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Peptides: an arrival point in cancer vaccinology

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1. ABSTRACT

During the past few decades, numerous approaches towards therapeutic vaccines have been investigated. In addition to traditional prophylactic vaccines against infectious microorganisms, there have been attempts to develop therapeutic vaccines for indications as complex as autoimmunity and cancer. Driven by an increasing understanding of the underlying mechanisms, researchers have attempted to interfere with complex molecular cascades during disease progression. Monoclonal antibodies have gained more importance, and their specificity has become more predictable. However, in spite of the advances in our knowledge, crucial problems linger unsolved in vaccinology, such as the major histocompatibility complex (MHC) degeneration phenomenon, the escape from immune surveillance of cancer and microbes, and the possibility of adverse events, perhaps linked to peptide cross-reactivity. In essence, it seems that in order to understand immune responses the peptide-peptide interactions have yet to be clearly defined. These issues will be discussed in the frame of current approaches to vaccine development with special focus on cancer vaccines.

2. VACCINES: AN HISTORICAL OUTLINE

Vaccine research began with the early vaccination trials of Edward Jenner and Louis Pasteur at the end of the 19th century. Since those early days, vaccines have been developed for many infectious diseases that were once major afflictions of mankind. For example, vaccine use has dramatically reduced the incidence of diphtheria, measles, mumps, pertussis, rubella, poliomyelitis, tetanus, and many more. Clearly, vaccination is a cost-effective weapon for disease prevention. An important example is the smallpox vaccine, the use of which has basically eradicated the disease (1). Experience with the smallpox vaccine has been recognized worldwide and has affected many contemporary disease control programs. Besides classical prophylactic vaccines, new treatment modalities have been explored.

At present, there is also growing interest in the development of therapeutic vaccines to treat already established infections or endogenous diseases, as is reflected in growing numbers of recent publications (2). In this respect, detailed knowledge of the differences in the

epitopes recognized by T cells and B cells is of utmost importance and has enabled immunologists to design vaccines that activate the humoural and/or the cell-mediated branches of the immune system (3). In order to be successful, the activation of the immune system has to provoke a response that specifically recognizes the disease-associated pathogen. Furthermore, vaccines should stimulate the immune system to develop a long-lasting response that is both curative and protective. An example of an experimental approach that combines both characteristics is an isolated human monoclonal antibody (MAb) against the influenza antigen M2 (4). The MAb was able to protect vaccinated mice from a lethal challenge with influenza virus and cured infected mice as long as the MAb was given 2 to 3 days after infection. However, when the numerous approaches and encouraging preliminary data are translated to clinical practice, the results have not been as successful. For example, vaccine-based attempts to reprogram the immune system so that it will control tumour cell growth or fight infectious diseases have been failing so far (5, 6). With this background, the current review aims to explore the many approaches that have led to the current state of the art in vaccine development with special focus on cancer vaccines.

3. GENERAL CONCEPTS OF VACCINES

3.1. Active versus passive immunization

Before focusing on specific treatments it is necessary to define the various vaccine types. Immunity to infectious microorganisms can be achieved by either active or passive immunization. Furthermore, immunity can be acquired by natural processes or by artificial means involving administration of Abs or vaccines. Detailed knowledge of the mechanism of action is a prerequisite for beneficial interference *in situ*ations where the immune system is out of balance.

Active immunization with a dead or attenuated viral pathogen generally provokes a long-term protective memory. Similar results are observed using vaccinations with virus-like particles (VLPs) against human papilloma virus (HPV) infections (7) or with bacterial toxoids, such as tetanus (8). Therefore, active vaccination has the goal of stimulating the immune system to generate a specific and sustained response. Such a response can consist of Abs (humoural immune response), T cells (cellular immune response), or both (combined immune response).

In contrast, passive immunization with polyclonal serum (9) or MAbs (10, 11) against the pathogen provides instant help *in situ*ations where the disease or infection has already manifested in the body. However, no long-term protection can be achieved since the treatment does not generate an immunological memory and the effect vanishes with the decay of the therapeutic (12).

3.2. Prophylactic versus therapeutic vaccines

Vaccinations can be distinguished according to the time point of application. An active vaccination can be performed prophylactically, that is before infection or with the purpose of treating an existing disease. In contrast to prophylactic vaccines that are extensively known and used (1, 7), therapeutic use of active vaccination is a relatively new approach. In this respect, it is important to closely examine the target antigens. Targets for therapeutic vaccines can be either foreign- or self-antigens. Depending on the nature of the antigen, the vaccine has to fulfil different criteria. A vaccine against tumour cells (self-antigen), for instance, should stimulate specific T cells in order to be effective (13), while a vaccine against microbes (foreign antigens) should lead to the generation of neutralizing Abs (14).

