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REVIEW

An Overview of Current Uses and Future Opportunities for Computer-Assisted Design of Vaccines for Neglected Tropical Diseases

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Abstract: Neglected tropical diseases are infectious diseases that impose high morbidity and mortality rates over 1.5 billion people worldwide. Originally restricted to tropical and subtropical regions, changing climate conditions have increased their potential to emerge elsewhere. Control of their impact suffers from shortages like poor epidemiological surveillance or irregular drug distribution, and some NTDs still lack of appropriate diagnostics and/or efficient therapeutics. For these, availability of vaccines to prevent new infections, or the worsening of those already established, would mean a major breakthrough. However, only dengue and rabies count with approved vaccines at present. Herein, we review the state-of-the-art of vaccination strategies for NTDs, setting the focus on third generation vaccines and the concept of reverse vaccinology. Its capability to address pathogens´ biological complexity, likely contributing to save developmental costs is discussed. The use of computational tools is a fundamental aid to analyze increasingly large datasets aimed at designing vaccine candidates with the highest, possibly, opportunities to succeed. Ultimately, we identify and analyze those studies that took an in silico approach to find vaccine candidates, and experimentally assessed their immunogenicity and/or protection capabilities.

Keywords: neglected tropical diseases, vaccines, reverse vaccinology, immunoinformatics, epitopes

Introduction

Neglected Tropical Diseases (NTDs) are high-morbidity, high-mortality infectious diseases associated with extreme poverty in tropical and sub-tropical countries, where people usually have limited access to health services.[1,2] They can be caused by bacteria, endo- and ecto-parasites, viruses and fungi. More recently, snake envenoming has also been included in the World Health Organization (WHO) list of NTDs.[3] At present, this list includes 20 diseases (Table 1), which altogether are estimated to affect over 1.5 billion people worldwide, standing as a major obstacle to health access, poverty reduction, and socio-economic development.[1–3]

Most NTDs are zoonotic infections, but there are also others whose main reservoir are humans like dengue.[2] Some are transmitted by vectors, while others spread by direct contact and/or oral and food-borne routes.[2] Originally confined to tropical and subtropical regions, under currently changing climate conditions, their potential to emerge in other areas due to migration of human populations and/or changes in vector distribution must be taken into consideration. This is what respectively occurred with Chagas disease and Chikungunya virus in the last few decades.[4,5] Therefore, it is very important

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Table 1 WHO List of NTDs and Their Corresponding Etiological Agents

Neglected Tropical Disease	Causative Pathogen
Buruli ulcer	*Mycobacterium ulcerans*
Chagas disease	*Trypanosoma cruzi*
Dengue, Chikungunya	Dengue virus, Chikungunya virus
Dracunculiasis	*Dracunculus medinensis*
Echinococcosis	*Echinococcus granulosus, Echinococcus multilocularis*
Food-borne trematodiases	Flat worms (trematodes)
Human African trypanosomiasis	*Trypanosoma brucei*
Leishmaniasis	*Leishmania* spp.
Leprosy (Hansen's disease)	*Mycobacterium leprae*
Lymphatic filariasis (elephantiasis)	*Wuchereria bancrofti, Brugia malayi, Brugia timori*
Mycetoma, Chromoblastomycosis and other deep mycoses	Fungi/Bacteria
Onchocerciasis	*Onchocerca volvulus*
Rabies	Rabies lyssavirus, Australian bat lyssavirus
Scabies and other ectoparasites	*Sarcoptes scabiei*
Schistosomiasis	*Schistosoma* spp.
Soil-transmitted helminthiases	Intestinal worms
Snakebite envenoming	Poisonous snakes
Taeniasis/Cysticercosis	*Taenia solium*
Trachoma	*Chlamydia trachomatis*
Yaws	*Treponema pallidum pertenue*

to invest in them, as human health is a global matter. Increasing our comprehension and knowledge of NTDs to pursue their cure, epidemiological control and eventual eradication should be a priority to reduce their economic and social impact as well as to limit their chances of expansion.

Effective control of NTDs impact depends on access to appropriate diagnostics and the availability of efficient and safe drugs.[6] In these regards, a "Preventive Chemotherapy and Transmission control (PCT)" approach is used on the group of NTDs that already have existing tools for their prevention and treatment, such as lymphatic filariasis, onchocerciasis or schistosomiasis.[3,7] Nonetheless, success of PCT depends on the ability to identify populations at higher risk of suffering the disease impact and treat them, as well as controlling the causative vectors or any other possibilities of transmission. By contrast, there are other NTDs for which the implementation of appropriate tools for their detection and management is still lacking. For these, the strategy followed is the "Innovative and Intensified Disease Management (IDM)",[3,8] but research investment is low, their burden is not fully known, and it is urgent to improve already available interventions to control them, as well as identifying and developing novel ones.[8] Several pharmaceutical companies, non-profit organizations, and governmental and academic agencies have provided economic aid to achieve this goal,[9] and hopefully all those efforts will not wane.

For controlling disease impact, vaccinology is a field with great opportunity. Contribution of vaccines towards societal development by improvement of health status and increasing life-expectancy has been paramount. Many communicable diseases that used to exert a very high toll have been controlled or even eradicated (like smallpox) thanks to implementation of generalized vaccination programs.

Initially, vaccines were made by growing the pathogen in question and injecting a killed or attenuated version of it to evoke a protective memory immune response, in what has been termed "vaccinology 1.0."[10] With the advent of recombinant DNA technologies, new vaccination approaches have been explored immunizing with pathogen subunits or chimeric moieties, which led to "vaccinology 2.0."[10] Nowadays, advances in genetic sequencing and omics sciences allow a tailored design of vaccine candidates based on the genetic information of the pathogen,[10] which coined the term "reverse vaccinology."[11] This methodology has already proved successful in the development of a vaccine against the challenging bacterial meningitis caused by meningococcus serogroup B (MenB) (trade name Bexsero).[10,11] Such an approach is also known as "vaccinology 3.0,"[10] and inspired by Bexsero, several vaccine candidates against other infectious diseases and cancer are being pursued following such approach.[12–14]

In that context, access to genomic and proteomic information of the pathogen is essential, and for the analysis and query of large genomic databases, a series of bioinformatic resources have been developed in the last few years (see Immunoinformatics Pipeline).[11] However, despite the clear biomedical advantages offered by vaccination over the highly toxic chemotherapies employed to treat NTDs, their vaccine landscape remains mostly unpopulated. The complex biology of the microorganisms responsible for these diseases (many of them eukaryotic protozoa or even multicellular pathogens), and the very scarce funding

dedicated to their research, have precluded vaccine development. With NTDs out of the scope of pharmaceutical industry portfolios, academic laboratories involved in the study of the biology and clinical outcome of these diseases stand as the main players in the field.

The aim of the present work was to review the state-of-the-art of vaccine development for NTDs, with a focus on technologically advanced third generation vaccines. These could represent the sought after solution to ameliorate their impact. We framed the information retrieved on the availability of genomic data of the pathogens, and systematically identified those studies that have actually surpassed the initial computational part of the work and included experimental data on immunogenicity and protection against infection.

