NOVEL STRATEGIES FOR ANTI-TUMOR VACCINES

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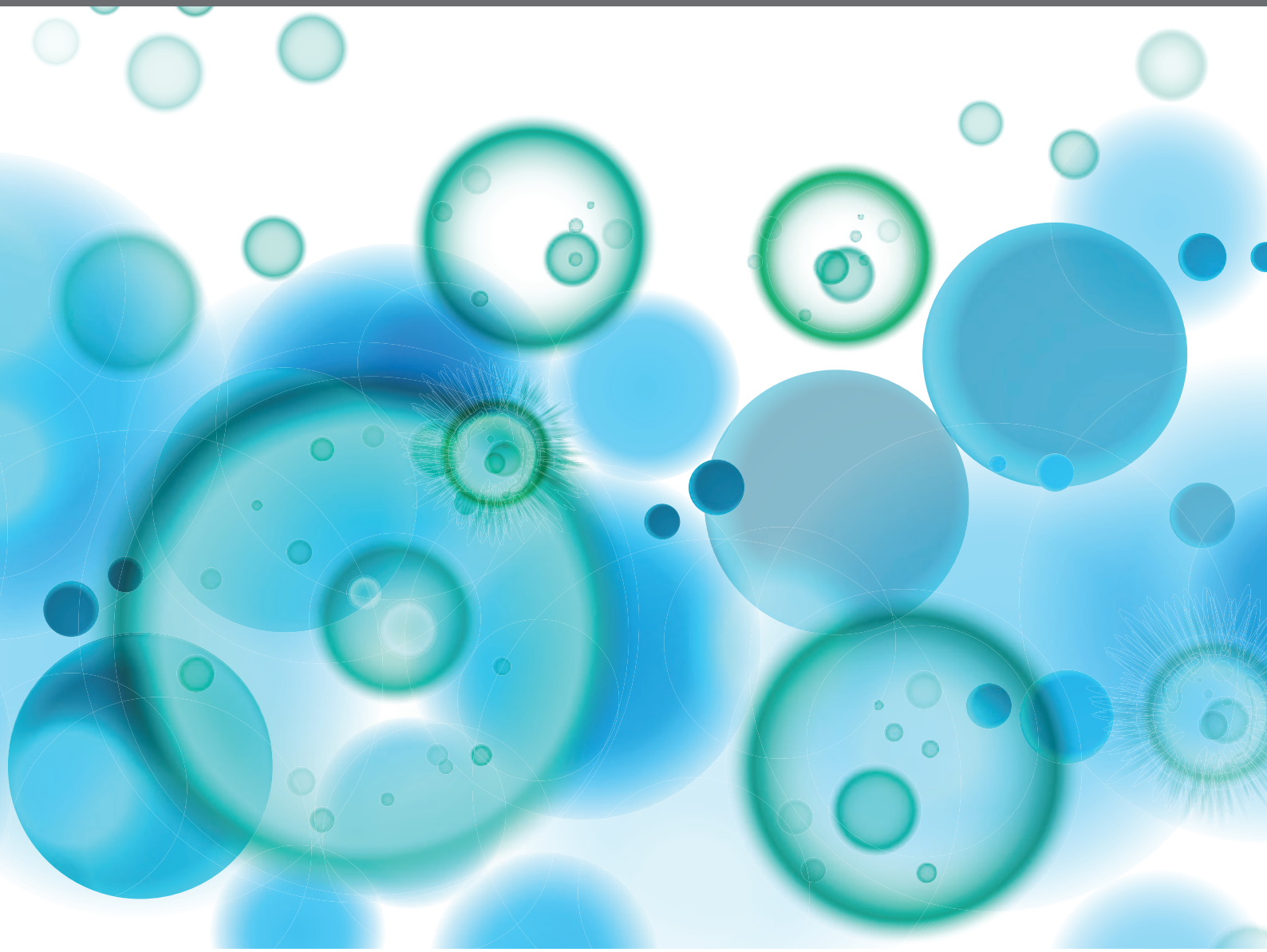

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NOVEL STRATEGIES FOR ANTI-TUMOR VACCINES

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EDITORIAL
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Editorial: Novel Strategies for Anti-Tumor Vaccines

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Keywords: tumor vaccine, HLA peptidome, tumor antigens, neoantigens, APC, T helper cells (Th)

Editorial on the Research Topic

Novel Strategies for Anti-Tumor Vaccines

The old dream of tumor immunologists is the construction of efficient anti-tumor vaccines to fight cancer. Anti-tumor vaccine strategies are necessarily focused, however, on patients who are already affected by the pathology in which the tumor not only has eluded the host initial immune response but often further creates suppressive mechanisms that keep counteracting the action of the immune system.

This Research Topic was intended to focus on several aspects of anti-tumor vaccinology and particularly on ways to increase the potency of anti-tumor vaccines by acting both on facilitating tumor antigen selection and presentation to cells of adaptive immunity and on reducing the effect of suppressive mechanisms on these immune responses. We are convinced, however, that to fight cancer single immune-based approaches cannot stand alone and thus vaccine approaches need to be complemented by other immune approaches.

Identification of the optimal repertoire of tumor antigens, in particular neoantigens, for the best use in anti-tumor vaccination is extensively discussed by Garcia-Garijo et al., who provide an overview of the existing strategies to identify neoantigens and to evaluate their immunogenicity. Indeed, only a small fraction of all tumor somatic non-synonymous mutations (NSM) identified represent *bona fide* immunogenic neoantigens, and even fewer mediate tumor rejection. Thus, the impact of neoantigens for vaccine purposes may be overestimated (1). A rich source of tumor antigens that are non-mutated but still highly tumor specific comes from analysis of the HLA ligandome landscape of tumors. To complement these studies, Fennemann et al. describe how personalized tumor vaccines containing multiple neoantigens can broaden and enhance the anti-tumor immune response. They also focus on the issue of the intratumor mutational landscape containing different tumor cell subclones, temporal and spatial diversity of neoantigen presentation and burden, and the relation to tumor immunogenicity, all parameters to be taken into account to improve clinical efficacy of personalized tumor vaccines.