3.3. B cell versus T cell responses

It is expected that Abs will deal with the pathogen itself (i.e., bacteria, free viruses, and parasites) and T cells act upon infected cells. With this paradigm in mind, what would be the best or appropriate attack against malignant cells or pathologically over-expressed self-structures such as tumour necrosis factor alpha (TNF-alpha) in inflammatory autoimmune diseases? Researchers are trying different approaches. Let us take a closer look at tumour cells. With their tumour-antigen expression profile, do they resemble more parasites, bacteria or viruses, or are they more like infected cells? In the first case, a humoural immune response would be appropriate to deal with the disease. In fact, numerous therapeutic Abs are under investigation (15). The success of these Abs, either passively given or actively stimulated, is diverse. It seems that Abs are a powerful weapon to identify and eliminate single circulating tumour cells, resulting in a prolongation of tumour-free time. However, as a therapeutic to decrease the tumour burden, they are rather disappointing (16).

However, if tumour cells are considered to be more like infected cells, then a cell-mediated immune response would be appropriate. As is the case for infected cells, the immune system requires the presentation of the tumour antigen in association with MHC class I or II; only then can T cells be activated. In this respect, a T cell vaccine is more likely to reduce a tumour mass. Taking both options into consideration, one comes to the conclusion that malignant cells bear both features, those of invading pathogens as well as of infected cells, thus suggesting that a combination therapy consisting of both humoural and cellular attacks will likely be the most effective.

4. ANTIGENS USED FOR VACCINATION

4.1. Carbohydrates

Carbohydrates or polysaccharide antigens are large molecules consisting of repeating epitopes that are not processed by antigen-presenting cells (APC), but interact directly with B cells, inducing antibody synthesis in the absence of T cells (thus designated T-independent antigens). T-independent responses are restricted in a number of ways. For example, they fail to induce significant and sustained amounts of antibody in young children (17). While polysaccharides are immunogenic in

older children and adults, the characteristics of the antibody responses are rather restricted. They are dominated by IgM and IgG2, are relatively short lived, and a booster response cannot be elicited on repeated exposure. This failure to induce immunological memory is also reflected in the absence of demonstrable affinity maturation.

4.2. Proteins

While attenuated pathogens provide effective protection from viral infections (18), it is rare to see an attenuated version of a bacterial or parasitic pathogen used as vaccine (19). Thus, the choice is generally limited to immunization with killed pathogens. Activation of both the humoural and cellular immune responses is important (20-22). As the genome sequences of many pathogenic bacteria have become available, a new systematic approach for identification of vaccine candidates, termed reverse vaccinology, has been developed (23). The process begins with the identification of all putative surface proteins, which are a logical choice for vaccine candidates. The surface proteins can be predicted from genomic sequences using computer programs based on signal peptides, transmembrane helices, and other surface protein prediction algorithms (24). The candidate genes are then cloned and the proteins expressed in *Escherichia coli*. The immune responses of animals are determined after injection of the purified proteins. This approach was successfully applied to identify vaccine candidates in *Neisseria meningitidis* and has since been applied to identify vaccine candidates for other bacterial pathogens. However, the developed vaccines are constantly challenged by continuous adaptation of surface meningococcal structures to external stimuli resulting in a genetic shift of the epitopes initially recognized by immune responses (25).

T cells influence antibody responses to protein antigens. The consequence of this T cell help is that antibody responses to protein antigens can be elicited in immature immune systems. In addition, immunity is long lived due to the generation of immunological memory. Antibody responses to protein antigens are dominated by the IgG1 and IgG3 subclasses, and affinity maturation influenced by B-cell receptor-antigen binding can be demonstrated over time (26).

4.3. Peptides

Small peptides and in particular self-peptides are poorly immunogenic by themselves and require co-administration with strong adjuvants. For example, Kel *et al.* (27) administered a self-peptide derived from the proteolipid protein together with complete Freund's adjuvant to mice with experimental encephalomyelitis and observed an inhibition of disease progression. In contrast, self-peptides derived from the glucose-6-phosphate isomerase protein have been shown to induce autoimmune arthritis in a murine model (28). The use of self-peptides also carries the risk of enhancing the pre-existing disease instead of healing it.

