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1 **CURRENT PERSPECTIVES**

2 **Neopitopes-based vaccines: challenges and perspectives**

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13

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16 **ABSTRACT**

17 First generations of cancer vaccines using shared tumor antigens have been associated with
18 disappointing clinical results. However, the paradigm shift introduced by immune checkpoint inhibitors has
19 led to a renewed interest on anti-tumoral vaccination based on mutation-associated neoantigens. First
20 clinical results are encouraging with some signs of clinical activity associated with induction of a specific
21 immune response. In advanced or metastatic diseases, vaccination may either enhance the response to PD-
22 1/-L1 antagonists by increasing the number of effectors within the tumor or induce an anti-tumoral T cell
23 response in immunologically "cold" tumors. There is also a strong rationale to use cancer vaccines in an
24 adjuvant setting to induce a long-term control of the residual disease. Prediction of neoepitopes efficiently
25 presented by HLA molecules remains a challenge, as well as identification of clonal neoantigens. Some
26 mechanisms of resistance are already identified, such as tumor loss of neoepitopes-presenting HLA class I
27 molecules. In this context, the role of CD4+ T cells induced by different cancer vaccines should be clarified.
28 Finally, while studies have focused on mutated epitopes corresponding to single nucleotide variants, other
29 neoantigens could be of strong interest such as those linked to tumor specific RNA-splicing abnormalities or
30 associated with insertions-deletions.

31

32 **Keywords:** Personalized vaccine, cancer, neoantigens, neoepitopes

33

34 **TOWARDS PERSONALIZED VACCINES**

35 First generation cancer vaccines have shown relatively disappointing clinical results, with less than
36 7% objective clinical responses and an overall clinical benefit rate estimated around 20% [1]. The use of non
37 tumor-specific antigens, which elicit low affinity T cell response due to elimination of the most reactive T
38 cells by central tolerance, is one of the multiple factors that explain these results. Tumor-specific antigens
39 (neoantigens) derived from tumor gene mutations or rearrangements have been reported to induce
40 stronger immune responses in the absence of central tolerance [2]. The abundant literature around
41 mutation-associated neoantigens (MANA) together with the paradigm shift introduced by immune
42 checkpoint inhibitors (ICIs) led to a renewed interest in the field of cancer vaccines. Indeed, multiple studies
43 showed a link between clinical responses to ICIs and tumor mutational burden in melanoma [3], lung cancer
44 [4] or colorectal cancer [5]. Furthermore, a linear relation was observed between anti-PD1/-L1 objective
45 response rate and the median number of coding somatic mutations per megabase of DNA [6]. Since
46 mutational burden is a reflect of the quantity of MANA [7], these latter may represent the main targets of
47 activated T cells after ICI therapy.

48

49 **NEOEPITOPES-BASED VACCINES: CURRENT CHALLENGES**

50 *Neoepitopes selection*

51 The identification of neoepitopes relies on different steps. First, a precise detection of tumor somatic
52 mutations is performed by combined whole exome sequencing of tumor and normal cells [8]. A matched
53 RNA expression analysis (like RNA sequencing) of tumor cells is added to select expressed mutations.
54 Prediction softwares are then used to identify potential neoepitopes with a high affinity for the individual's
55 major histocompatibility complex molecules (MHC) [9]. However, classical algorithms have a limited
56 predictive value as they do not take into account the different steps of epitope processing. Moreover, the
57 affinity threshold needed to elicit a cytotoxic T-cell response has been mostly validated on viral epitopes and
58 may not be adapted for mutated self-peptides [10]. In a set of 448 potential CD8-T-cell epitopes identified in

59 a patient with melanoma, Rooij et al. found that less than 1% were effectively recognized by the patient's T
60 cells [11]. The positive predictive value of such tools is therefore pretty low and different additional steps
61 (such as proteasome cleavage/TAP transport predictions or self-proteins cross-reactivity verification) can be
62 added to the prediction [12,13]. Recently, a quality-control step considering the probability of recognition of
63 a neoepitope by a T-cell receptor has been proposed, using a model based on sequence similarity with that
64 of known antigens [14]. It is also possible to verify predicted epitopes' MHC affinity by binding assay or to
65 test their immunogenicity by *in vitro* stimulation assays.

66 The fact that a majority of predicted class I epitopes may not be effectively presented by MHC class I
67 molecules on tumor cells remains a major concern. A validation step has been added by some authors, using
68 mass spectrometry for identification of peptides eluted from tumor MHC class I molecules [15]. The
69 sensitivity of such approach is however limited to neoepitopes with relatively high expression, preventing
70 the detection of less abundant but still immunogenic peptides [16]. The optimization of MHC class II
71 neoepitopes prediction is another challenge (MHC class II epitopes harbour a high diversity with variables
72 sizes rendering prediction more complicated than for MHC class I epitopes), considering the potential role of
73 CD4+ T cells response after neoepitopes-based vaccine [17,18].

