capsid proteins of prototype HAdV-5. All regions containing T and B-cell epitopes or immunomodulatory sequences were highlighted (supplementary fig. 3). The sequence present in HAdV-5 fiber protein was selected to display foreign epitopes, due to the low conservancy of this protein across the species.

The T and B cell epitopes present in the hCRs and pCRs from set three are demonstrated in supplementary tables 3–11. The scores obtained by the prediction of immunomodulatory sequences present in set three were plotted and the predicted induction potential of the hCRs and pCRs from each HAdV species could be compared (supplementary fig. 6–13).

Table 3
Prediction of immunomodulatory sequences present in SARS-CoV-2 proteins.

Protein	SARS-CoV-2 Induction modules sequences	Predicted induction potential (VaxinPAD, IL10pred, IL4pred, IFNepitope, IL17eScan, ProInflam)
Spike glycoprotein	GVYFASTEKSNIIRGWIFGTTLDSKTQSLLIV	IFN-gamma; IL-10
	ALEPLVDLPIGINITRFQTLLALH	Proinflammatory
	TRFASVYAWNRKRISNCVADYS	APCs activation; IL-10
	KPFERDISTEIYQAG	IL-4
	NFTISVTTEILPVSMTKT	IL-4
	DVDLGDISGINASVVNIQKEI	IL-4
	KWPWYIWLGFIAGLIAIVMVTIMLCCM	IFN-gamma; IL-10
Membrane protein	TLACFVLAAVYRINWITGGIAIAMACLV	Proinflammatory
	LFARTRSMWSFNPETNILLNVPLHGTILTRPLLESELVIGAVILRGHLRIA	IFN-gamma; Proinflammatory; IL-10
	KDLPKEITVATSRTLSYYKLGASQRVAGDSGFAAYSRYR	Proinflammatory; IL-4
Envelope protein	MYSFVSEETGTLIVNSVLLFLAFVVFLLVTLAIL	IL-4; IL-10; IFN-gamma
	NIVNVSLVKPSFYVYSRVKNLNSSRVPDLLV	IL-10; Proinflammatory

The CRs of the hexon and penton proteins of HAdV were defined as "vector modules". Based on the prediction data of T and B cell epitopes and immunomodulatory regions, the user of this platform can select CRs of different species to create versions of the hexon or penton proteins more suited to enhance and modulate the immune response against foreign epitopes displayed in the VRs of the HAdV capsid (Fig. 2a).

If desired, editing of the CRs can be furthered pursuit and it is also possible to merge sequences from different species in the same CR, in order to gather different immunomodulatory properties (Fig. 2c). An example to improve the predicted IFN-gamma induction of a CR, can be observed in the hCR1 of species C. Two peaks (scoring above the 0.5 threshold) were found in the graphs plotted with the scores from the IFN-gamma prediction of the hCR1 of all species and are located from aa 8–26 and 80–96 (Fig. 2b). In the first peak, species C had the best scoring sequences, however in the second peak, species B, E and G scored better than C. To apply the modular approach in the construction of a better IFN-gamma inducing hCR1, the user can replace the aa sequence (80–96) of HAdV-5 to the sequence present in species B, E or G. This editing can also be performed to merge peaks of different immunomodulatory properties, such as IFN and IL-10 or IL-4 and proinflammatory potential, among other applications.

3.7. Modular design of foreign epitopes for display in the major capsid proteins of HAdV-5C

The same modular design can be used for the selection of foreign epitopes to be displayed is the VRs of the major capsid proteins of HAdV-5C. We propose two categories of modules to be inserted in the VRs of the major capsid proteins. The first category was defined as "target modules" and is composed of sequences containing T or B-cells epitopes, overlapped or not. These "target modules" can be generated after applying the analysis described in 2.2 and 2.3 in the aa sequence of proteins from any chosen pathogen.

The second category was defined as "induction modules" and is composed by sequences capable of any immunomodulatory activity, such as induction of cytokines or APCs activation. Such modules can be obtained by applying the analysis described in 2.4 in the aa sequence of proteins from any selected pathogen. The regions of the major capsid proteins in which is possible to display foreign epitopes is better depicted in supplementary fig. 4.