4.4. Conjugates

A conjugate vaccine is created by covalently attaching a poorly immunogenic antigen to a carrier protein, thereby conferring the immunological attributes of the carrier on the attached antigen. This technique for creating an effective immunogen can be applied to peptides, small chemical entities, and last but not least to bacterial polysaccharides for the prevention of invasive bacterial disease.

The ability to enhance the immunogenicity of polysaccharide antigens was first noted by Avery and Goebel in 1929 (29, 30). They demonstrated that the poor immunogenicity of purified *Streptococcus pneumoniae* type 3 polysaccharide in rabbits could be enhanced by conjugation of the polysaccharide to a protein carrier. Their observations have formed the foundation for the modern development of conjugate vaccines.

Recent investigations of HIV-1 infections have demonstrated that virus-like particle (VLP)-carbohydrate conjugates are even more immunogenic when the carbohydrate motif has been slightly altered (31). A vaccine composed of a VLP-carrier conjugated to gp120-derived glycans was able to elicit specific Abs that recognized the altered gp120. Additionally, binding could be inhibited by the known anti-HIV-1 MAb 2G12. However, generated Abs did not show cross-reactivity with wild-type gp120 (31).

Following animal studies, initial human infant studies confirmed the immunogenicity of *Haemophilus influenzae* type b (Hib) capsular polysaccharide (polyribosylribitol phosphate [PRP]) conjugate vaccines. Formulations of Hib conjugates with different protein carriers, including tetanus toxoid, diphtheria toxoid, mutant diphtheria toxin, and outer membrane protein, have been developed and have been shown to vary both quantitatively and qualitatively in their immunogenicity (32). For example, the PRP antigen conjugated to outer membrane protein has been shown to be immunogenic following a single dose in infancy, while the other formulations have only demonstrated significant immunogenicity after two or three doses. Antibody avidity induced by different Hib conjugates has also been shown to vary, as has Hib variable region gene usage. Prototypes of the pneumococcal and meningococcal conjugate vaccines demonstrated enhanced immunogenicity compared with plain polysaccharide formulations in infants and young children. Furthermore, formulations using different carrier proteins have similarly been shown to vary in their avidity.

The relative importance of memory versus circulating antibody levels for clinical protection by conjugate vaccines is unclear; however, it is interesting to note that even the least immunogenic of the Hib conjugates, PRP-D, has been shown to be efficacious in reducing the incidence of invasive Hib infection in Finland. The efficacy of such formulations may thus be related to the ability of the conjugate vaccines to prime for memory, even in the face of poor primary immunogenicity (33). Demonstration of the presence of immunological memory is thus increasingly being used in the evaluation of further formulations. The success

of the Hib conjugate vaccines in reducing the incidence of invasive Hib disease in childhood has accelerated the development of conjugate vaccines designed to prevent infection by other encapsulated bacteria. The imperative driving the development of such vaccines has been the need to find a vaccine formulation that renders bacterial capsular polysaccharides immunogenic in those patients who are most at risk for infection.

5. TREATMENT MODALITIES

5.1. Antibodies against self-antigens

During the past few decades, increasing numbers of MAbs against self-structures have been developed to cope with various diseases. Initially very successful, these MAbs turned out to carry increasing risks of unwanted side effects. One of the best-investigated examples is rheumatoid arthritis (RA) where MAbs against TNF-alpha reduce inflammatory symptoms in the patient, but also increase the patient's susceptibility to infections like tuberculosis (34). In this context some researchers are sceptical of the safety and efficacy of such approaches (35).

Vaccination against self-structures is an old concept adopted from the area of cancer therapeutics. Here, researchers tried for a long time to stimulate Abs effective against cancer cells. Cancer cells are basically self-structures with a misdirected cell program and the immune system has learned to tolerate such altered tissue. This tolerance was originally thought to be manifested by the absence of autoreactive immune cells; however, recent research has shown that it is more likely due to regulation/suppression of existing autoreactive cells (36, 37). This self-tolerance has to be overcome if an effective anti-tumour therapy is to be achieved. As a consequence, one has to try to develop an artificial autoimmune disease in the cancer patient where immune cells are directed against self-structures. If the self-tolerance has been broken, the important task in this scenario is how to control the resulting immune response, so that it is not overacting and/or causing negative side effects.