74

75 *Formulation*

76 Different formulations or antigen sources currently exist. DNA vaccines consist in transfecting a DNA
77 sequence encoding for the neoepitopes of interest. While unmethylated CG-rich DNA was supposed to
78 provide an immune adjuvant by TLR stimulation, poor immunogenicity and clinical activity were observed
79 [19]. In the opposite, RNA vaccines harbour a convincing profile with a potential adjuvant effect (by TLR 7 / 8
80 stimulation) and multiple epitope encoding possibility [20]. However, this TLR stimulation precludes the
81 combination with other adjuvants. Conversely, peptide-based vaccine can be combined with any adjuvant.
82 Of note, the diversity of possible adjuvants has already been reviewed elsewhere and is beyond the scope of
83 this article [2,21]. As single peptide formulation is associated with a higher risk of immune escape, multi-

84 peptides approaches are usually developed [22]. With their potential to stimulate both CD4+ and CD8+ T cell
85 response, long peptides (20-30 mer) are indeed being more and more considered [23]. Other formulations
86 such as viral vectors, *ex vivo* generated dendritic cells or whole cell have also being developed. The
87 impressive results obtained in infectious diseases with viral vectors, eliciting a strong cytotoxic T-cell
88 response in terms of quality and number, merit a particular attention [24]. Considering the importance of T
89 cells numbers in clinical responses after adoptive T cell therapies, it makes no doubt that vectors inducing a
90 higher number of effector T cells compared to peptides or mRNA approaches would be of a great interest.

91

92 *Tumor heterogeneity*

93 Given tumor heterogeneity, especially at an advanced stage, there is a high risk of selecting epitopes
94 present in certain tumor clones only ("subclonal" epitopes). In Carreno's study a majority of the identified
95 neoepitopes were not found at the different tumor sites [25]. Assigning a clonal (shared by all the tumor
96 clones) or subclonal (specific to a subclone) status to a mutation remains difficult and requires data of
97 excellent quality. Moreover, as they do not confer any survival advantages, passenger mutations could be
98 lost without any counterpart for tumor cells.

99 Since most mutations occur randomly in passenger-type genes, the frequency of mutated
100 neoantigens shared between different tumors is very low. It was found that out of a total of 911,548
101 mutated neoantigens, only 24 were shared in at least 5% of patients [7]. A recent study found that, among
102 3,760 predicted neoantigens, only 0.42% were found in more than one tumor [13]. It is therefore necessary
103 to define for each patient a panel of neoepitopes in a purely personalized approach. Considering the impact
104 of the presence of clonal versus subclonal neoepitopes in the quality of the anti-tumor immune response
105 [26], it will theoretically be appropriate to favour clonal neoepitopes or a mixture of neoepitopes
106 representative of the main subclones, prioritizing when possible neoepitopes derived from driver mutations,
107 which adds complexity to the neoepitopes selection method. The delay related to this complex process of

108 production may be an issue for the use in advanced progressive diseases. One possibility is to start with a
109 vaccine containing shared tumor antigens before combining the personalized vaccine [27].

110

111 *Mechanisms of resistance*

112 A possible limitation of this approach is the availability of the patient's T repertoire. It was observed
113 that the T-cell repertoire of healthy donors contained T cells recognizing MANA for which specific intra-
114 tumor T lymphocytes were not found in the corresponding tumors [28].

115 A resistance mechanism already identified in Sahin's study is the β 2-microglobulin loss leading to the
116 absence of MHC class I molecules on tumor cells [27]. This mechanism has also been identified in acquired
117 resistance to PD1/-L1 antagonists [29]. More subtly, the loss of heterozygosity (LOH) of HLA alleles
118 corresponding to MHCs presenting mutated neoepitopes could represent an initial or acquired resistance
119 mechanism. HLA LOH has been shown in 40% of non-small cell lung cancers, and is associated with a large
120 amount of subclonal neoantigens [30]. It will therefore be necessary to verify the quality of MHC expression,
121 and ideally to select neoepitopes according to the corresponding MHC expression. Optimisation of high
122 affinity MHC class II neoepitopes prediction will also help mounting a diversified response by adding CD4+ T
123 cells anti-tumoral effects.

124 Finally, advanced tumor-associated immunosuppression represents a general resistance mechanism
125 to immunotherapy. In this context, there is a strong rationale for combining vaccine approaches with anti-
126 PD-1/-L1 from the outset, so as to inhibit the resistance induced by IFN- γ response [31] or even favour a *de*
127 *novo* immune response and initial T cell activation [32].