Search Strategy and Eligibility Criteria

We undertook the search of publications in PubMed/MEDLINE database. We included publications on clinical studies, clinical trials, reviews, and journal articles that assessed NTDs in the WHO list and/or their causative agents, according to the search terms depicted in Table 2. Searches were performed in March 2020, and we restricted the publications retrieved to those published within the last 20 years. Figure 1 depicts the number of publications found per NTD or pathogen according to the search terms used. We performed a manual revision to discard any non-relevant publications, while full texts presenting experimental data were individually curated to select those that included immunogenicity and/or protection data of the vaccine candidates under research. Biological information available for each pathogen was retrieved at the corresponding NCBI repositories. Identification of the number of available genomes for each of the NTDs etiological agents was carried out in the NCBI Genome data base.

Table 2 Keywords Used in the Electronic Searches

Search Term	Subheading
[Name of the corresponding NTD]	AND Pathogen AND infection AND vaccine AND vaccine AND epitopes AND epitope-based vaccine AND immunoinformatics AND reverse vaccinology
[Name of the corresponding Pathogen]	AND infection AND vaccine AND vaccine AND epitopes AND epitope-based vaccine AND immunoinformatics

The Vaccine Landscape for NTDs: Where are We?

Some NTDs already have efficient chemotherapies at hand while others do not. Although these are not absent of limitations, like short supply and lack of distribution, Buruli ulcer, foodborne trematodiases, leprosy, onchocerciasis, scabies, schistosomiasis, soil-transmitted helminthiases, snakebite envenoming, taeniasis/cysticercosis, trachoma and yaws belong to a group of NTDs that generally count with effective therapeutics available. On the contrary, a second group of diseases encompassing Chagas disease, dengue, chikungunya, dracunculiasis, echinococcosis, human (and animal) African trypanosomiasis, leishmaniasis, lymphatic filariasis and rabies, could largely benefit from improved therapeutic solutions. For the treatment of some of the latter (like Chagas disease, African trypanosomiasis, leishmaniasis and lymphatic filariasis) there are drugs available, but they are either too expensive, entail invasive administration, render variable efficacies, and/or have frequent side-effects. For others, mainly viral infections like dengue, chikungunya or rabies, but also dracunculiasis, there are no drugs at all. Mycetoma and chromoblastomycosis would fit in both groups, depending on their causative agent: if it is bacteria, treatments are usually secure and effective; if it is fungi, available therapies are more expensive and not exempt from numerous adverse effects. Anyhow, for all those NTDs that lack of an appropriate and accessible treatment, vaccination represents a very valuable solution. In fact, two of those NTDs, dengue and rabies, currently have approved vaccines to avert them.

Dengue and Rabies Vaccines

Dengue vaccine was developed by Sanofi Pasteur, which commercializes it under the trade name Dengvaxia® (CYD-TVD).[15] It is based on live attenuated dengue viruses and was approved in 2015 for vaccinating people between nine and 45 years old.[15] CYD-TVD is a tetravalent vaccine that targets the four existing serotypes of the virus causing dengue disease (DENV-1, DENV-2, DENV-3, DENV-4), but yields serotype-variable efficiencies.[15] In fact, efficacy against the highly virulent serotype 2 was found to be the lowest of the four.[15] Its induced immunogenicity depends on several factors such as age, immune status of the patient, other underlying diseases, and/or previous exposure to the virus.[15] In