Immunosuppressive MAbs act by one of two general mechanisms. Some MAbs trigger the destruction of lymphocytes *in vivo*, and are referred to as depleting [e.g., Rituximab (38)], while others are non-depleting and act by blocking the function of their target protein without killing the cell that bares it [e.g., Ipilimumab (39)].

Autoimmune disease is only detected once the autoimmune response has caused tissue damage or disturbed specific functions. There are three main approaches to treatment. First, anti-inflammatory therapy (e.g. IL1) can reduce tissue injury caused by an inflammatory autoimmune response; second, immunosuppressive therapy (e.g. steroids) may be aimed at reducing the autoimmune response; and third, treatment may be directed specifically at the organ systems damaged by the disease. The diabetes induced by autoimmune attack on pancreatic cells is treated with insulin. Insulin is used to directly compensate for the loss of beta-cells while most recently anti-IL-1b therapy has shown additional benefit of anti-inflammatory action. Anti-inflammatory therapy for autoimmune diseases includes the use of anti-cytokine Abs; anti-TNF-alpha, or more recently anti IL-17 Abs induce striking temporary remissions in rheumatoid arthritis (40, 41). Abs can also be used to block cellular migration to sites of inflammation. For example, anti-CD18 Abs prevent tight leukocyte adhesion to vascular endothelium and reduce inflammation in animal models of disease (42). In contrast to passive immunization with MAbs, several approaches have explored to break self-tolerance and thereby to stimulate a therapeutic immune response. Approaches, such as a combination of chemotherapy and protein vaccination [murine cancer model (43)] or a virus-like particle conjugate [human hypertension (44)] positively interfere with the immune system. Additionally in cancer, the tumour microenvironment can be modified with transforming growth factor-beta to increase susceptibility of the immune system towards the vaccine (45-47). Selective inhibition of IgE may benefit patients with allergies. In animal models, for example, MAbs to IL-4 have been used to decrease IgE production (48).

5.2. Cellular vaccines

Cellular vaccines using antigen-presenting cells, such as dendritic cells (DCs), are known to reliably generate effective T cell immunity. Recently, virus-infected DCs that express Her-2/*neu* have been reported to induce stronger Her-2/*neu*-specific cytotoxic T lymphocytes (CTLs) than did DNA vaccination (49). Furthermore, several reports have shown that mature DCs can break self-tolerance against tumour-associated antigens, thus inducing activated self antigen-specific CTLs (50-53). Although a peptide-loaded DC immunization can break self-tolerance at the cellular level by activating autoreactive CTLs, host levels of antitumor responses are governed by a diverse regulatory mechanism established between the host and tumour environments.

6. APPLICATION TO CANCER VACCINES

6.1. Peptide-based vaccines

Often a peptide is not sufficient to immunize, but needs to be combined with T cells and cytokines (54). In this way, the tolerant (silent) stage of autoreactive T cells can be converted (55), which is vital for development of an effective vaccine against pathologic autoantigens. Although many adjuvants have been investigated for peptide immunotherapies, to-date current strategies such as particulates, oil emulsions, toll-like receptor ligands, immunostimulatory complexes, and other biologically sourced materials utilize chemically or structurally heterogeneous materials, making their characterization, mechanistic understanding, and anticipation of side-effects challenging (56-59). In general, the development of peptide vaccines has been challenged by imprecise antigen display and the use of heterogeneous immune adjuvants whose mechanisms of action are both complex and incompletely understood (45).

Within the last decade, we and others (60-67) proposed vaccines based on low-similarity peptides (e.g., peptide sequences not present or scarcely represented in the

host proteome). This approach promises to be specific, flexible, and universal. Indeed, synthetic peptides are useful as antigens because their precise chemical definition allows one to specify the exact epitopes against which an immune response has to be raised (68, 69). Moreover, short peptide modules can be easily synthetized and administered, are less likely to induce collateral adverse events (70, 71), may be selected for activity against a broad range of (sub)strains and species of a given microbe, and are quickly modifiable to fight emerging mutated types (72-74).

Finally, and of no less importance, short epitopic amino acid modules might also be of help in autoimmune disorders. Specifically, they have the potential to block circulating autoantibodies against recognizing self-molecules and tissues (75).