3.8. Applying the modular design in the development of a SARS-CoV-2 HAdV-based vaccine

The prediction analysis of HLA class I and II epitopes in the SARS-CoV-2 spike glycoprotein, membrane and envelope proteins indicated the presence of promiscuous epitopes in all three proteins. The B-cell epitope prediction also indicated the presence of such epitopes in all proteins. Two regions of the spike glycoprotein indicated as containing overlapping HLA class I, II, and B cell epitopes were selected as target modules. In one of these regions, the most promiscuous HLA class I and II epitopes (FAMQMAYRF and ALQIPFAMQMAYRFN predicted to 66 and 22 alleles respectively) and the top scoring B cell epitope (AGTITSGWTFGAGAAL prediction score 0.97) were found to overlap.

In the membrane protein, two regions were indicated as containing overlapped HLA class I, II and B cell epitopes and were selected as target modules. The first region contains overlapped the most promiscuous HLA class I and II epitopes (FAYANRNRF and LLQFAYANRNRFLYI predicted to 62 and 20 alleles). The top scoring B cell epitopes (RSMWSFNPETNILLNV prediction score 0.89) is present in the other region, also selected as a module.

In the envelope protein, two regions were indicated as containing overlapped HLA class I, II and B cell epitopes and were selected as target modules. One of these regions contains overlapped the most promiscuous HLA class II epitope (LVTLAILTALRLCAY, predicted to 11 alleles) and the top scoring B-cell epitope (TLAILTALRLCAYCCN, prediction score 0.85). All the SARS-CoV-2 T and B cell predicted epitopes are displayed in supplementary table 12 and supplementary fig. 5.

The prediction of immunomodulatory sequences indicated the presence of such sequences in all analyzed proteins. No sequences capable of inducing IL-17 were predicted in any of the three analyzed SARS-CoV-2 proteins. A total of 12 induction modules were predicted (Table 3), seven in the spike glycoprotein, three in the membrane protein and two in the envelope protein. Some of the induction modules were predicted for more than one immunomodulatory potential, such as the one selected to be displayed in the HAdV-5 capsid, predicted as a APCs activator and IL-10 inducer.

After being selected, the induction modules were inserted in the VRs of the HAdV major capsid proteins. The sequence of the HAdV-5C hexon protein was altered with the approach here described to enhance the induction of IFN-gamma (hCR1, 3 and 4) and enhance the activation of APCs (hCR2). Two target modules generated from the spike glycoprotein were inserted in the HVR-1 and 5 of this modified hexon protein. The RGD motif and sequences flanking it, present in the spike glycoprotein receptor binding of SARS-CoV-2, were inserted in the place of the HAdV-5 penton RGD motif. One induction module generated from the spike glycoprotein and predicted as capable of inducing IL-10 and activating APCs, was inserted in the HI loop of the HAdV-5C fiber protein. The resulting sequences are available at Fig. 3.

4. Discussion

The development of vaccines based on the display of foreign epitopes in the VRs of the HAdV-5 major capsid proteins has been shown as a promising method to elicit a protective immune response against different pathogens (Hoelscher et al., 2006; Tang et al., 2017; Wu et al., 2015a). Many strategies have been developed such as the construction

>Edited hexon protein aa sequence – Improved IFN-gamma induction and APCs activation / Display of two target modules from the spike glycoprotein