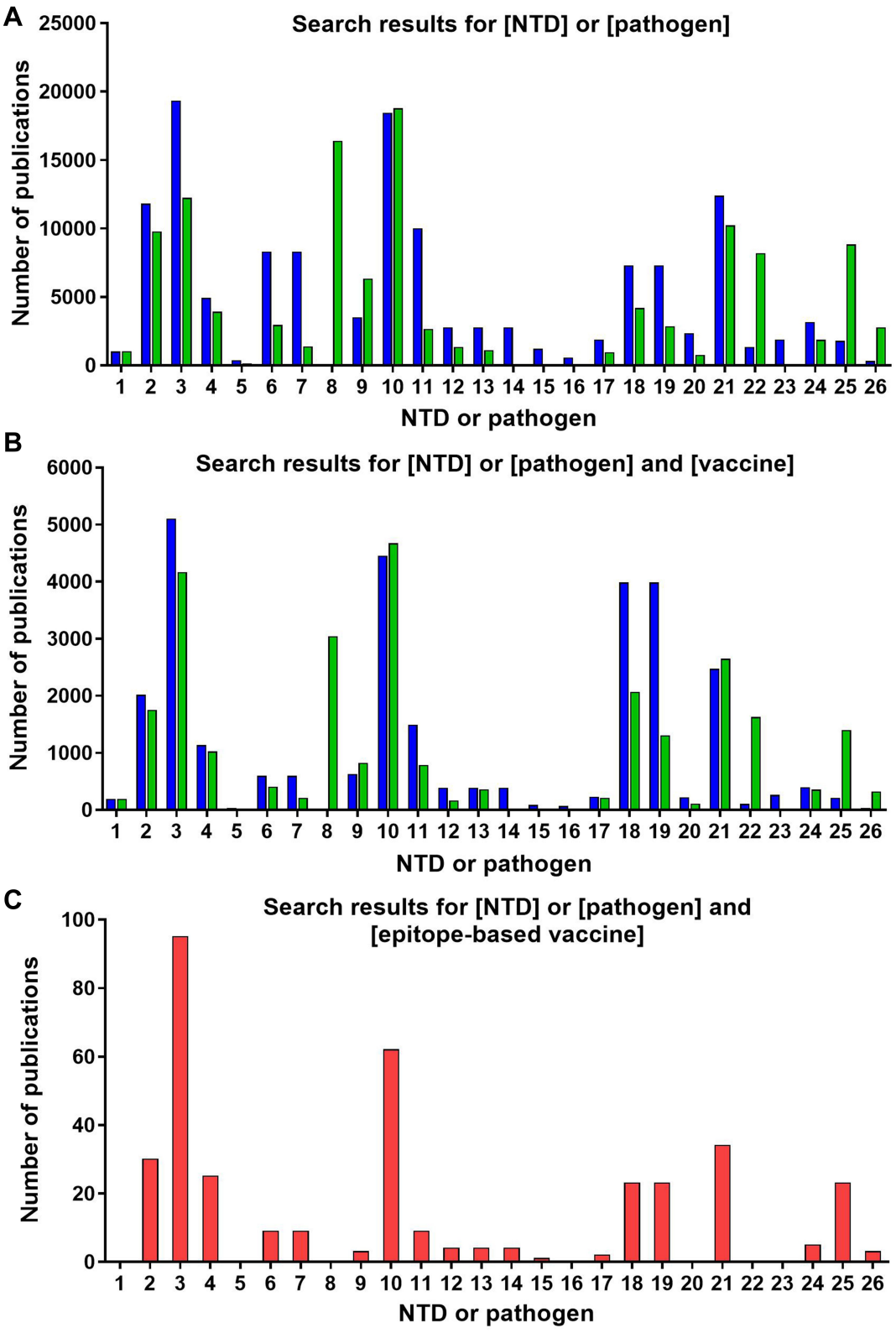

Figure 1 (**A**) Search results for [NTD] or [pathogen] in question. (**B**) Search results for [NTD] or [pathogen] in question and [vaccine]. (**C**) Search results for [NTD] or [pathogen] in question and [epitope-based vaccine]. Blue bars represent NTDs, green bars pathogens, and red bars both. Numbers refer to: 1) Buruli ulcer; 2) Chagas disease; 3) Dengue; 4) Chikungunya; 5) Dracunculiasis; 6) Echinococcosis (*Echinococcus granulosus*); 7) Echinococcosis (*Echinococcus multilocularis*); 8) Food-borne trematodiases; 9) Human African trypanosomiasis; 10) Leishmaniasis; 11) Leprosy (Hansen´s disease); 12) Lymphatic filariasis (*Wuchereria bancrofti*); 13) Lymphatic filariasis (*Brugia malayi*); 14) Lymphatic filariasis (*Brugia timori*); 15) Mycetoma, chromoblastomycosis and other deep mycoses (fungi); 16) Mycetoma, chromoblastomycosis and other deep mycoses (bacteria); 17) Onchocerciasis; 18) Rabies (Rabies lyssavirus); 19) Rabies (Australian bat lyssavirus); 20) Scabies and other ectoparasites; 21) Schistosomiasis; 22) Soil transmitted helminthiases; 23) Snakebite; 24) Taeniasis/Cysticercosis; 25) Trachoma; and 26) Yaws.

relation to the latter, a recent study reported it was less efficient in seronegative people, and worryingly, in some cases it was found that its use could entail severe forms of dengue upon occurrence of posterior infections.[15,16]

In the case of rabies, there are several vaccines commercialized. The first rabies vaccine was developed by Louis Pasteur in 1885 and consisted of inactivated viruses extracted from spinal cords of rabbits (Pasteur vaccine).[17] It had safety risks due to the possible presence of live viruses, and for this reason the Semple rabies vaccine was developed adding phenol to completely inactivate them.[17] Either way, both vaccines caused severe side effects due to the presence of myelin from the rabbits´ nervous system that was the source of the viruses.[17] A third attempt to have a safer rabies vaccine was that of Fuenlazida product, obtained from newborn or embryonic mice, it contained less quantity of myelin.[17] Nonetheless, it was not free from side-effects because there was still some presence of myelin in it.[17] More recently, rabies vaccines have been obtained from infected cell cultures, which yielded products with increased immunogenicity and fewer adverse effects, so vaccine production is presently made in duck or chicken embryonic cells.[17] All licensed options are based on inactivated viruses because the administration of attenuated live rabies virus to humans is not approved due to the possibility of developing the disease.[18] Rabies vaccines like RabAvert, Rabipur or Rabivax efficiently induce long-lasting immunity triggering the production of neutralizing antibodies but several doses are required.[18] Regrettably, rabies vaccination is not included in the vaccines covered by the Expanded Programme on Immunization, and they are expensive and not affordable in developing countries.[19] Furthermore, real demand is difficult to calculate, which leads to shortage of doses and purchasing of poor-quality vaccine alternatives at higher costs and without quality assurance.[20] Thus, investigations are ongoing to find cost-effective and efficient alternatives, but most new vaccine candidates have not been clinically tested yet.[18]

Next Generation Vaccinology for NTDs

Most NTDs are caused by biologically complex eukaryotic parasites or even multicellular organisms, and current vaccination approaches may not be sufficient to tackle such complexity. Fortunately, vaccinology is a dynamically evolving research area and new vaccine development strategies such as reverse vaccinology are now available. Principles of vaccinology 1.0 and 2.0 led to the development of numerous vaccine products for several infectious diseases, but their whole undertaking is time consuming, arduous and very expensive.[10] Moreover, they have been unable to confer protection against numerous emerging and re-emerging infections.[21] Therefore, thinking of NTDs context of high medical urgency and low investment, new strategies involving shorter development times and lower costs would be required.

In the last few decades, linked to the latest advances in sequencing and omics sciences, we have witnessed the expansion and perfecting of a plethora of in silico tools, which application could contribute to the design of vaccine candidates at a fraction of the cost of more traditional methods. Experimentally screening large genomes to find out the antigens of vaccine interest would be highly time-consuming and costly.[22] In this regards, implementation of vaccinology 3.0 and its related immunoinformatics methodologies to in silico pre-screening of the pathogens genomes would guide the selection of those antigens with higher interest.[22] Such tools are also very valuable if the pathogen in question is isolated and grown with difficulty. In addition, in silico-based approaches may permit seeking "pan-genome" protective antigens, exploring vaccine candidates that can immunize against the different pathogenic strains or genetic variants of the pathogen.[22–24] Another advantage of identifying evolutionary conserved sequences would be that conservation might respond to the importance of such sequences in the pathogen life cycle, making of them remarkable vaccine targets. Thus, combination of in silico methods with omics to prioritize the most suitable sequences to be specifically investigated experimentally, in vitro and in vivo, stands as a promising approach to develop new vaccines. Nowadays there are several efforts following these principles to design vaccines for NTDs, their number varying depending on the research background and specificities of each disease (Table 3). The strategy has also been used to investigate vaccine candidates against protozoan parasites like *Toxoplasma gondii* and *Neospora caninum*.[25,26]

When correlating NTDs vaccinology research with the number of published studies, we can see that the commitment is very unequal depending on the disease being considered. Some seem to receive little to no interest when it comes to the development of a vaccine or vaccine-like approach, like Buruli ulcer, trachoma or Yaws, because understandably they already dispose of treatments

Table 3 Studies Following a Reverse Vaccinology Approach to Develop Vaccine Candidates for NTDs

Disease	In vivo Model and/or Clinical Samples	Type of Immunogen	Epitopes	Route of Inoculation; and Number of Them	Time Between Inoculations (in Days)
Dengue	BALB/c and C57BL/6 mice (in-vivo model)	Plasmids containing ns1 gene (pcTPANS1) and ectodomain of E protein (pE1D2)	T-cell epitopes	Intramuscular (IM); 2	14
	HLA-A2 transgenic mice (in-vivo model)	6 HLA-$A2^+$, $A2^+$/$A24^+$ dual binding Dengue virus peptides formulated in calcium phosphate nanoparticles (individually or as pools)	T-cell epitopes	Not specified; 3	7
	BALB/c mice, AG129 mice, C3H mice and macaques (non-human primates) (in-vivo model)	Recombinant DSV4, based on DENV envelope protein domain III which contains cross-reactive epitopes	Not specified	IM (mice)/Not specified (macaques); 3	Days 0, 30 and 90 (mice)/0, 28 and 84 (macaques)
	BALB/c mice (in-vivo model)	Tetravalent recombinant ED3 (envelope protein domain III) in plasmid (pTDV-ED3)	$CD4^+$ T-cell epitopes	Subcutaneous (SC)(samples)/ IM (control); 3	14