6.2. Immunization with mimetic peptides (mimotopes)

The definition of epitope peptide mimics, i.e., mimotopes, was made feasible by the screenings of random peptide phage display libraries (76). Immunization with mimotopes may induce epitope-specific anti-cancer Abs (77). They are capable of eliciting Abs with biologic properties comparable to those of the original MAbs, with the advantages of production by the patient him- or herself. Additionally, the resulting Abs are not restricted to one given isotype, but are of various antibody classes, and thus able to mediate the full range of immune effector mechanisms. Moreover, the induction of immunological memory could prove beneficial in the event of disease recurrence. So far only animal studies are available, but these are very promising for a variety of antigens, and warrant translation of this approach into humans.

Recent investigations have transposed the principle of vaccination against tumour antigens using carbohydrates, particularly GD2. GD2 is a ganglioside expressed on tumour cells of neuroectodermal origin, and the antigen used for vaccination is a peptide mimic of GD2. This so-called mimotope elicited GD2 cross-reactive IgG Abs, as well as MHC class I-restricted CD8+ T cells, to syngeneic neuroblastoma tumour cells (78). Furthermore, the same principle is applied to certain other carbohydrate tumour antigens. It was shown in non-human experiments that immunization with a carbohydrate-peptide conjugate resulted in a substantial humoural immune response specific for antigen-expressing tumour cells (79, 80). Additionally, small chemical entities profit from conjugation to a carrier and gain immunogenicity in humans (81).

One special type of conjugate contains a carrier component that resembles viral structures. So-called 'virus-like-particles' (VLPs) are typically protein shells with an ordered structure that displays the antigen of choice in a repetitive way. Unlike 'real' viruses, VLPs do not replicate or integrate into the host's genome. The strong immunogenicity of VLPs helps to break the self-tolerance to stimulate a humoural immune response, at least in animal disease models of clinical relevance (82, 83). Several VLP-based vaccines have been shown pre-clinical efficacy (84-86) and some have entered clinical development (44, 87, 88).

6.3. Towards low-similarity peptides in the design of vaccines

A wide array of themes has unfolded in the previous paragraphs, although they do not cover all research areas due to space constraints. Nevertheless, we have shown several pathways that scientists have explored to find 'the vaccine'. The theoretical vaccine formulation platform must be able to fight/neutralize a disease, be safe for the patient, and be globally applicable. However, looking at the numbers, it seems that despite the high hopes, the results have been scarce in terms of global health. In 2011, we still witness the emergence of new infectious diseases, the re-emergence of old diseases, and the persistence of intractable diseases. For example, influenza pandemics and West Nile virus outbreaks represent constant threats. HIV/AIDS, HCV, human B19 erythrovirus, malaria, and tuberculosis – to cite only a few diseases - show an increasing incidence, and most attempts to develop vaccines have failed (89-91). Cancer continues to be a plague (92, 93). With the due caveats and proper proportions, the current clinical situation is not so much different from that of 1905, when Ehrlich shifted from immunology to chemotherapy: *"I have, generally speaking, the impression that it is necessary that I concentrate all of my energy, consistent with my innate ability, to chemical therapy. Now is the moment to confront the major types of illness (protozoan diseases) from the direction of chemical approaches, which are not very open to immunization therapy"* (94), as cited by Silverstein (95). In other words, the immunological armamentarium accumulated during the last century and outlined above has not defeated cancer and infectious diseases. In this regard, the main obstacles to the translation of this immunological theoretical framework into effective clinical applications are represented by unsolved phenomena such as the heavy degeneracy of MHC molecules (96-98).

The broad binding capacity of MHC molecules and the consequent lack of discrimination expected in their peptide-binding capability are obvious obstacles to specific immune targeting. To complicate the issue, Buus *et al.* (99) observed that only 5-10% of affinity-purified MHC class II molecules are available to bind, the others being constitutively occupied by self-peptides. It seems that evoking effective T cell responses implies careful tests such as analysis of the TCR affinity threshold delimiting maximal CD8 T cell function (100, 101), identification of factors which accelerate the dissociation of the peptide from an unstable intermediate of the binding reaction, thus mediating the binding of the high-affinity peptide to class I (102), and measurement of the peptide-MHC class II complex stability that possibly governs CD4 T cell clonal selection.

An additional major safety question is how to control an immune response to self-peptides in a way that does not lead to overacting autoimmune disease. One major objection to vaccination protocols consists of the potential adverse effects that are ascribed to adjuvants (103-105).