The use of embryonic and pluripotent stem cells for the identification of additional suitable antigens to induce an optimal anti-tumor response are described by Ouyang et al. Approaches that combine an autologous induced pluripotent stem cells (iPSC) vaccine with an immune adjuvant

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have demonstrated great promise to elicit potent anti-tumor responses for cancer treatment. Insights on future directions are described by the authors.

Targeting tumor-associated antigens with specific antibodies in association with stimulating cytokines in novel formulations can be beneficial particularly in case of neoplastic diseases characterized by the pronounced expression of these antigens as it is the case of the unique tumor-associated form of MUC1 in pancreatic ductal carcinoma (PDA) described by Dreau et al.. Interestingly, the treatment results in infiltration of cells that can mediate ADCC function of phagocytes and reduction of suppressive regulatory cells, stressing the fact that effective anti-tumor treatment can also include a drastic modification of the tumor microenvironment.

Mimicking tumor antigens by approaches of anti-idiotype responses is revisited by Kohler et al.. They discuss advantages and limitations of this approach and explain how this old/novel strategy can be adapted in Biotech-standard production of therapeutic antibodies. As all nominal antigens, tumor antigens become immunogenic only if appropriately processed and presented by antigen presenting cells (APC). A novel therapeutic strategy of combining personalized vaccines in combination with standard therapy and anti-PD1 checkpoint inhibitors is proposed by Bassani-Sternberg et al. as a Phase1b study in resected pancreatic adenocarcinoma (PDAC) patients. The vaccine platform is based on autologous dendritic cells (DCs) loaded with mutated neoantigens and tumor-specific antigens identified through their original proteo-genomics antigen discovery pipeline. The addition of nivolumab to boost and maintain the vaccine effect underscores once more the belief that multiple immunological approaches should be used for optimal triggering and maintenance of the anti-tumor immune response.

Ameliorating and/or selecting the optimal DC subpopulation to present tumor antigens are discussed also by Zeng et al. who focus on a new type of DC, designated CD137 ligand-induced DC (CD137L-DCs), that induce strong cytotoxic T cell responses. They show that superior potency of CD137L-DCs in APC activity compared to other types of DCs is due to their intrinsic increased Akt-driven glycolysis, thus suggesting that Akt-driven glycolysis could be a therapeutic target to manipulate the function of CD137L-DCs for better clinical efficacy.

Increasing tumor antigen availability and T cell priming are crucial parameters for the efficient response to anti-cancer vaccines. Accolla et al. reviewed their work on tumor cells genetically modified by transfection with the MHC class II transactivator CIITA. These modified tumor cells can not only process and present antigens to naïve Th cells but they can also prime virgin tumor specific T cells. Their experimental approach has been extended to isolate MHC-II-bound relevant tumor peptides to formulate novel multipeptide vaccines (MHC-I + MHC-II- bound) against human hepatocarcinoma, presently in clinical trial[1].

Alternative procedures of tumor antigen presentation and T cell priming are also discussed by Schluck et al. who concentrate on artificial APC methods employing biomaterials, highly promising tools to activate T cells and evoke robust *in vitro* and *in vivo* immune responses. In this perspective, they presented an overview of molecular cues that could be used to selectively expand T cell subsets that are beneficial for strong anti-tumor immune responses.

Ways to facilitate triggering of anti-tumor immune response *in vivo* have been also analyzed by Loeffler et al. who studied the immune response of patients undergoing radio-frequency ablation (RFA) of liver metastasis from colorectal cancers. RFA induced and/or boosted T cell responses specific for individual tumor antigens were frequently detectable, but not sufficient for the rejection of established tumors, indicating once more that combination therapies, such as immune checkpoint inhibitors should be considered.

Oncolytic virus (OV) therapy is also becoming an interesting strategy not only to treat but also to trigger and/or increase the anti-tumor immune response for vaccination purposes as Marchini et al. summarized in their review. They also provide information about OV-mediated immune conversion of the tumor microenvironment. As a case study they focus on the rodent protoparvovirus H-1PV and its dual role as an oncolytic and immunomodulatory agent.

Construction of therapeutic vaccines against tumors must take into account reduced or loss of MHC-I expression in tumor cells as an important mechanism of immune escape. Abdelaziz et al. stress this point and present intriguing new data on unexpected block of MHC-I-restricted tumor antigen presentation without reduction of cell surface expression of MHC-I molecule by using a Human Cytomegalovirus (HCMV)-based vector including a HPV E6/E7 fusion protein in a murine glioblastoma model. The molecular mechanism of lack of MHC-I presentation is not fully clear but it seems to correlate with defects of proteasome function generated by the HCMV vector. Of course this should be taken in serious considerations when approaches of tumor peptide vaccination using HCMV as host for gene sequences of tumor peptides are used.

In conclusion, we are confident that this Research Topic will help to better delineate the past and present problems related to the efficacy of anti-tumor vaccines and, based on this background, develop new ideas and strategies to improve their construction and efficacy for tomorrow.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Promise of Anti-idiotype Revisited

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The promise of idiotype-based therapeutics has been disappointing forcing a new look at the concept and its potential to generate an effective approach for immunotherapy. Here, the idiotype network theory is revisited with regard to the development of efficacious anti-idiotype vaccines. The experience of polyclonal anti-Idiotype reagents in animal models as well as an understanding of the immune response in humans lends to the proposition that polyclonal anti-Idiotype vaccines will be more effective compared to monoclonal-based anti-Idiotype vaccines. This novel strategy can be adapted in Biotech-standard production of therapeutic antibodies.