Amount of Inoculum per Animal and Inoculation	Adjuvant	Detection Technique	Percentage of Protection	Responsiveness	Ref.
50 μg in 50 μL PBS	–	ELISPOT (measurement of INF-gamma and identification of positive peptides) + Cytokine staining (TNF-alfa and INF-gamma production)	For BALB/c mice, 95% of survival rate in immunized mice with pE1D2, 85% in mice immunized with pcTPANS1 vs 30% of survival rate in mice immunized with plasmid control vector pCTPA and 20% of non-immunized mice.	–	[27]
10 μg pooled free peptides with ISA51 (10 μg of each peptide/150 μL); 50 μg pooled free peptides with ISA51 (50 μg of each peptide/150 μL); 10 μg of particle/multipeptide formulation (10 μg of each peptide/150 μg with nanoparticle); 50 μg of particle/multipeptide formulation (50 μg of each peptide/150 μL with nanoparticle)	N-acetylglucosamine and Montanide ISA51	ELISPOT (measurement of INF-gamma)	Peptides formulated with nanoparticles were seen to induce a strong $CD8^+$ T-cell response. The lower concentration of nanoparticles/multipeptide formulation generated the highest T-cell responses.	–	[28]
20 μg of DSV4 adsorbed in 500 μg of alhydrogel (BALB/c mice; 20 μg of DSV4 adsorbed in 500 μg of alhydrogel with MPLA (AG129 and C3H mice); 100 μg of DSV4 in 500 μg of alhydrogel and 100 μg of MPLA + 100 μg of DSV4 in 500 μg of alhydrogel (macaques)	Alhydrogel/MPLA	ELISA (antibody detection) + FACS-based neutralization test (neutralizing titers)	Immunogenic potential seen of DSV4 with alhydrogel as adjuvant in BALB/c mice (tetravalent seroconversion). DSV4 was shown to elicit neutralizing antibodies. These antibodies confer protection against DENV infection. In macaques, the adjuvants together increased the immunogenicity of the antigens. All 6/6 macaques seroconverted against at least three serotypes when immunized with the antigen with MPLA/alhydrogel adjuvant.	–	[29]
10 μg ED3 of each DENV serotype with adjuvant/200 μg of vector control/100 μg of tetravalent DNA vaccine pDV13-ED3 and pDV24-ED3 (200 μg total)	Alum	ELISPOT (measurement of INF-gamma) + ELISA (antibody detection) + FRNT	pTDV-ED3 induced an increase in the production of INF-gamma to DENV-1, 2 and 3 but not DENV-4. Increase in the IL-4 production was also detected in the rTED3 vaccine-immunized mice. The mice immunized with the dengue vaccines showed a significant antibody response against the four serotypes of DENV when compared to the control. pTDV-ED3 vaccine confers no protection against DENV infection.	–	[30]

(*Continued*)

Table 3 (Continued).

Disease	In vivo Model and/or Clinical Samples	Type of Immunogen	Epitopes	Route of Inoculation; and Number of Them	Time Between Inoculations (in Days)
	Male BALB/c mice (in-vivo model) and DENV infected patients (clinical samples)	Recombinant non-structural protein 5 (NS5)	B-cell epitopes + T-cell epitopes ($CD4^+$ and $CD8^+$)	SC; 3	14
	Female C57BL/6 mice (in-vivo model)	DENV-3 E protein	$CD8^+$ T-cell epitopes	Intradermal (ID); 2	28
	Female HLA-A*0201 transgenic mice, HLA-A*1101 transgenic mice and HLA-A*2402 transgenic mice (in-vivo model)	15 DENV-1 derived synthetic peptides engineered together forming a multi-epitope chimeric gene (DNA vaccine)	$CD8^+$ T-cell epitopes	IM; 3	14
	HLA-A*0201, HLA-A*2402 and HLA-B*3501 transgenic mice on the C57BL/6 background (in-vivo model)	Plasmid encoding nucleotide sequence of DENV1-NS poly-epitope + lipid nanoparticle encapsulated mRNA vaccine (four regions of NS3, NS4B, NS5)	$CD8^+$ T-cell epitopes	ID (DNA) + IM (mRNA); 2	21–28

Amount of Inoculum per Animal and Inoculation	Adjuvant	Detection Technique	Percentage of Protection	Responsiveness	Ref.
10 µg NS5 in PBS or in 22 µg of Alum	Alum	ELISA (antibody detection) + ELISPOT (measurements of INF-gamma and TNF-alfa)	Immunization with NS5 induced specific antibody production, being the ratio IgG1/IgG2a bigger when adjuvanted with Alum. NS5 was seen to induce production and secretion of TNF-alfa by T $CD8^+$ cells and both TNF-alfa and INF-gamma by T $CD4^+$ cells. NS5 immunization also induced protection against DENV2 NGC strain and JHA1 clinical isolate. Immunized mice showed 80% and 60% protection against NGC strain and JHA1 respectively.	Antibodies from infected patients recognized NS5 (number of reactive samples not specified).	[31]
10^7 PFU of Vaccinia Ankara Vaccine or 10 µg of recombinant E protein plus adjuvant in 10 µL of PBS	Saponin	ELISA (antibody detection) + Cytokine staining (measurements of INF-gamma and TNF-alfa)	Immunization with rMVASg-/E induced clear humoral response, whereas rMVA/E induced no cellular response.	–	[32]
50 µg of recombinant or control plasmid in 50 µL of PBS	-	INF-gamma ELISPOT + Cytokine ELISA + LDH cytotoxicity assay	The immunization with the recombinant plasmid induced significant INF-gamma responses directed to each epitope. In immunized mice, epitope-specific T cells were able to kill infected splenic monocytes as well as epitope-pulsed cells. No protection data.	–	[33]
50 µg plasmid DNA or 10 or 2 µg mRNA vaccine or control	-	INF-gamma ELISPOT + Virus neutralization assay (quantification of neutralizing antibodies) + Cytokine staining	mRNA vaccine induces strong immunogenicity by stimulating CD8 T cells. 5/6 HLA-A*0201 control vaccinated mice developed significant viraemia at day 2 whereas 3/6 vaccinated mRNA mice did (2 of them close to detection limit). 5/6 vaccinated HLA-A*2402 mice had no viraemia at days 1.2,3 in front of 4/5 control vaccinated did. 8/10 vaccinated with mRNA HLA-B*3501 mice had no detectable viremia at day 2, whereas 9/9 control vaccinated mice did. Data shows efficiency of vaccine from NS-proteins.	–	[34]

(*Continued*)

Table 3 (Continued).