128

129 **PERSPECTIVES**

130 *Addition of other tumor antigens*

131 While ongoing studies have focused on mutated epitopes corresponding to single nucleotide
132 variants (SNVs), other neoantigens could be of great interest such as those derived from tumor specific RNA
133 splicing abnormalities [33] or from insertions-deletions (indels). Indels could be a major source of
134 neoantigens and a recent study suggests a higher frequency of epitopes with high MHC affinity for epitopes
135 derived from indels compared to SNVs. In addition, the localisation of indels in tumor suppressor genes
136 could also be associated with a higher probability of obtaining shared antigens [34]. The combination with
137 PD-1/-L1 antagonists also highlights the possibility of using some cancer germline antigens [35].

138

139 *Improving T-cell response*

140 Targeting multiple neoepitopes, together with stimulating a CD4+ T cell response, is a promising way
141 to induce an efficient immune response against the tumor. Sahin *et al.* reported the feasibility and
142 immunogenicity of a mRNA-based vaccine targeting multiple selected neoepitopes in melanoma [27].
143 Responses were detected against 60% of the predicted neoepitopes, with 57% of isolated CD4+, 17% of
144 CD8+ and 26% of combined CD4+ and CD8+ responses. Ott *et al.* also demonstrated the immunogenicity and
145 feasibility of a vaccine that targets up to 20 predicted neoantigens in patients with melanoma [36]. Using
146 long synthetic 15-30 mer peptides with poly-ICLC (Hiltonol) as adjuvant injected subcutaneously, the authors
147 reported both CD4+ and CD8+ T cell responses, respectively targeting 60% and 16% of the vaccine
148 neoantigens. These results underline the importance of the CD4+ T cells in supporting CD8+ response and
149 providing additional anti-tumoral effects. Of note, CD8+ T cell response was evaluated after 2 weeks of *in*
150 *vitro* re-stimulation in Ott's study. It is thus not possible to conclude that the vaccine really induced a specific
151 response in patients since an *in vitro* priming against the tested antigens cannot be formally excluded. Still,
152 four out of six vaccinated patients had no recurrence 25 months after vaccination. The two others with
153 recurrent diseases were successfully treated with anti-PD-1 therapy inducing a complete tumor regression.

154 Combination of cancer vaccine with ICI can also help improving T cell response by promoting T cell
155 activation and epitope spreading [37,38]. In Sahin's study, the only complete response was observed in

156 combination with PD1 blockade [27]. Several studies are currently underway based on this scheme: for
157 instance, NCT02897765 study adds a personalized vaccine after 3 months of nivolumab in different types of
158 cancers. The advantage in this context of advanced disease is the possibility of performing sequential tumor
159 biopsies to evaluate changes in the immune infiltrate following vaccination, in addition to demonstrate the
160 induction of a specific immune response detected in blood. Furthermore, neoepitopes-based vaccines could
161 be an interesting approach in combination with ICIs for diseases with low or no response to ICIs alone
162 despite a significant mutational burden. This is the case for microsatellite stable colorectal cancer, where
163 response to immunotherapeutic approaches (eg T-cell recruiting bispecific antibody with atezolizumab) have
164 shown clinical responses in favor of a functional immune system [39]. This may represent an interesting
165 proof of concept of efficacy.

166 Depleting the immunosuppressive milieu (by using cyclophosphamide for regulatory T cells depletion
167 or gemcitabine for myeloid derived suppressor cells) or promoting T cell expansion by the use of cytokines
168 such as IL-7 are other ways for improving T cell response [40,41].

169 Finally, combination of different immunotherapies will probably be required to eradicate advanced
170 tumors. Moynihan *et al.* evaluated in a murine syngeneic tumor model, a combination including a tumor-
171 targeting antibody, recombinant IL-2, anti-PD-1 antibody and specific vaccine [42]. This combination induced
172 tumor infiltration of both innate and adaptive immune cells, mediating a strong anti-tumoral effect with
173 eradication of large established tumors. While the tolerability of such combination must be confirmed in
174 humans, it makes no doubt that enhancing the vaccinal response by different simultaneous ways is a
175 promising approach.