Keywords: idiotype, polyclonal, vaccines, polyreactive, multi-epitope binding, therapeutic, mimetic

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1. INTRODUCTION

The strategy of using anti-idiotype (anti-Id) antibodies as surrogate antigens stems from the Idiotype cascade proposed by Niels Jerne (1). Accordingly, anti-Id antibodies were originally described as Ab2α and Ab2β, whereby the former does not block antigen binding and the latter can inhibit binding of the corresponding Ab1 to its antigen. This lent to the conclusion that Ab2β mimics structurally the antigen for Ab1. The concept and its experimental use have been extensively reviewed, (2–5). Noted advantages of using anti-Ids over nominal antigens as therapeutic vaccines include difficulties to produce vaccines containing non-protein antigens. Anti-Ids can be produced that mimic lipid, carbohydrate or nucleic acid epitopes or even drugs. Tolerance to antigens is a major hurdle in vaccine development. Antibody-B Cell Receptor binding occurs at multiple sites, while antigen strictly binds to Complementary Determining Regions (CDRs) of antibodies. This allows stimulation of a broader determinate targeting antibody response that might include epitope spreading. Finally, anti-Ids can be persistent in inducing an immune response against antigens while avoiding autoimmune responses triggered by nominal antigen based vaccines (6).

A major obstacle both theoretically and practically is reconciling the immunization concept with the postulated restriction of the putative idiotypic network of natural antibody producing B cell clones (7). Natural antibodies, in the strictest sense, are constitutively produced (8), but this strict definition leaves out some polyreactive antibodies induced in marginal zone B cells and in T-cell independent responses, which can also be defined as natural antibodies in a broader sense (9–11). The gray zone of the natural antibody concept probably contains the answers to some of the paradoxes of idiotypy. Thus, several animal studies using anti-Id antibodies support their utility, as vaccines while human trials with monoclonal Ab2β were disappointing and have failed in later phase trials. Here, we analyze this failure and propose an alternative strategy for an idiotype-based immunotherapy.

2. SETTING THE STAGE FOR THE IDIOTYPE INTERACTIONS IN REGULATING AN IMMUNE RESPONSE

In 1963 two laboratories reported evidence for a new marker on antibodies distinct from allotypes (12, 13). The term IDIOTYPE for determinants recognized by antibodies was adopted. Recognizing that antibodies against antibodies exist and playing a number game on the multitude of B-cells producing antibodies, Jerne concluded that there must be a functional network of idiotype (Id) and anti-idiotypes (anti-Id) (14). Thus, the idiotype network hypothesis was born. Yet, evidence was lacking for network interactions during an induced immune response and that an anti-Id response might have a regulating function. In 1972 several reports appeared on the potential of anti-Id antibodies to suppress a specific immune response (15–17). Such results suggested that anti-Ids can affect an immune response, but did not establish that immune-modulation is part of an antigen-induced immune response. Two reports supported this latter premise (17, 18). An idiotypic cascade was perceived: Ab1>Ab2β >Ab3. Ab3 would resemble Ab1 and were labeled Ab1'. Jerne distinguished two types of anti-Ids (1, 14): Based on this concept, Ab2β's resemble structurally the antigen; thus the term *Internal Image* of antigen emerged as an explanation for this mimicry.

Shortly after this concept emerged several laboratories put this to the test by using Ab2β as antigen to induce target-specific immune responses (19–23). The dual functional property of Ab2 was demonstrated as either suppression (15) or induction of a specific response (24) to be dependent on the IgG-class (25). The idiotypic cascade implies that Ab1 used therapeutically might induce an antigen specific antibody response (26). Clinically, support for the idiotypic cascade is suggested in that patients developing low-level Human Anti-Mouse Antibody (HAMA) to a GD2 reactive Ab1 were shown to have higher long-term survival rates than those who did not (27, 28). GD2 is a disialoganglioside expressed on tumors of neuroectodermal origin, including human neuroblastoma and melanoma, with highly restricted expression on normal tissues, principally to the cerebellum and peripheral nerves in humans. The relatively tumor specific expression of GD2 makes it a suitable target for monoclonal antibody therapy and potentially a proving ground to probe and dissect network interactions.

The idiotype cascade has been suggested to be part of the functional utility of at least one monoclonal antibody presently approved by the US FDA [dinutuximab targeting the GD2 antigen: (29)]. The FDA approved Dinutuximab (Ch14.18, trade name Unituxin) and Dinutuximab beta (trade name Isquette), a monoclonal antibody used as a second-line treatment for children with high-risk neuroblastoma. However, differences in immune responses to Ab1 might be attributed to differences in Germline origins of the selected monoclonal Ab1 used in therapeutic application. A clinical trial with Ch14.18, a chimeric, in combination with IL-2, while showing a strong activation of antibody effector functions, did not show a better clinical outcome (30). Development of human anti-chimeric antibody (HACA) (21% of patients) did result in strong reduction of ch14.18 levels, abrogating complement dependent cytotoxicity and antibody dependent cellular cytotoxicity (31). The monoclonal studied in Cheung et al. (27, 28) is of the IGVH2-9*02 germline while the ch14.18 variable region is derived from the IGHV1S135*01 germ line. Little attention is paid to such difference yet we know that no two antibodies need to be alike immunologically.