Disease	In vivo Model and/or Clinical Samples	Type of Immunogen	Epitopes	Route of Inoculation; and Number of Them	Time Between Inoculations (in Days)
	BALB/c mice (in-vivo model)	Recombinant DENV-2 E ectodomain assembled into VLP using *Pichia pastoris*	Not specified	Intra-peritoneal (IP); 3	Days 0, 30 and 90
	Transgenic mice HLA-A*0201, HLA-B*0702, HLA-A*2402, HLA-DR2, HLA-DR3 and HLA-DR4 (murine H-2 class II-deficient) (in-vivo model) + PBMCs from 7 infected patients, A02, B07 (CD4 depleted) and DR2 (CD8 depleted) (clinical samples)	Pool of synthetic peptides from proteins E and NS1, NS3, NS5 of DENV-3	T-cell epitopes ($CD8^+$ and $CD4^+$)	SC; 2	14
	8 patients positive for anti-dengue serotype 3 and 21 negative (clinical samples) + BALB/c mice (in-vivo model)	11 synthetic peptides from envelope glycoprotein of DENV-3	B-cell epitopes	SC; 2	21
	Male BALB/c mice (in-vivo model)	7 vaccine constructs consisting on 5 to 7 epitopes predicted from E protein of DENV-2	B-cell epitopes synthesized with a helper T-cell epitope	SC; 2	28

Amount of Inoculum per Animal and Inoculation	Adjuvant	Detection Technique	Percentage of Protection	Responsiveness	Ref.
20 µg of purified recombinant DENV-3 E antigen	Alum	ELISA and indirect IFA (analysis of antibodies) + FACS-based assay (determine homotypic and heterotypic neutralizing antibodies)	DENV-3 VLPs elicit virus-specific antibodies and induce immunogenicity in form of neutralizing antibodies against serotype 3 (serotypes 1, 2 and 4 weakly neutralized).	–	[35]
Pool of peptides (1 µg of each peptide)emul in adjuvant or only adjuvant (control)	Titermax gold	INF-gamma ELISPOT (analyze immunogenicity of each peptide)	Out of the 477 tested peptides, 13 were shown to be immunogenic in HLA Class-I transgenic mice; 173 peptides were immunogenic in HLA Class-II transgenic mice.	For peptide NS3 (399–407), 2/2 A07 patients were responsive. For peptide NS5 (318–326), 1/2 A02 patients were responsive. 0/2 A02 patients responded to peptides NS5 (325–333) and Env (106–114). 2/2 B07 patients responded to NS5 (389–398) peptide, whereas only 1/2 subjects responded to peptides Env (226–234) and NS3 (593–601). 3/3 DR2 patients were responsive to peptides Env (126–140) and NS1 (85–99). 1/3 patients was responsive to Env (231–245), NS1 (69–83) and NS3 (357–371). To the remaining peptides there was no response.	[36]
50 µg of peptide emulsified in adjuvant per epitope region. If the epitope region has more than one peptide, pooled together so final amount is 50 µg. Controls only immunized with adjuvant.	Titermax	ELISA (peptide epitope identification and immunogenicity study of B cell epitopes in mice) + INF-gamma ELISPOT	T $CD4^+$ was the only T cell response seen. All regions tested elicited specific IgGs except for 71–90. Regions 131–170, 196–210 and 246–260 induced significant INF-gamma production in splenocytes.	11 peptides were selected as responsive when analyzed with patients sera.	[37]
100 µL of solution consisting on 50 µg of peptide immunogen resuspended in PBS in a 1:1 ratio with adjuvant	Freund's adjuvant, complete and incomplete	ELISA + Neutralization assay	6/7 vaccine constructs showed antibody response, but with differences in the cross-reactivity depending on the recombinant protein being tested; 3/7 vaccine constructs elicited production of neutralizing antibodies against DENV-2, 2/7 showed production of neutralizing antibodies against DENV-2 and DENV-3; 1/7 elicited neutralizing antibodies against DENV-1 and DENV-2; 1/7 did not elicit neutralizing antibodies.	–	[38]

(*Continued*)

Table 3 (Continued).

Disease	In vivo Model and/or Clinical Samples	Type of Immunogen	Epitopes	Route of Inoculation; and Number of Them	Time Between Inoculations (in Days)
	BALB/c mice (in-vivo model)	Recombinant DENV-4 E protein assembled into virus-like particles (VLPs)	Not specified	IP; 3	Days 0, 30 and 90
Chagas disease	C3H/HeNsd Female mice (in-vivo model)	Synthetic peptide derived of MASP family member XP_820771.1 and conjugated with KLH - DAENPGGEVFNDNKKGLSRV	B-cell epitopes + T-cell epitopes ($CD4^+$ and $CD8^+$)	Not specified; 3	Days 0, 15, 30 (aprox.)
	PBMCs of *T. cruzi*-infected adults and uninfected control adults (clinical samples)	28 Trans-sialidase (ts) derived synthetic peptides from six different HLA I supertypes (HLA A01, HLA A02, HLA A03, HLA A24, HLA B07, HLA B44)	$CD8^+$ T-cell epitopes	-	-
Echinococcosis	Rabbit and infected patients anti-sera (in-vivo and clinical samples)	Recombinant Eg95 molecules obtained from the transformation of *E. coli*	3 Tcells and Bcells combined epitopes	ID multipoint; 4	Not specified
	Female C57BL/6 and BALB/C mice (in-vivo model)	Chimeric antigen made of 18 antigenic peptides from 5 different proteins (EgGST, EgA31, Eg95, EgTrp, P14-3-3). Protein obtained from the transformation of *E.coli*	T-cell epitopes	SC; 3	14

Amount of Inoculum per Animal and Inoculation	Adjuvant	Detection Technique	Percentage of Protection	Responsiveness	Ref.
20 µg of recombinant DENV-4 E protein in adjuvant	Alhydrogel	ELISA and IFA (analysis of antibody production) + FNT	Mice immunized with E VLPs was seen to produce antibodies able to recognize EDIII and DENV-4. Not significant recognition in the case of the other serotypes. Antiserum of immunized mice recognized infected cells with DENV-4. The antibodies elicited had neutralizing capacity against DENV-4 but not against DENV-1, 2 and 3.	–	[39]
20 µg in 200 µL PBS per animal per immunization	$Al(OH)_3$	CL-ELISA (determination of IgG levels) + ELISA (determination of cytokines) + Lysis assay (measurement of lytic capacity of antibodies) + qRT-PCR (testing of protection)	86% of survival rate in MASPpep-KLH immunized mice; 0% survival rate in MASPpep-KLH/Al and control groups. Production of protective antibodies. Decrease in parasite load in immunized mice (97% decrease compared to controls in the heart, 85% decrease in the liver and 92% in the spleen).	–	[40]
-	-	ELISPOT (measurement of INF-gamma and IL-2 levels, individually and simultaneously) + Cytokine staining assay (detection of cells producing both INF-gamma and IL-2)	-	INF-gamma production only, responses to ts-derived peptides: HLA A01: 2/15; HLA A02: 7/25; HLA A03: 6/25; HLA A24: 4/25; HLA B07: 2/25; HLA B44: 2/25. IL-2 production only, responses nearly undetectable. Production of both INF-gamma and IL2 found in 5 patients. Control group showed no positive responses.	[41]
Not specified	No	M13KE Phage display system + Western Blot (analyze immune reaction) + ELISA (to analyze the antigen-antibody reaction)	No protection data. Antigenicity reported of epitope Eg95–2 and 3 is much higher than Eg95–1, but detected in all three of them.	Not specified.	[42]
50 µg	Freund's adjuvant, complete and incomplete	Measurement of mesenteric lymph nodes and spleen with a micrometer + ELISA (detection of cytokine levels)	99,6% of immunized mice with adjuvant were found to present no cysts in spleen, kidneys and liver. In those immunized with no adjuvant one lesion was detected in a single mouse (1/5). High level of protection induced (99,6–100%). Lesions present in control groups.	–	[43]

(*Continued*)

Table 3 (Continued).