176

177 *Optimisation of the timing of vaccination*

178 Most of studies have used cancer vaccines in an advanced or metastatic setting. Considering the risk
179 of higher immunosuppression and of higher resistance associated to clonal heterogeneity in case of high
180 tumor burden, it has been suggested that cancer vaccination should be used in an adjuvant setting in a

181 context of low residual disease [1]. Vaccination could be also used in a neoadjuvant setting. In this context, it
182 has been shown in murine models that T cell stimulation by ICI was more efficient in preventing metastasis
183 when applied before tumor resection [43]. In fact, more and more studies are currently being performed in
184 an adjuvant situation (Table 1). Triple negative breast cancer, in non-complete pathological response
185 following neoadjuvant chemotherapy, is well suited to this type of approach. The risk of relapse is high and
186 the vaccine is set up in a context of low tumoral mass after conventional treatment combining neoadjuvant
187 chemotherapy, surgery and radiotherapy. Several phase I trials are underway in this context, such as
188 NCT02348320 study. It will be necessary to set up control groups in this setting to demonstrate a benefit in
189 terms of survival. Furthermore, the absence of biopsy-accessible tumors restrains the immunological
190 analyses to the blood level. Nevertheless, it is likely that the adjuvant setting in pathologies at high risk of
191 relapse represents a positioning of choice for vaccine approaches, used alone or in combination with an anti-
192 PD-1/-L1.

193

194 **CONCLUSION**

195 Vaccination based on specific neoantigens in a personalized approach opens new therapeutic
196 perspectives in oncology. Recent studies have indeed provided a clinical proof of concept in melanoma
197 [25,36]. Immunogenicity is however too often considered as a surrogate marker of efficacy, and objective
198 responses remain low. In advanced metastatic diseases, vaccination may either enhance the response to
199 PD-1/-L1 antagonists by increasing the number of effectors within the tumor, or induce an anti-tumoral T
200 response in immunologically "cold" tumors (characterized by the absence of T infiltration), a prerequisite for
201 the activity of an anti-PD-1/-L1. The lack of HLA class I expression by tumor cells represent an important
202 limitation of this approach. Nevertheless, CD4⁺ T cells induced by vaccination may promote an effective anti-
203 tumoral response by IFN- γ secretion and stimulation of other cells from the immune environment. This
204 aspect is barely known and needs to be clarified given the importance of the CD4⁺ T cell response generated
205 during the first neoepitopes-based vaccination trials [17]. Finally, adjuvant therapy represents a privileged

206 positioning for vaccination, so as to allow long-term control of residual disease following the generation of
207 an immune response.

208

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213

214 **CONFLICT OF INTEREST STATEMENT**

215 SD is employee for Collectis and reports personal fees from AstraZeneca, Elsalys, Erytech Pharma, Netris
216 Pharma. The other authors declare no potential conflict of interest.

217

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TABLES

Study number	Promotor	Cancer	Vaccine type	Position
NCT02301611	Cancer Insight, LLC	Melanoma	DC	Adjuvant
NCT02348320	Washington University School of Medicine	Breast (TN)	DNA	Adjuvant
NCT02600949	M.D. Anderson Cancer Center	Pancreatic, colorectal	Long peptide	Advanced/metastatic
NCT02721043	Nina Bhardwaj, Icahn School of Medicine at Mount Sinai	Solid tumors	Long peptide	Adjuvant
NCT02808364	Guangdong 999 Brain Hospital	Glioblastoma	DC	Adjuvant
NCT02808416	Guangdong 999 Brain Hospital	Solid tumors with brain metastases	DC	Adjuvant
NCT02933073	UConn Health	Ovarian	Long peptide	Neoadjuvant or adjuvant
NCT02956551	Sichuan University	Carcinoma, Non-small cell Lung	DC	Advanced/metastatic
NCT02933073	UConn Health	Ovarian	Long peptide	Adjuvant
NCT03122106	Washington University School of Medicine	Pancreatic	DNA	Adjuvant
NCT03289962	Genentech, Inc.	Melanoma, Non-Small Cell Lung, Bladder, Colorectal, Breast (TN), Renal, Head and Neck	RNA	Advanced/metastatic
NCT03480152	National Cancer Institute (NCI)	Melanoma, Colon, Gastrointestinal, Genitourinary, Hepatocellular	RNA	Advanced/metastatic
NCT03552718	NantBioScience, Inc.	Colorectal, Breast, Head and Neck, Melanoma, Non-Small Cell Lung, Pancreatic, liver	DC	Adjuvant
NCT03633110	Genocea Biosciences, Inc.	Melanoma, Non-small cell Lung, Head and Neck, Urothelial Carcinoma, Renal Cell Carcinoma	Long peptide	Adjuvant
NCT03558945	Changhai Hospital	Pancreatic	NA	Adjuvant
NCT03645148	Zhejiang Provincial People's Hospital & Hangzhou Neoantigen Therapeutics Co., Ltd	Pancreatic	Long peptide	Advanced/metastatic

341 **Table 1:** Ongoing recruiting studies using neoepitopes for personalized therapeutic vaccine.

342 *DC: Dendritic Cells, NA: Non Available*