3. LESSONS LEARNED FROM THERAPEUTIC ANTI-ID ANTIBODIES

While the earlier anti-Id data were generated with polyclonal antibodies, later experiments used monoclonal anti-Ids (32, 33). The successful use of monoclonal anti-Ids as vaccines in inbred mice prompted several clinical trials with monoclonal Ab2β antibodies. The early studies on the immunomodulatory activities of Ab2, while consistently demonstrating immunological activity in animals, clinical trials with anti-Ids in the cancer space proved to be mixed (34). Herlyn and coworkers demonstrated that humoral immune reactivity against a tumor can be enhanced upon active anti-id vaccination (35). In these studies 30 patients with advanced colorectal carcinoma (CRC) were treated with alum-precipitated polyclonal goat anti-Id antibodies to monoclonal anti-CRC antibody CO17-1A (Ab1) in doses between 0.5 and 4 mg per injection. All patients developed Ab3 with binding specificities on the surface of cultured tumor cells similar to the specificity of Ab1. Furthermore, the Ab3 competed with Ab1 for binding to CRC cells. Fractions of Ab3-containing sera obtained after elution of the serum immunoglobulin from CRC cells bound to purified tumor antigen and inhibited binding of Ab2 to Ab1. Six patients showed partial clinical remission and seven patients showed arrest of metastases following immunotherapy (35). Therefore, it was concluded that the Ab3 could share binding similarities with Ab1.

In other studies, an anti-Id vaccine to induce anti-Carcinoembryonic antigen (CEA) antibodies (Ab3) was tested in non-human primates (36). CEA is a tumor marker largely utilized for the detection of minimal disease associated with colon cancer and considered a target for immunotherapy. The murine monoclonal antibody specific for CEA, was generated via hybridoma technology and selected for inhibition of the binding to CEA. These successful preclinical studies led to clinical trials in humans with CEA positive tumors (37). In this trial, 9 of 12 patients demonstrated an anti-anti-idiotypic (Ab3) response. All nine patients generated specific anti-CEA antibody demonstrated by reactivity with radiolabeled purified CEA. Toxicity was limited to local reaction with mild fever and chills. However, in all 12 patients the tumor progressed after completion of the trial. Four of seven responding patients were reported to have T cell responses to purified CEA suggesting that there was an antigen specific T cell response after immunization (37). A patent was filed for the anti-Id (Chatterjee et al. 5,977,315). Yet, a phase II trial with anti-Id did not improve relapse of tumor (38) and a phase III study with the anti-Id and 5-Fluorouracil (5-FU) did not improve the overall outcome of the study (39). In preclinical

models CEA was found to be up-regulated after exposure of cancer cells to 5-FU (40). Therefore, the premise for combination therapy would be to increase the expression of the target antigen for Ab3 to bind to.

Further anti-Id-based vaccine studies in humans have included those associated with Tumor Associated Carbohydrate Antigens (TACAs), particularly the ganglioside targets GD3 and GD2. The anti-Id BEC2, a mimic for GD3, was found not to be highly immunogenic in melanoma patients suggesting adjuvants might be necessary (41–43). More recently BEC2 was considered as a therapeutic intervention in GBS by neutralizing specific pathogenic anti-ganglioside antibodies (44). The murine monoclonal anti-Id antibody 1A7 (TriGem), a mimic of GD2, has been tested in pre-clinical studies and in the clinic (45). In pre-clinical studies, active immunization of mice, rabbits, and monkeys with TriGem induced polyclonal IgG anti-GD2 responses and TriGem specific T cell proliferative responses suggesting the generation of CD4+ T cell help. In clinical trials, it was demonstrated that patients with advanced metastatic melanoma and patients with high-risk melanoma in the postsurgical adjuvant setting generated active immune responses against GD2 following immunization with TriGem. IgG subclasses were shown to be predominately IgG1 and IgG4, suggesting the possibility of the generation of CD4+ T cell help. Median survival was 16+ months for 47 patients with advanced disease. Eighty-two percent of 69 patients with stage III disease were alive at a median follow up of 2 years.

An anti-Id vaccine has reached the market. Racotumomab (Vaxira) is now the first approved anti-Id vaccine—with approval in Cuba and Argentina. Vaxira was shown to increase the survival of Non-Small Cell Lung Cancer patients in recurrent or advanced stages (IIIB/IV). A phase III trial is currently ongoing (NCT01460472). The vaccine was initiated by the Center for Molecular Immunology in Havana, Cuba. Racotumomab, an Ab2γ, was raised against the murine anti-ganglioside N-glycolyl (NGc) GM3 (NGcGM3) (46). The safety of Racotumomab was established in several phase I trials in melanoma, breast and lung cancers (47, 48). In the lung trial, patients developed antibodies against NGcGM3 and had longer medium survival times (49). Results from a randomized trial with Racotumomab showed necrosis of tumor cells as a mechanism for efficacy (50).

While preclinical studies suggested that anti-Ids could mediate cellular responses, little evidence in humans demonstrates this aspect (51, 52). The most direct example for the activation of CD8+ Cytotoxic T Lymphocytes (CTL) involvement comes from a clinical trial testing a combination of the murine anti-id monoclonal antibodies MEL-2 and MF11–30 that are mimics of the high molecular weight melanoma-associated antigen (HMW-MAA) (53). The two anti-ids mimic two distinct epitopes of HMW-MAA. This combination called MELIMMUNE was shown to induce HLA-A2-restricted CTLs that lyse melanoma cells expressing both HLA-A2 antigen and HMW-MAA (53). Collectively, preclinical and clinical trials, albeit very limited, indicate that anti-Id vaccines can induce B and T-cell immune responses both in general terms supporting CD4+ T cell activation for IgG production and tumor antigen specific CD8+ CTLs if the anti-Ids are properly chosen.