MATPSMMPQWSYMHISGQDASEYLSPGLVQFARATETYFSLNNKFRNPTVAPTHDVTTDRSQ
RLTLRFIPVDREDTAYSYKVRYTLAVGDNRVLDMASTYFDIRGVLDRGPTFKPYSGTAYNALAP
KGAPNPCEWDEA**GPGPG**IGINITRFQTLLALHRSYLTPGDSSSGWTAGAAAYYVGYLQPRTF
LLKYGPGPGQQKTHVFGQAPYSGINITKEGIQIGVEGQTPKYADKTFQPEPQIGESQWYETEIN
HAGGRALKKDTKMKPCYGSFAKPTNKEGGQGILVKQQNGKLESQVEMQFFSTT**GPGPGAGTI**
TSGWTFGAGAALQIPFAMQMAYRFNGIGPGPGKVVLYSEDVDIETPDTHISYMPTIKEGNSRE
LMGQQSAPNRPNYIAFRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDS
IGDRTRYFSMWNQAVDSYDPDVRIIENHGTEDELPNYCFPLGGVINTETLTKVKPKTGQENGW
EKDATEFSDKNEIRVGNNFAMEINLNANLWRNFLYSNIALYLPDKLKYSPSNVKISDNPNTYDY
MNKRVVAPGLVDCYINLGARWSLDYMDNVNPFNHHRNAGLRYRSMLLGNGRYVPFHIQVPQK
FFAIKNLLLLPGSYTYEWNFRKDVNMVLQSSLGNDLRVDGASIKFDSICLYATFFPMAHNTASTL
EAMLRNDTNDQSFNDYLSAANMLYPIPANATNVPISIPSRNWAAFRGWAFTRLKTKETPSLGS
GYDPYYTYSGSIPYLDGTFYLNHTFKKVAITFDSSVSWPGNDRLLTPNEFEIKRSVDGEGYNVA
QCNMTKDWFLVQMLANYNIGYQGFYIPESYKDRMYSFFRNFQPMSRQVVDDTKYKDYQQVGI
LHQHNNSGFVGYLAPTMREGQAYPANFPYPLIGKTAVDSITQKKFLCDRTLWRIPFSSNFMSM
GALTDLGQNLLYANSAHALDMTFEVDPMDEPTLLYVLFEVFDVVRVHRPH**RGV**IEAVYLRTPFS
AGNATT

>Penton protein aa sequence – Display of the spike glycoprotein RGD motif

MRRAAMYEEGPPPSYESVVSAAPVAAALGSPFDAPLDPPFVPPRYLRPTGGRNSIRYSELAPL
FDTTRVYLVDNKSTDVASLNYQNDHSNFLTTVIQNNDYSPGEASTQTINLDDRSHWGGDLKTIL
HTNMPNVNEFMFTNKFKARVMVSRLPTKDNQVELKYEWVEFTLPEGNYSETMTIDLMNNAIVE
HYLKVGRQNGVLESDIGVKFDTRNFRLGFDPVTGLVMPGVYTNEAFHPDIILLPGCGVDFTHSR
LSNLLGIRKRQPFQEGFRITYDDLEGGNIPALLDVDAYQASLKDDTEQGGGGAGGSNSSGSGA
EENSNAA**GPGPGNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIAD**GPGPGAAEAAAPAAQPE
VEKPQKKPVIKPLTEDSKKRSYNLISNDSTFTQYRSWYLAYNYGDPQTGIRSWTLLCTPDVTCG
SEQVYWSLPDMMQDPVTFRSTRQISNFPVVGAELLPVHSKSFYNDQAVYSQLIRQFTSLTHVF
NRFPENQILARPPAPTITTVSENVPALTDHGTLPLRNSIGGVQRVTITDARRRTCPYVYKALGIVS
PRVLSSRTF

>Fiber protein aa sequence – Display of the induction modules from the spike glycoprotein

MKRARPSEDTFNPVYPYDTETGPPTVPFLTPPFVSPNGFQESPPGVLSLRLSEPLVTSNGMLA
LKMGNGLSLDEAGNLTSQNVTTVSPPLKKTKSNINLEISAPLTVTSEALTVAAAAPLMVAGNTLT
MQSQAPLTVHDSKLSIATQGPLTVSEGKLALQTSGPLTTTDSSTLTITASPPLTTATGSLGIDLKE
PIYTQNGKLGLKYGAPLHVTDDLNTLTVATGPGVTINNTSLQTKVTGALGFDSQGNMQLNVAG
GLRIDSQNRRLILDVSYPFDAQNQLNLRLGQGPLFINSAHNLDINYNKGLYLFTASNNSKKLEVN
LSTAKGLMFDATAIAINAGDGLEFGSPNAPNTNPLKTKIGHGLEFDSNKAMVPKLGTGLSFDST
GAITVGNKNNDKLTLWTTPAPSPNCRLNAEKDAKLTLVLTKCGSQILATVSVLAVKGSLAPISGT
VQSAHLIIRFDENGVLLNNSFLDPEYWNFRNGDLTEGTAYTNAVGFMPNLSAYPKSHGKTAKS
NIVSQVYLNGDKTKPVTLTITLNGTQET**GPGPGTRFASVYAWNRKRISNCVADYSGPGPG**DTT
PSAYSMSFSWDWSGHNYINEIFATSSYTFSYIAQE