Disease	In vivo Model and/or Clinical Samples	Type of Immunogen	Epitopes	Route of Inoculation; and Number of Them	Time Between Inoculations (in Days)
Leishmaniasis	PBMCs of six patients successfully treated for visceral Leishmaniasis (VL; clinical samples)	Five synthetic peptides of LdODC protein used individually or as a cocktail	$CD4^+$ T-cell epitopes	–	–
	PBMCs of 16 patients successfully treated for VL (clinical samples)	Four peptides from Thiol-specific antioxidant (TSA) and five peptides from *Leishmania* eukaryotic initiation factor (LelF)	$CD8^+$ T-cell epitopes	–	–
	Male BALB/c mice and PBMCs of six active and six treated VL patients (in-vivo and clinical samples)	Six epitopes derived from six-stage dependent overexpressed antigens, individually or as a cocktail	$CD4^+$ T-cell epitopes	SC; 3	Days 0, 7, 15
	PBMCs and skin of five mongrel adult dogs infected with *L. infantum* and female BALB/c mice	38 synthetic peptides divided into 24 pools (three/pool)	B-cell epitopes + T-cell epitopes ($CD4^+$ and $CD8^+$)	ID multipoint (dogs); 1 SC (mice); 3	14
Onchocerciasis	Plasma samples from 97 nodule positive subjects	2 Linear peptides (Ov-RAL-2, Ov-103) with immuno-reactive stretches	Not specified	–	–

Amount of Inoculum per Animal and Inoculation	Adjuvant	Detection Technique	Percentage of Protection	Responsiveness	Ref.
–	–	BD OptEIA kit (detection of INF-gamma and IL-10 cytokines) + ELISA (measurement of humoral peptide efficiency)	Increase in INF-gamma production in the cells in contact with the immunogen. No significant production of IL-10 was observed.	6/6 PBMCs samples showed increase in iNF-gamma production when compared to control. Only 1/6 samples showed a significant increase in IL-10 production when compared to control.	[44]
–	–	ELISA (analysis of the induction in the production of INF-gamma)	PBMCs stimulated with peptide cocktail produced higher amounts of INF-gamma when compared to control groups non-stimulated, both in positive and negative samples. Also seen significant levels of GrB in the stimulated cells.	Not specified.	[45]
50 µg	Freund's adjuvant, complete and incomplete	ELISA (detection of anti-peptide antibody) + T cell proliferation assay (analysis of production of cytokines and chemokines)	Vaccination induced higher T cell proliferation in the spleen. Epitopes evoked a immuno-dominant proliferative T-cell response. PBMCs from immunized BALB/c mice showed elevated levels of $CD4^+$ IL-12, IL-17, IL-22 and INF-gamma, but not IL-10.	Not specified.	[46]
10^8 cells-mL in 1mL syringes (dogs) + 100 µL of vaccine formulation-animal-immunization (12.5 µg of each peptide of the mix + 60 µg adjuvant) (mice)	Saponin	Proliferation assay (study of T-cell proliferation) + Intracellular cytokine stain +Flow cytometry (study of T-cell memory phenotypes)	Pools of peptides promoted production of $CD4^+$ and $CD8^+$ T cells, as well as INF-gamma in dogs' PBMCs. Erythema was generated in dogs' skin after injection of peptides, no reaction when injected only with saline. Peptides 25, 30 showed reaction in 4/5 dogs, peptides 33, 34 in 5/5. Peptide 4 induced reaction in 1/5. The rest induced no reaction. Peptides stimulated $CD4^+$ and $CD8^+$ proliferation, as well as production of INF-gamma in vaccinated mice, as well as reduction of 70% in the parasite load.	–	[47]
–	Not specified	Peptide array analysis (confirmation of positive or negative samples) + ELISA (determination of peptide-specific antibody levels)	–	Stretch in Ov-RAL-2 recognized by 9/12 individuals; in Ov-B20 recognized by 9/12; and in Ov-103 recognized by 8/12. The rest was recognized by less than half of individuals. Analysis of Ov-RAL-2 showed that 75% of infected individuals recognized it. Good for diagnosis.	[48]

(*Continued*)

Table 3 (Continued).

Disease	In vivo Model and/or Clinical Samples	Type of Immunogen	Epitopes	Route of Inoculation; and Number of Them	Time Between Inoculations (in Days)
Schistosomiasis	Swiss female mice, C57BL/6 mice and BALB/c mice and human sera from 40 individuals living in endemic area for *S. mansoni* and 15 healthy donors	Recombinant Sm200 protein	B-cell epitopes	SC; 3	15
	Female C57BL/6 mice and 18 patients infected with *S. mansoni* and 13 normal individuals	Epitopes Smp043300e, Sm204830e, Sm151290.1e and Sm151290 obtained from transmembrane proteins (Smp043300e results in chimeric molecule)	B-cell epitopes	SC; 3	15
	Female BALB/c mice	Five synthetic peptides derived from secretory and transmembrane protein sequences	$CD4^+$ T-cell epitopes	SC multipoint; 3	14

against them (Figure 1B). With respect to the retrieved studies on NTDs vaccinology 3.0 that included wet lab experimental results, most have been performed in mice and rabbits as pre-clinical models of infection, with the exception of a study performed on non-human primates (Table 3). Other works relied on patients' peripheral blood mononuclear cells (PBMCs) to run epitope identification experiments. When it comes to the epitopes selected to perform the immunizations, both T cell epitopes and B cell epitopes were used, which in the majority of the cases were administered in the presence of an adjuvant (Table 3). Techniques to perform the analysis of immune responses by either in vivo infection models or samples collected from patients included enzyme-linked immunosorbent assays (ELISAs), intracellular cytokine staining and ELISPOTs.

In connection with the higher funding and interest received, dengue is the disease with more studies exploring a reverse vaccinology approach (Table 3). Most of them are based on the use of peptides from the envelope (E) glycoprotein as immunogens (Table 3). This protein constitutes the primary target for inducing neutralizing antibodies since it interacts with receptors of host cells to mediate virus internalization. Moreover, it triggers a T cell response after virus infection that plays a vital role in providing protection against it. A few other studies are based on targeting non-structural proteins (NS) which are highly immunogenic. Commonly, all report some sort of protection and immunological response, but in those where different serotypes were studied, immunogenicity and protection was not induced to all of them, highlighting the relevance of selecting invariable regions to call the immunity (Table 3).

Publications on leishmaniasis are the second most numerous overall (Figure 1). When it comes to vaccinology 3.0 efforts, most are in vitro works using patients´ PBMCs stimulated with a variety of antigens. A remarkable one is thiol-specific antioxidant (TSA), a promising vaccine candidate due to its protective capacity against different *Leishmania* spp. (Table 3).