4. SOLVING THE PROBLEMS WITH CURRENT ANTI-ID VACCINES

While showing promise, to date no anti-Id-based vaccines has been approved by the US FDA for use in patients. Reasons for the failure of anti-Id vaccines against tumors are similar to generalized failures of other cancer vaccines. On the one hand it is possible that such failures reflect the patient populations used in the studies. We have now come to realize that checkpoint inhibitors are necessary to take the brakes off the immune system. On the other hand a major problem in cancer is the complexity and heterogeneity of antigen expression, the antigens that are potential targets of T and B-cells are multiple, diverse and endlessly adaptable. This reduces the ability of responding immune cells to consistently carry out their task to recognize, bind and destroy. A lesson might be forthcoming from consideration of the "normal" immune response to pathogens as many viruses, bacteria, and parasites induce a strong polyclonal B cell response, which can be crucial for early host defense against rapidly dividing microorganisms. In certain situations the response is restricted such as in HIV infections (54, 55). Interestingly, this clonal-restricted antibody response shares an idiotypic marker (56), termed Ab2δ. The polyclonal and sometimes oligoclonal antibodies in immune reactions would suggest that, in order to stimulate the polyclonal Ab1 spectrum, Ab2 should also be polyclonal. Early vaccine experiments were performed in rabbits and not subject to potential monoclonal anti-Id restrictions (25, 57). Later experiments suggested a strategy to simulate polyclonal immunization by combining monoclonals that are functional anti-Ids in that they compete with antigen clearly are not distinguished in their ability to activate functional T cell responses *a priori* (53, 58, 59). Yet making a panel of hybridomas by screening and selecting only high affinity binders may not be enough to distinguish between protective and non-protective anti-Ids (59).

The advantages of polyclonal vs. monoclonal antibodies has recently been reviewed (60). Previous discussions have suggested a soluble antigen reflective of multiple epitopes can be a more potent modulator of humoral and cellular immune responses than Ab2 that represents a singular epitope (61). Counter arguments have been made (62). However, these arguments often neglect a possible influence of a network and the structural basis for antibody recognition. The major characteristic of polyclonal responses is their clonal and structural diversity. Multi-epitope binding increases the overall avidity to the target. For optimizing the targeting of Ab2 to idiotype expressing B-cell receptors all classes of anti-Id, (Ab2α, Ab2β, Ab2γ, and Ab2δ) should be involved. Thus, a polyclonal or oligoclonal anti-Id vaccine would improve targeting, by invoking a "normal' polyclonal immune response. Polyclonal B cell response is a natural mode of an immune response in adaptive immunity. It is a practical and functionally important element of a healthy immune system, with considerable evidence to support its role in protection from at the least infectious agents. Consequently, we are proposing to change the strategy of monoclonal-based anti-Id vaccine development

and use. Immunizing with polyclonal–based anti-Ids has the capacity to induce humoral antigen spread in patients by engaging multiple BCR's with the potential to activate both targeted and non-targeted antibody producing B and T cells. Immunizations with selected polyclonal anti-Ids to one or multiple target antigens might be a plausible strategy to amplify preexisting B cells and potentially preexisting T cell responses in addition to *de novo* generation of novel responses. This strategy abandons the concept that the idiotype vaccine represents the "Internal Image" of the antigen and supports our earlier suggestion of being a "Network Antigen" (63).

5. RECIPES FOR MAKING POLYCLONAL ANTI-ID-BASED VACCINES

A key prerequisite for an idiotypic network is poly/autoreactivity of some B cell clones. Moreover, it implies positive selection on existing variable regions for which there is evidence (64–66). Positive selection of the B cell repertoire has been demonstrated numerous times over a span of years (67–71) but the nature and the intensity of the self-signal define the choice between elimination, annergy and survival. This implies that a certain range of signal intensities including from existing antibody variable regions can probably recruit the emergent repertoire (7). A constant component of natural IgM would provide the necessary signal exposing idiotopes in the CDR3 regions (72), albeit other regions can be defined as idiotope containing (73), of the required concentration. The unique structures would be too dilute but those shared by a number of clones or sets of clonal products recognized cross-reactively by the same paratope would provide signal sufficient either for positive selection or for negative if the signal were too strong. Maybe this precludes the selection by too broadly distributed public idiotopes. It is interesting to speculate that every strong antibody response might temporarily provide a similar signal. During this time of optimal intensity it may recruit corresponding anti-idiotypic immature B cells. This mechanism may constitute an indirect way to elicit anti-Ids by (inadvertently) manipulating the existing natural antibody network and its capacity to recruit anti-Ids. It may reconcile the "second generation" network concept (7) with experimental induction of anti-Ids as well as introduce the notion that a set of clones rather than a single antibody may be necessary to put this machine in motion.

To stimulate and simulate a polyclonal response, Ab2s can be a mixture of monoclonal antibodies stimulating B and T-cells (53, 58). There are examples of anti-Ids containing both B and T cell epitopes (59, 74). Admixing them might broaden a response. An alternative concept of inducing antibodies against multiple tumor-associated antigens is a pan-immunogen, which harbors "fuzzy" mimicking determinants to induce a polyclonal response to multiple antigens. This concept has not been developed with an Ab2-based vaccine but antigen-mimicking peptides of glycans and TACA have shown such an ability in preclinical (75–80) and clinical studies (81, 82) where a carbohydrate mimetic peptide can induce polyclonal responses to two or more TACAs (81–83). This can be due both to shared epitopes as well as to a multifaceted mimotope exposing diverse antigenic determinants—a structural substrate of immunological polyspecificity.

The advantage of monoclonal antibodies over polyclonal is its consistency and excellent characterization. Monoclonals are produced by cell cultures seeded from a reference cell bank. In contrast polyclonal antibodies are derived from immunized animals producing a unique batch-specific biochemical and biophysical property. For use in humans, each batch must be validated satisfying the advertised criteria. The call for polyclonal or oligoclonal anti-Id antibodies must be answered with novel production strategies. The final step in monoclonal antibody production by hybridoma or recombinant technologies is the selection of the most potent clone or cell line. This is performed under so-called limiting dilution conditions. Suppose one reduced the stringency of selection and mixed a number of clones including ones with lower affinity. The number of antibodies in this polyclonal mix can be controlled. A master cell bank can be established, similar to the master banks in monoclonal production. However, since there is no experience with the clonal stability of cell lines growing in large cell culture tanks research will be required to maintain the original cell culture mix.