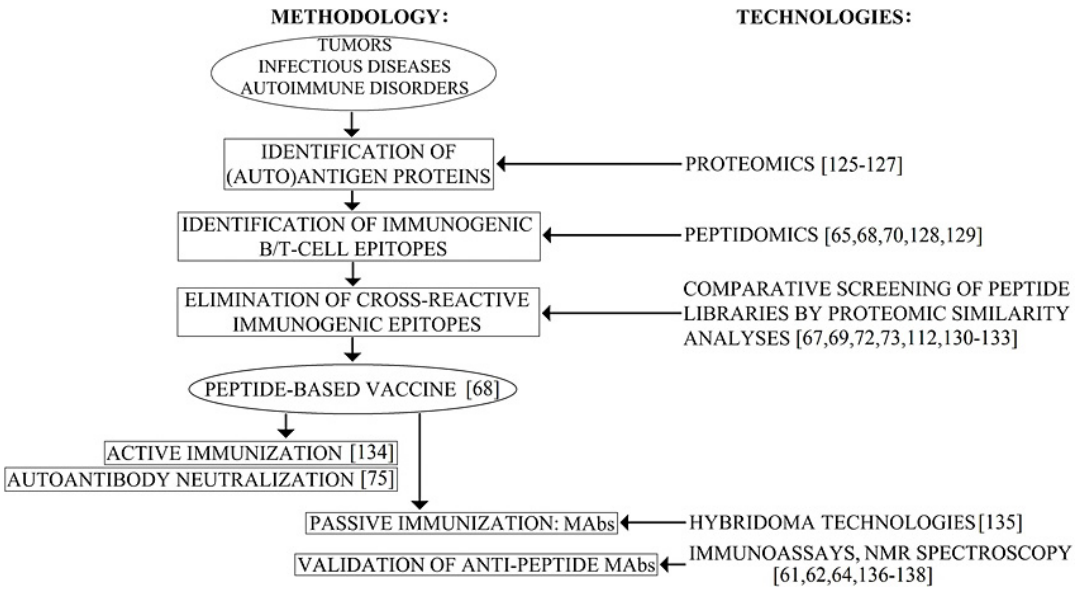

Figure 1. Peptide-based vaccines: methodology and technologies

It is possible that, as recently suggested (106-109), adverse effects ascribed to adjuvants and cross-reactions with the host proteins might be related. Indeed, a massive peptide overlap exists between microbes and human proteins (73, 106, 107, 110-117). Hence, it is logical to hypothesize that human anti-microbial immune reactions are prevented by the tight self-tolerance mechanisms that protect our organism. That would also explain the well-known phenomenon of microbial escape from immune surveillance and the consequent necessity of adding adjuvants to anti-microbial vaccine formulations in order to evoke immune responses. In parallel, the highest peptide redundancy exists among human proteins, thus underlying tumour escape mechanisms (113, 118). In addition, adjuvant-induced immunogenicity might also derange immune system activity and its fine modulation, therefore explaining autoimmunity, which usually accompanies cancer regression and microbe neutralization following adjuvanted vaccination (108, 119-122).

In such a complex context, analysing the structural and molecular features of the interactions between effector cells, Abs, and antigens at the peptide level might provide the tools for designing targeted vaccines. Indeed, analysis of peptide commonality between the antigen and the human host appears to be a practical method for designing safe, targeted, and effective peptide vaccines (65, 66, 71-74, 123). Selection of peptide sequences unique to microbes or tumour-associated-antigens would specifically hit the microbial agents or tumours without cross-reacting with host proteins. That is, the risk of autoimmunity would be nullified in such low-similarity peptide vaccines. The positive implications of this approach for clinical practice would obviously be paramount.

In conclusion, notwithstanding our increasing knowledge of the mechanism of vaccination, there is still a long way to go until therapeutic vaccines can be broadly used. The most critical obstacles are firstly, the multiple measures the immune system can take to prevent or circumvent autoimmunity; it is of course of eminent importance for the survival of the organism to avoid self-attacks. Secondly, one has to be able to control a potential therapeutic autoimmune response; otherwise the effects are even worse (124). This can be accomplished via neutralizing Abs or immune-suppressant treatments. However, in any case the benefits and risks have to be judged before a therapeutic vaccination is started.

Within this framework, based on the scientific and clinical problems that have constellated the field of vaccine research, Figure 1 illustrates a methodological pathway and describes already available technologies for designing safe and effective immunotherapeutic approaches against cancer, infectious diseases, and autoimmune pathologies.

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