Amount of Inoculum per Animal and Inoculation	Adjuvant	Detection Technique	Percentage of Protection	Responsiveness	Ref.
25 µg of rSm200/animal/ immunization	Freund's adjuvant, complete and incomplete	Indirect ELISA (determination of antibody response) + Cytokine analysis	Mice immunized showed no significant reduction in worm burden compared to the control, as well as no reduction in the number of eggs. Immunized mice did present higher amounts of IgG1, INF-gamma and IL-10, but not IgG2, IL-4 or TNF-alfa.	No significant differences in the IgGs anti-rSm200 in the anti-sera of immunized and non-immunized mice. Sera from infected individuals presented significant recognition of rSm200 when compared to the healthy donors. No number specified.	[49]
10 µg of each epitope diluted in 100 µL of PBS and 100 µL of adjuvant/animal/immunization	Freund's adjuvant, complete and incomplete	T-CD4$^+$ lymphocyte proliferation assay + ELISA (detection of IgG in human sera)	Epitopes Sm043300e and Sm204830e induced proliferation of T-CD4$^+$ compared to the control group.	Recognition of Sm043300e was seen in patients infected with *S. mansoni*. Number of patients that recognized it are not specified.	[50]
50 µg per animal per immunization	Freund's adjuvant, complete and incomplete	Proliferation lymphocyte assay + ELISA (cytokine detection) + Flow cytometry (identification of cytokine-producing cells)	Peptides 1, 2, 3 and 5 were able to stimulate lymphocyte proliferation, with peptide 2 inducing the strongest reaction. All five peptides induced INF-gamma production. Low IL-4 levels were detected in the samples of immunized mice. Lower levels of IL-10 were also detected when compared to unstimulated groups, except for peptide 3.	–	[51]

Studies on vaccines for schistosomiasis are mostly based on Sm200, a tegument protein, and different secretory and transmembrane proteins. While Sm200 protein was unable to reduce worm or egg burdens, secretory and transmembrane proteins increased T-cell proliferation and production of interferon gamma (Table 3). These results make of the selected epitopes within them promising candidates for a possible future epitope-based vaccine.

Echinococcosis is another NTD endowed with work on vaccinology as a therapeutic approach. Eg95 antigen is the common molecule used in the two available studies (Table 3). Thought to influence host specificity and affect vaccine efficiency due to its increased expression on the parasite during invasion of host cells, results revealed the antigenic capability of this antigen. When combined with other proteins of the parasite in a chimeric molecule, it led to an increased protection and a reduced number of cysts when compared to a control group in the in vivo models of infection (Table 3).

Studies on Chagas disease were also identified, mostly based on immunization with peptides obtained from MASP family members and trans-sialidases (Table 3). Proteins from these families are essential for a successful infection as they play an important role in the host-parasite interaction and in the establishment of the disease chronic stage.[40] In vivo studies showed some kind of immunization in mice, increasing their survival rate, production of antibodies and managed to decrease the parasitic load (Table 3).

Finally, a single study was found related to the development of a vaccine for onchocerciasis (Table 3). It was performed using PBMCs isolated from patients and led to the discovery of promising peptides for a possible diagnosis of the disease, as there is evident recognition on the part of the infected patients' cells. For obtaining an immunization output, though, further investigations will be required.

Aforementioned diseases with studies that bestow real data on the development of in silico designed epitope-

based vaccines are characterized by the fact that they lack efficient and safe treatments, and a real solution is yet to be discovered. In comparison to dengue, Chagas disease, echinococcosis, leishmaniasis, onchocerciasis and schistosomiasis are caused by parasites with much larger genomes. A feature reflected in the wide variability of available immunogens for each parasite that results in an increased difficulty to ultimately develop a vaccine for them. Their genomes have been sequenced (eg, for *T. cruzi* there are several strains already sequenced), and there are several immunoinformatic tools that can aid in the selection of those antigens or epitopes of interest to be further studied for their inclusion in potential vaccine candidates. Notably, there are improved in vivo models of disease where those can be evaluated, in some cases even including non-human primates. Plus the analysis of in silico identified epitopes can also be made with clinical samples from patients and controls, which may contribute to a rapid translation of results reducing the attrition rates.

Immunoinformatics Pipeline

The largest publicly available repository of epitopes is the Immune Epitope Database (IEDB), funded by the United States of America National Institutes of Health (NIH).[52] This open resource holds data on more than 900,000 epitopes, products of the curation of immunologic assays in the scientific literature, and includes their reactivity, type and grade of response, antigens of origin, among information of relevance.[53] In addition, one of the available options when querying the database is the selection of those epitopes that have been validated experimentally. It also features the IEDB Analysis Resource, a hub of immunoinformatics tools for epitope prediction and assessment.

In search of potentially good epitopes to encompass a vaccine ensemble, protein invariability has been considered to be a major attribute.[23,24,54,55] A hypothetical vaccine product providing pan-protection against multiple strains of a circulating pathogen would indeed be a much desirable characteristic considering the very limited resources for vaccine development, especially for NTDs. Therefore, an initial step to find out the best epitopes often entails using CD-HIT or other BLAST-like tools to cluster together all the proteins from the different proteomes or strains of the pathogen in question, in order to reduce their redundancy (Figure 2).

Each of these protein clusters will then undergo a multiple sequence alignment step (eg, using MUSCLE), so that the variability within each amino acid position can be further analyzed. A measurement that is applied to parametrize the variability is the Shannon entropy,[56] which quantifies it and allows masking of any position with variability over a certain entropy threshold.[57] The outcome will be a conserved consensus proteome of our pathogen of interest, against which the list of experimentally validated epitopes retrieved from the IEDB repository can be parsed following a legacy experimentation approach.[58] However, basic research on NTDs and their etiological pathogens has historically been neglected, and the amount of information on their epitopes is usually insufficient. As a consequence, a legacy experimentation approach cannot be pursued. Fortunately, there is a whole series of tools designed and trained for de novo prediction of epitopes.

Adaptive immunity main players are T cells and B cells, so epitopes are consequently classified as T-cell or B-cell epitopes. The former can be further differentiated into $CD8^+$ or $CD4^+$ T-cell epitopes, the two main subpopulations of these cells. Predictions for T-cell epitopes mainly focus on MHC binding.[59] The IEDB Analysis Resource hosts several prediction tools for both types of T-cell epitopes, with a default "IEDB recommended" method that is periodically updated based on benchmarks of predictors for a specific allele.[60] Generally speaking, binding prediction tools for MHC I ($CD8^+$) molecules are more accurate than for MHC II ($CD4^+$).[59] Additionally, MHC I predictions can be somewhat enhanced by taking into account intracellular antigen processing steps, such as proteasomal degradation and the importation of potential epitopes into the endoplasmic reticulum through TAP, the transporter associated with antigen processing.[61] This allows the creation of multi step prediction methods for $CD8^+$ T-cell epitopes, which can be combined into a single tool (http://tools.iedb.org/processing/), or used separately.[62,63]

On the other hand, B-cell epitope predictions are more unreliable than those of T-cell epitopes.[59] B-cell epitopes drive humoral immune responses based on antibodies. Some are conformational (discontinuous) epitopes, while others are linear (continuous). Most tools are focused on predicting the latter because they can be directly predicted out of the protein primary structure and delivered independently from their context in order to induce an immune response, while that is much more complex to achieve with discontinuous epitopes.[59] An alternative approach for B-cell epitopes prediction is to rely on 3D protein structures where it is possible to identify regions accessible to antibody recognition through relative solvent accessibility calculations (eg, using NACCESS[64]), as well as determining the