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2. Ab1 can also be used to initiate idiotype cascades - Section 2.
3. Lessons learned - Section 3
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   b. Utility in combination therapy
   c. Clinically, Anti-Ids can induce B and T-cell immune responses against antigens.
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6. Introduction of a Master Bank for Polyclonal anti-Ids - Section 5.

AUTHOR CONTRIBUTIONS

HK originally laid out the framework and draft of this review. AP contributed to the discussion on polyclonal immunology. TK-E provided clinical assessment of previously published work. All authors contributed to the writing of the manuscript.

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Attacking Tumors From All Sides: Personalized Multiplex Vaccines to Tackle Intratumor Heterogeneity

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Tumor vaccines are an important asset in the field of cancer immunotherapy. Whether prophylactic or therapeutic, these vaccines aim to enhance the T cell-mediated anti-tumor immune response that is orchestrated by dendritic cells. Although promising preclinical and early-stage clinical results have been obtained, large-scale clinical implementation of cancer vaccination is stagnating due to poor clinical response. The challenges of clinical efficacy of tumor vaccines can be mainly attributed to tumor induced immunosuppression and poor immunogenicity of the chosen tumor antigens. Recently, intratumor heterogeneity and the relation with tumor-specific neoantigen clonality were put in the equation.In this perspective we provide an overview of recent studies showing how personalized tumor vaccines containing multiple neoantigens can broaden and enhance the anti-tumor immune response. Furthermore, we summarize advances in the understanding of the intratumor mutational landscape containing different tumor cell subclones and the temporal and spatial diversity of neoantigen presentation and burden, and the relation between these factors with respect to tumor immunogenicity. Together, the presented knowledge calls for the investment in the characterization of neoantigens in the context of intratumor heterogeneity to improve clinical efficacy of personalized tumor vaccines.

Keywords: tumor vaccines, personalized vaccines, neoantigens, intratumor heterogeneity, multiplex neoantigen vaccines

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INTRODUCTION–A SHORT HISTORY OF TUMOR VACCINES

The beginnings of immunotherapy date back to the late Nineteenth century. In 1891, the American bone surgeon William B. Coley started to treat cancer patients with bacterial injections with the rationale to stimulate the immune system and thereby enhance tumor cell killing. With varying success "Coley's Toxins" were accepted as treatment for inoperable bone cancers but could never fully be clinically established (1). In 1909, Paul Ehrlich established the concept of protective vaccinations of mice by transplanting them with foreign tumors. He described that in 50–100% of vaccinated mice an acquired tumor-directed immunity could be observed (2). The genetic basis of this type of rejection was discovered in 1914 by Clarence Little (3), and later linked to histocompatibility antigens on transplanted tumors by Gorer et al. (4). In line with these findings, Foley (5) and Prehn et al. (6) investigated the mechanism of protection in mice against carcinogen-induced sarcomas after rechallenge with the same tumor, thereby formulating the rational of using

tumor tissue as a vaccine. In the following years more pieces of the immunological puzzle were solved finally culminating in the concept of immunological surveillance, formulated by Burnet in 1970, further justifying the use of cancer vaccines (7).

Nowadays, it is well-established that tumor vaccines can effectively mount an anti-tumor immune response. These vaccines can be comprised of whole tumor lysates, recombinant tumor proteins, tumor antigen derived epitope peptides, or antigen encoding mRNA and DNA (8). Once injected, dendritic cells (DCs) play a crucial role by taking up the vaccine and presenting the vaccine-derived tumor epitope in the context of major histocompatibility class (MHC) I or II complexes to $CD8^+$ or $CD4^+$ T cells, respectively (9, 10). In turn, ideally tumor specific $CD8^+$ T cells will be activated, proliferate and infiltrate into the tumor to exert cytotoxic functions. $CD4^+$ T cells are skewed toward T helper cell subsets and support the anti-tumor immune response by the release of cytokines (11, 12) or tumor cell killing (13).

Despite promising results obtained in 1995 with a DC vaccine pulsed *ex vivo* with the melanoma tumor antigen 1 (MAGE-1) (14, 15), it was not until 2010 before the first DC-based vaccine Sipuleucel-T was approved by the Food and Drug Administration (FDA). This revitalized the tumor vaccine field resulting in the initiation of clinical trials to test new formulations and delivery methods of tumor vaccines across multiple types of cancers.

Especially, recent developments in the understanding of the nature of tumor antigens have attributed to the improvement of tumor vaccines. Together with new insights into the mutational landscape of tumors and their evolution these findings are instrumental for the rise of novel, multiplex and personalized tumor vaccines.

THE IMPORTANCE OF ANTIGEN-SPECIFICITY FOR TUMOR VACCINES

Since Edward Tyzzer coined the term "somatic mutation" in 1916 for describing "modifications of the somatic tissue" that determine foreignness and antigenicity of a transplanted tumor, it took until 1991 to discover the first tumor antigen MAGE-1(16, 17). MAGE-1 was shown to be expressed on patient-derived melanoma cells and in immune privileged sites, such as testis, hence the name cancer/testis antigen (CTA), and therefore qualified as a good target for immunotherapy (17). Although CTAs are specific targets on tumor cells and therefore classify as candidates for tumor vaccines, their expression is limited to a small number of tumors and patients (18). In contrast to cancer/testis antigens, tumor-associated antigens (TAAs), such as gp100, tyrosinase or EGFR, are overexpressed on tumor cells and are shared in a bigger patient population (19). Their concurrent expression on healthy cells however, will result in a weaker antigen-specific immune response, due to negative selection as a consequence of central tolerance. Furthermore, DCs and regulatory T cells will dampen the immune response by inducing peripheral tolerance and inhibiting effector T cells (20). Together with the potential of inducing auto-immune reactions, these features underline that TAAs are not ideal candidates for effective tumor vaccination and that therapy targets are preferably expressed exclusively on tumor cells.