Fig. 3. HAdV-5 edited major capsid proteins displaying SARS-CoV-2 spike glycoprotein sequences. The aa sequences of the HAdV-5 major capsid proteins were edited to display sequences obtained from the predictions. The underlined regions in the hexon protein were edited with the modular approach to enhance IFN-gamma induction and APCs activation. In the hexon and penton protein, the highlighted sequences represent conserved regions. In the Fiber protein, the highlighted sequences represent characterized sites for the insertion of foreign epitopes. The sequences of the SARS-CoV-2 in display on the HAdV major capsid proteins are in bold and the GPGPG linkers were colored in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of vectors that besides displaying foreign epitopes in their VRs, also carry a transgene encoding the sequences in display (Vujadinovic and Vellinga, 2018). This strategy was shown to enhance the development of the immune response against the epitopes in display (Shiratsuchi et al., 2010; Tang et al., 2017; Zhou et al., 2013). Various HAdV genotypes, such as HAdV-3, 35, 48, have been used in epitope display based vaccines, but the HAdV-5 is the most characterized thus far (Hossain et al., 2018; Leen et al., 2007; Onion et al., 2007; Vujadinovic and Vellinga, 2018).

The selection of sequences to be displayed in the HAdV vector has focused on antigens that are already known or on regions that are targets of neutralizing antibodies (Curiel, 2016; Vujadinovic and Vellinga, 2018). It is also necessary to consider which of the HAdV major capsid protein is best for the display of a foreign epitope, once demonstrated that the same epitope displayed in different HAdV proteins induced different levels of immune response against the foreign epitope (Krause et al., 2006).

The hexon and penton proteins are known to contain regions conserved across species, and in this analysis the conservations ranged from 57.14% (hCR2) to 90.1% (hCR3) and 79.67% (pCR3) to 83.08% (pCR2) for the hexon and penton proteins, respectively. The conservation analysis of the amino acids involved in hydrogen bonds and salt bridges in the interface of the HAdV homomers also demonstrated the contribution of the CRs to the structural stability of these proteins, which is a crucial point for the vector editing herein proposed (Rux et al., 2003; Zubieta et al., 2005).

The targets of the anti-HAdV immune response have been demonstrated to be present in both conserved and VRs. The T-cell anti-HAdV immune response has been shown to have as targets, HLA class I and II epitopes present in the CRs of the hexon protein (Leen et al., 2007; Leen et al., 2004; Onion et al., 2007). Antibodies directed against the VRs of the hexon protein act efficiently on virus neutralization (Sumida et al., 2005; Sumida et al., 2004; Teigler et al., 2014). In the context of HAdV-based epitope display vaccines, the pre-existence of an anti-HAdV humoral immune response was shown to increase the induction of a humoral response against an epitope in display on the fiber protein, whereas in the hexon protein the reverse effect was observed (Lanzi et al., 2011). This is an important finding because it is estimated that 70% to 80% of the population have serological evidence of prior exposure to HAdV (Hiwarkar et al., 2018).

Based on the predictions of immunomodulatory properties and T and B-cell epitopes present in this study, it is possible to suggest that this induction of higher levels of humoral response against epitopes in display on the fiber protein might be related to the lack of promiscuous HLA class II epitopes or sequences capable of activating APCs in the HAdV-5 fiber protein.

For some of the epitope display strategies used in HAdV-based vaccines, the foreign epitopes are displayed in the VRs of the major capsid proteins (Palma et al., 2011; Wu et al., 2015a,Wu et al., 2015b). Thus, the study of the antigenic determinants presents in the hCRs and pCRs can aid in the development and characterization of HAdV vectors.

To address the aspects inherent to the vector and its contribution to the generation of immune responses against foreign epitopes in display, we first identified T and B-cells epitopes present in the HAdV major capsid proteins. HLA class I, II and B-cell epitopes were identified in these proteins across the genotypes representing all seven species (set two). The prediction protocol was able to locate regions containing epitopes already characterized experimentally, such as the HLA class I epitope LPGSYTYEW described by Leen et al. (2007), and present in a region indicated by this analysis as containing overlapped T and B-cell epitopes. In the HAdV-5, regions of overlap between T and B-cells epitopes were found in the hexon and penton protein, however no HLA class II epitope scored above the cutoff in the fiber protein.