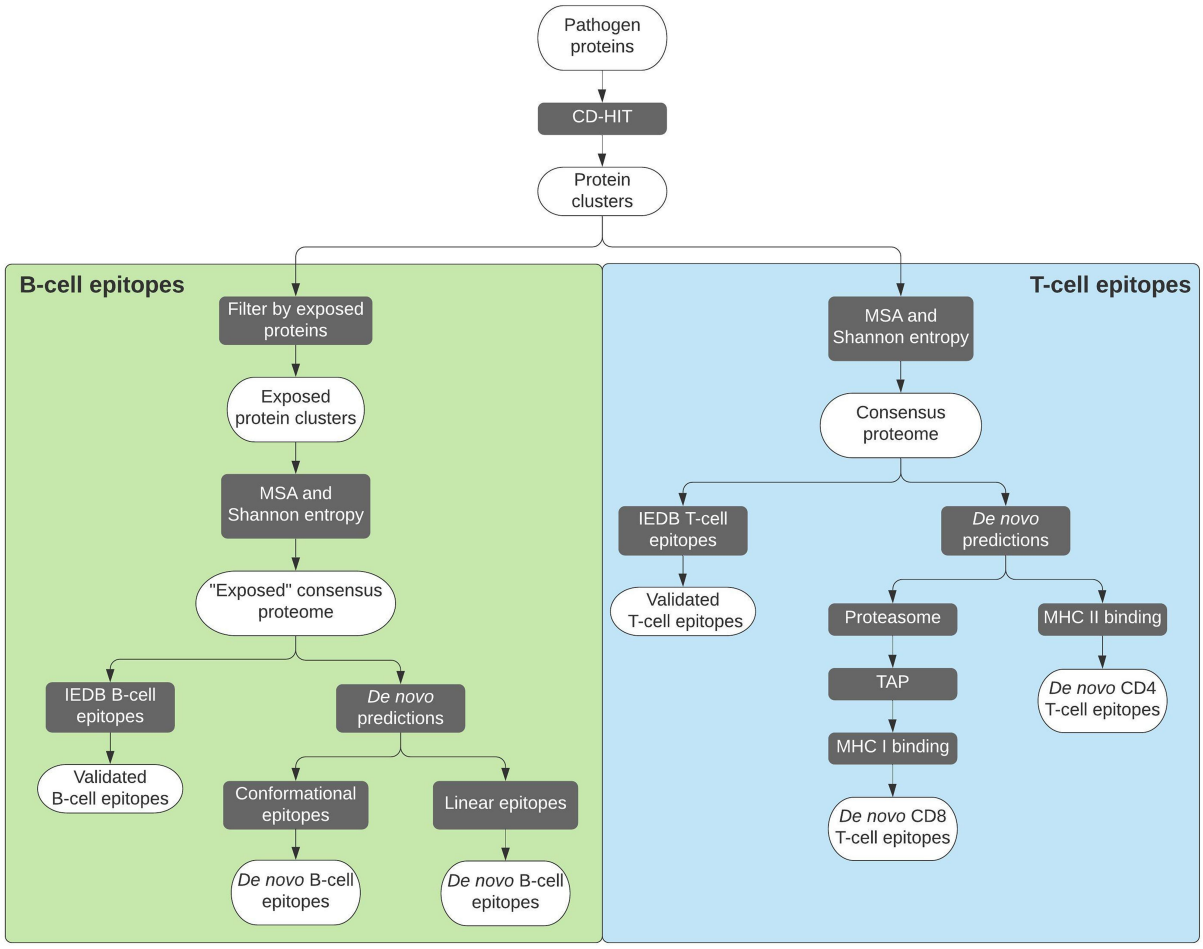

Figure 2 Flowchart depicting the procedures followed to reach selected B-cell and T-cell epitopes. In each case, the legacy experimentation approach is indicated on the left.

flexibility of such regions by analyzing the temperature factors, or B-factors, of residues within 3D structures.[23,24] These factors are included in .pdb files downloaded from the Protein Data Bank,[65] and are a measure of the smearing in a protein structure due to the motions of its atoms: the higher this value is, the higher the flexibility.[66]

Predicted epitopes can then be packaged together to construct a new vaccine ensemble. However, when designing a new epitope-based vaccine, two main issues have to be taken into account: which combination of epitopes will offer the highest immune coverage, and how are these administered.[67,68] The former can be answered using HLA coverage tools, which output is the best combination of epitopes for a wider coverage taking into consideration the population frequencies of HLA alleles. Two advantages of prioritizing epitopes following this approach are envisaged: first, ensuring selected epitopes are recognized by HLA alleles with a high population frequency,[68] and second, targeting specific HLAs considering different frequencies for different human populations.[68] Examples of these tools include the Population Coverage tool at the IEDB Analysis Resource,[53] PEPVAC,[69] and PREDIVAC.[70] Regarding the delivery of the epitope-based vaccine candidates, poor immunogenicity has been described when administering them as peptides,[54] so the use of genetic delivery has also been proposed.[23,24] In any case, the use of vaccine encoded adjuvant sequences or the co-administration of the vaccine constructs with an adjuvant would be desired. Finally, selected epitopes have to be delivered in a vaccine formulation, usually in the form of a multi-epitope "string of beads" genetic construct.[67] Any theoretical construct must take into account the order of the epitopes, as well as the separation between them, in order to avoid the appearance of neo-epitopes, which could have negative effects.[71] The most common approach is to use "linker" or "spacer" sequences that

promote proteasomal cleavage, such as the commonly used AAY sequence.[67,71] Additionally, some algorithms have been developed to facilitate this task, such as the one developed by Schubert and Kohlbacher,[71] or VaccineCAD.[72]

All these tools could play a critical role in the search for vaccines against NTDs due to their effects in the reduction of costs. "Traditional" epitope identification involves costly time-consuming screenings of large arrays of candidates, and being able to reduce the potential list of epitopes via the methodologies described above might provide a huge benefit.

Conclusions

No doubt implementing vaccination programs would be a breakthrough for controlling the impact of several NTDs. Nonetheless, this has been generally poorly explored due to scarcity of funding resources and the biological complexity of the pathogens causing them. Nowadays, new vaccinology approaches are at hand opening a field of great possibilities for the cost-effective design and assessment of vaccine candidates against complex pathogens carrying thousands of potential antigens. Yet, investments will be needed to progress on this complicated challenge. To this day, dengue and rabies are the only NTDs that can be vaccine prevented, but issues like cost and availability still limit their more widespread use.

Ongoing research efforts onto NTDs vaccines are unevenly distributed. If it is true that some NTDs would not require a vaccine due to the current existence of efficacious treatments, others could greatly benefit from them. For that, a computer-assisted strategy to screen the pathogens´ extensive antigen repertoire could largely contribute to save developmental costs. This might also be applied to the design of epitope-based vaccine candidates which could be at the same time a major approach to counteract the high biological complexity of the pathogens by allowing inclusion of multiple epitopes from multiple antigens.

Either way, progress towards NTDs' control and elimination mostly rest on academic groups under the umbrella of international partnerships funds and donations from pharmaceutical companies, but there is still a long road ahead. Strong and continued commitment by all actors involved in this process will be fundamental to eventually succeed in the control of NTDs impact.

Consent for Publication

We give our consent for the publication of this paper and its contents.

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Author Contributions

All authors made a significant contribution to the work reported, whether that was in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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