In 1994, the first report of such tumor-specific antigen (TSA) was published, being a mutated version of the membrane protein Connexin-37 in Lewis lung carcinoma (21). In the following years the rise of next-generation sequencing techniques led to the discovery of more TSAs or so called neoantigens. Neoantigens are seen as highly specific tumor antigens that arise due to somatic mutations exclusively in tumor cells while being absent in healthy cells [for extensive reviews about neoantigens see (22, 23)]. Except of mutations in driver genes, such as isocitrate dehydrogenase 1 (IDH1)(24) or KRAS(25) and in a rare form of hereditary colon cancer, called Lynch syndrome(26), neoantigens are not shared between individual patients and can have differential expression in tumor clones within one patient, as will be discussed later. Moreover, the load of neoantigens has been positively correlated with the presence of tumor-infiltrating, neoantigen-specific T cells and a good prognosis for checkpoint inhibitor therapy and survival across different types of cancers (27, 28). In turn researchers have made use of sequencing and peptide-based assays combined with computational filtering and prediction algorithms for the selection of candidate neoantigens to design the first generation of personalized tumor vaccines (29, 30). An example of such, is a point mutation in the gene encoding IDH1, which is shared by about 70% of diffuse grade II and III glioma patients, as mentioned above. Using the mutant IDH1 as synthetic long peptide vaccine, Schumacher et al. observed reduced tumor growth in vaccinated mice carrying tumors with the IDH1 point mutation compared to mice carrying IDH1 WT tumors (**Table 1**) (24).

FROM SINGLE TO MULTIPLEX PERSONALIZED NEOANTIGEN VACCINES

The IDH1 synthetic long peptide is an example of a rationally designed neoantigen vaccine based on a tumor-specific point mutation shared by a large patient population with a mildly immunogenic tumor. For more immunogenic tumors with higher mutational load such as melanoma, high throughput genome screens are needed. One of the first studies applying this strategy used massive parallel sequencing of mouse tumor and healthy tissue combined with RNA expression profiling and immunogenicity tests to obtain potential neoantigen sequences for vaccination purposes. Eventually, vaccinations were performed with two to five neoantigens in the form of synthetic long peptides or mRNA. These led to significant delay of tumor growth and protection of mice in a prophylactic (29) or therapeutic setting (29, 30). Predicted mutations for the B16F10 melanoma, CT-26 colon cancer or 4T1 mammary carcinoma models by Castle and Kreiter et al. were subsequently used and extended to generate neoantigen vaccines targeting more than one epitope simultaneously, and delivered as synthetic long peptide (43), mRNA (41), by carrier molecules such as nanoparticles (31, 34, 36, 39), nanodiscs (38, 45) or other modalities (33, 35, 46) (**Table 1**). In addition to this, in

TABLE 1 | Summary of neoantigen vaccine studies ordered by the amount of neoepitopes that are incorporated in the vaccine.

Tumor type	Organism	Neoantigen identification (origin, method)	Neoantigen prediction	Vaccine format	Amount of neoantigens used for vaccination	Year	Publication
Melanoma, B16F10	Mouse	(29) Mass spectronomy	-	PLGA capturing endogenous neoantigen containing proteins	n.m.	2017	(31)
Colon cancer CT26, TC-1, melanoma B16F10	Mouse	(29)	-	SLP in polyethyleneimine mesoporous silica microrods	n.m.	2018	(32)
Colon cancer MC-38, TC-1	Mouse	(33)	-	Ferritin nanoparticle neoantigen conjugates	1 (TC-1) 3 (MC-38)	2019	(34)
Sarcoma, A2.DR1	Mouse	(Most frequent point mutation in glioma)	-	SLP	1	2014	(24)
Colon cancer CT26, TC-1, melanoma	Mouse	(29)	-	RNA lipoplex	1	2016	(35)
Colon cancer, MC-38	Mouse	(33)	-	RNA-DNA nanostructures	1	2018	(36)
Sarcoma, d42m1-T3 and F244	Mouse	Complement DNA capture sequencing,	3x MHCI epitope binding, processing by immunoproteasome	SLP	2	2014	(37)
Melanoma, B16F10	Mouse	B16F10 cells, DNA/RNA sequencing	Expression, Location, mutation type, immunogenicity	SLP	2	2012	(29)
Melanoma B16F10, colon cancer MC-38	Mouse	(29, 33)	-	RNA nanodisc	2	2016	(38)
Colon cancer MC38	Mouse	Whole exon and RNA sequencing, Mass spectronomy	netMHC binding prediction, solvent exposure in MHC	SLP	3	2014	(33)
Melanoma, colon cancer, HPV E6/E7	Mouse	(29)	-	PC7A nanoparticle	3	2017	(39)
Melanoma B16F10	Mouse	Exome/RNA sequencing	MHCII Class binding	mRNA	5	2015	(30)
Melanoma	Human	Resected tumor, Exome sequencing	Binding to HLA-A, cDNA expression	SLP	7	2015	(40)
Stage III/IV Melanoma	Human	Tumor biopsy, Exome and RNA sequencing	Binding affinity to HLA class II and expression of mutation encoding RNA, and HLA class I binding	mRNA	10	2017	(41)
Lewis lung carcinoma, TC-1, ovarian cancer ID8	Mouse	Cell lines and lysed tumor, Whole exome and RNA sequencing	Binding affinity to HLA class I and II, proteasomal processing	Plasmid DNA	12	2019	(42)
Melanoma Stage IIIB/C Stage IVM1a/b	Human	Whole exon sequencing and RNA sequencing	Binding to HLA-A and -B	SLP	20	2017	(43)
Glioblastoma	Human Phase I/Ib	Whole exon sequencing and RNA sequencing	Binding to HLA-A and -B	SLP	20	2019	(44)

n.m, not mentioned.

pre-clinical mouse studies, the concurrent use of checkpoint inhibitors programmed cell death-1 (PD-1) or cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) at the time of vaccination worked synergistically and enhanced the treatment outcome (31, 34, 39, 45).