The in silico characterization of immunomodulatory sequences of HAdV capsid proteins in this study revealed that the major capsid proteins of each species contains distinct induction or cell activation potential. If the immunomodulatory properties of the HAdV-5 CRs are associated with the development of an immune response against foreign epitopes in display on the HAdV capsid, by characterizing the CRs of other species, it is possible to improve the design of the vectors and induce immune responses more suited to the selected foreign epitope in display. The modular approach here described is an effort to merge immunomodulatory properties of the CRs from different HAdV species. The “vector modules” consist of the CRs of the hexon and penton protein from each species and by analyzing the graphs produced with the scores each prediction program returned for the CRs, it is possible to design a HAdV vector with different induction potential.

Specific SARS-CoV-2 vaccines are of great interest to reduce the impact in the economy and health systems burden around the world (Ralph et al., 2020). Our study demonstrated how the HAdV modular platform could be used to create novel vaccines strategies and also to enhance some aspects of the immune response generated by HAdV vectors. The hCRs of the HAdV-5 hexon protein were edited to contain the sequences from other species that were best evaluated in the prediction of IFN-gamma induction potential and APCs activation. Since a high similarity is shared by the CRs of different species, this enhanced predicted IFN-gamma induction potential and APCs activation could be obtained by changing just a few amino acids in the CRs of the hexon protein.

The prediction of T and B-cell epitopes in HAdV sequences was performed in order to determine which epitopes could be affected by the aa changes introduced in the hCRs and pCRs. In this altered version of the hexon protein, one region predicted as containing overlapped HLA class I epitopes (hCR1) and another two B-cell epitopes (hCR2 and 3) from HAdV-5 were affected. The alterations in the hCR1 HLA class I epitopes resulted in the replacement of the original HAdV-5 epitopes (aa 76–86: TAYSYKARFTL) to the ones present in species G (TAYSYKVRYTL). The two aa variations present in these epitopes were responsible for the better scores obtained by this region of the hCR1 from species G. For the B-cell epitopes present in the HAdV-5, the alterations resulted in sequences that are not present in other HAdVs. The hCR2 of HAdV-5 was almost completely replaced by the hCR2 of species D, which scored better in the prediction of APCs activation potential, resulting in a disruption of a B cell epitope located in the end of VR-1 and the beginning of hCR2. The same occurred in a B-cell epitope present in the first aa of hCR3, where the change of one aa resulted in the better score of IFN-gamma prediction obtained by species G in this region. In the hCR4 one aa was modified, replacing a sequence conserved only species C for a sequence conserved in all other species. These changes in the original HAdV-5 epitopes must be detailed in order to provide a better understanding of the immune response obtained by this sequence, if it is experimentally tested at some point.

Through the predictions of T and B-cell epitopes and immunomodulatory sequences, target and induction modules were generated from the SARS-CoV-2 spike glycoprotein, membrane and envelope proteins. In order to make the most out of the sizes of the sequences that could be inserted in each HAdV VRs, these modules were created from regions containing overlapped T and B cell epitopes or sequences predicted for different immunomodulatory properties. Due to the sizes obtained by the target modules, the hexon protein was selected to display them, whereas the induction modules were displayed in the fiber protein.

Another strategy was adopted for the selection of sequences from the SARS-CoV-2 to be displayed in the penton protein. The RGD motif present in the penton protein is known to interact with cellular integrins and promote viral entry (Vigne et al., 1999). The RGD domain of the penton protein is also target of non-neutralizing antibodies (Hong et al., 2003). When this motif was inserted in the VRs of the hexon protein, a new pathway of infection independent on any other viral component was obtained (Vigne et al., 1999). By analyzing the sequence and structure of the SARS-CoV-2 spike glycoprotein, a RGD motif is present at aa 403 (reference sequence QHD43416.1) and structurally located at the receptor biding domain (Wrapp et al., 2020b). In 2009, Poh et al. designed sequences for a DNA vaccine coding the SARS-CoV spike glycoprotein with a HIS-tag and a RGD domain inserted in its C-terminal and used it to immunize mice. The results indicated that the insertion of the RGD domain polarized a cellular response in mice, increased IFN-gamma secretion and induced fewer antibodies than the spike glycoprotein construct containing only the HIS-tag. The RGD motif present in the SARS-CoV-2 spike glycoprotein, along with the sequences flaking it, were selected to replace the penton RGD motif as a target module. Even though this sequences were not indicated in any of the predictions. This strategy was adopted due to the contributions to viral entry shown in the HAdVs and the previous study demonstrating the better IFN-gamma induction obtained by the spike glycoprotein containing the RGD motif.

The insertion of the target and induction modules in the HAdV proteins' VRs can be performed with or without linkers, and many have been described for use in vaccines, such as GPGPG, AAY and EAAAK (Nezafat et al., 2014; Sayed et al., 2020). It is noteworthy that without the linkers, the regions of contact between the foreign epitope and the HAdV VRs may randomly result in sequences with different immunogenic properties and compromise the efficacy of the vaccine. The finding that the RGD motif polarized a cellular response against the spike glycoprotein in mice (Poh et al., 2009) led us to hypothesize if HAdV-based vectors could induce a better humoral response against the epitopes in display, by removing the RGD motif of the penton protein. If so, this motif could also be used as another alternative tool in the design of vectors that would help to direct an immune response to certain epitopes.

Alternatively, we suggest a concept that can be used as a strategy to replace the linkers used to insert foreign sequences in the HAdV capsid. This strategy is based on the use of a protease cleavage site instead of linkers flanking the foreign epitope in display, along with the incorporation of a genetic sequence in the HAdV late genes coding a viral protease capable of recognizing the cleavage sites inserted in the capsid proteins. By doing so, the epitopes in display in the HAdV VRs could be cleaved and presented for immune receptors, while also providing the presentation of possible epitopes present in the sequence of the viral protease.

This case of study was performed to exemplify the application of a HAdV-based modular platform of vaccination. For the SARS-CoV-2

vaccine, a HAdV capsid was designed considering only the target and induction modules produced from the SARS-CoV-2 spike glycoprotein. For better results, our suggestion is that one HAdV capsid (hexon, penton and fiber) should be constructed displaying the modules of each target protein, in order to make the best use of the aa sizes accepted by each VRs without compromising the structure of the vector. The target and induction modules of the membrane and envelope proteins and the sequences of the HAdV-based SARS-CoV-2 construct are available at Table 3 and Fig. 3, respectively. The design of our HAdV-based SARS-CoV-2 construct focused in obtaining a cellular response with stronger IFN-gamma induction, obtained through the modular editing of the hexon protein and by using the RGD motif (Fig. 3). In our construct, the induction module from the spike glycoprotein in display on the fiber protein was also predicted as capable of inducing IL-10, which is not a desired response in the vaccine strategy we developed. This module was selected because it was the only module predicted as containing sequences capable of activating APCs.

Recently, Anchim et al. (2018) demonstrated that the induction of a humoral response against foreign epitopes in display on the HAdV VRs is not dependent on the infection process. A replicating HAdV vector would probably not benefit from the modifications proposed by this modular approach, since the major capsid proteins are the main targets of the anti-HAdV immune response, and an escape from this immune response could occur as a consequence of these modifications made to the vector. Homologous recombinations with other HAdVs could also be favored, introducing recombinant CRs to wild type HAdVs (Robinson et al., 2013). Thus, formulations containing replication defective HAdV or virus like particles might constitute alternatives to induce an immune response against the foreign epitopes in display.

It is clear that experimental validation needs to be performed in order to verify the efficiency of the platform. However, this is a promising strategy not only in the design of new vaccines as shown here for SARS-CoV-2, but also to other HAdV related studies such as HAdV gene vectors in which the anti-HAdV immune response appears as a great obstacle to the efficiency of the technique (Ahi et al., 2011; (Anchim et al., 2018; Leen et al., 2007; Leen et al., 2004; Leen and Rooney, 2005).

5. Conclusions

To our knowledge, this is the first study proposing an epitope display HAdV-based modular platform developed through in silico analysis. The results obtained by this study demonstrates the feasibility of the modular approach capable of enhancing the induction of the immunomodulatory parameters (Proinflammatory potential, activating APCs and induce IFN-gamma, IL-10, IL-4) according to what is more suited to the foreign epitopes in display. Applying the modular approach, we developed a HAdV-5 based SARS-CoV-2 construct that aims to enhance the IFN-gamma induction and APCs activation. The modular platform is a promising strategy not only for the SARS-CoV-2 construct, but also in the development of new vaccines against different pathogens.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

This study was financed in part by the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* – Brasil (Capes).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2020.104489.

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