In clinical studies of late stage melanoma patients, multiplex personalized neoantigen vaccines have achieved significant results in clinical studies of late stage melanoma patients, as nicely summarized by Hellmann and Snyder (47). Recently, a similar approach has been presented for phase Ib glioblastoma patients. These patients received personalized neoantigen vaccines, covering 20 neoepitopes in the form of long peptides, based on mutational profiling and RNA expression analysis of surgically resected tumors. Although all patients died due to progressive disease, neoantigen-specific $CD8^+$ and $CD4^+$ T cells could be observed which were able to infiltrate into the tumor (44).

Finally, also DNA has been used as a delivery vector for encoding neoantigen vaccines. Duperret et al. used a combination of intramuscular injection and electroporation of plasmids with strings of up to twelve 9-mer neoepitopes, derived from lung carcinoma or ovarian cancer. Neoantigen-specific immune response were predominantly guided by $CD8^+$ T cells and resulted in a delay of tumor growth and increased survival in prophylactically or therapeutically vaccinated mice (42).

Concurrently, a general trend that can be observed in recent studies is an increase in neoepitope incorporation into tumor vaccines (**Table 1**). This is mainly the result of the increasing improvements of next-generation sequencing and computational tools for prediction of neoantigens, providing a more detailed view on the mutational landscape of tumors.

Argumentation supporting multiplex neoantigen vaccines can be found in a more fundamental aspect of tumor evolution which has been elucidated, especially within the last 10 years. It relates to the understanding of the identity of individual tumor cells within specific regions in the tumor mass. Although it is well-known that tumors are heterogeneous, comprised of different cell types, such as immune cells and stromal cells, more knowledge had to be gained at the single cell- and genotypic- level of malignant cells. As such, it was elucidated that a certain degree of tumor cell evolution takes place within one tumor leading to the formation of subclones separated not only spatially, but also by mutational patterns (48–52). The latest key findings on the deciphering of intratumor heterogeneity (ITH), its relation to neoantigen expression and its effect on the immune system and immunotherapy present an essential milestone toward the next generation of multiplex personalized neoantigen vaccines and offer an outlook on the challenges we face in the future.

INTRATUMOR HETEROGENEITY CHALLENGING MULTIPLEX PERSONALIZED NEOANTIGEN VACCINES

The concept of ITH was first introduced in the 1970's by Prehn et al. who investigated the immunogenicity of methylcholanthrene-induced murine sarcomas. Paired cancer cell subclones from different regions of a primary tumor showed differential induction of immune responses after transplantation into recipient mice. These differences were thought to be caused by distinct antigenicity and immunogenicity of subclones, hence a heterogeneous distribution within one tumor (53). In the following years other groups extended this knowledge by delineating the types of immune responses toward heterogeneous tumors based on subclonal expression of tumor antigens (54).

The first human study to investigate the extent of ITH within one tumor mass was performed in 2012 by Gerlinger et al. (48). In this study, several biopsies from a patient-derived primary renal-cell carcinoma were analyzed by whole-exome sequencing and aligned to healthy tissue. Next to several shared mutations between different subclones, ca. 23% of the mutations were only found in specific regions of the tumor. Strikingly, a single biopsy of that same tumor only covered around 55% of the total mutational diversity, underlining the need for multi-region sampling. Tracing the order of mutations in different subclones revealed that they develop in a branching fashion from the primary tumor clone, harboring the driver mutation, rather than in a linear model. Remarkably, these differentially branched subclones harbored different mutations in the same gene which suggests a mode of convergent evolution (48). These findings emphasize the importance of multi-region sampling of tumor samples, as it can explain the mutational ancestry of a tumor and thereby aid in the selection of neoantigens for tumor vaccination, which ideally target mutations from the trunk of the phylogenetic tree.

Besides being able to reconstruct the mutational history, it is also important to correlate this to the developmental stage of the tumor as shown by De Bruin et al. (49). In patients with non-small cell lung cancer (NSCLC) mutational events in the primary tumor coupled to known driver genes could be identified in the context of tobacco-induced carcinogenesis, bearing typical C>T transitions in early development. Mutations in driver genes were also observed in subclones of later development, however these clones also acquired other somatic mutations indicative of a branched evolution and supporting the idea of ITH in NSCLC. Knowing that these tumors carry driver mutations in late stage development, in different regions of the tumor, emphasizes the benefit of multi-dimensional sampling and sequencing for developing tumor vaccines that target these driver mutations (49). Importantly, in this study <5% of the tumor tissue could be analyzed, which probably underestimates the extend of observed ITH.

As growing evidence suggests that diverse sets of mutations occur in subclones in distinct regions of one tumor, McGranahan et al. asked to what extent these mutations translate into neoantigens and how neoantigen ITH (NITH) relates to the anti-tumor immune response (50). Analysis of neoantigen burden and NITH in single biopsies from roughly 200 cases of different types of lung cancers was performed. Using whole-genome and -exome sequencing and bioinformatic processing revealed that a high clonal neoantigen burden (upper quartile of total neoantigen burden) combined with a low NITH (smaller than 1%) correlates with a longer survival in lung adenocarcinoma patients. In contrast, a lower neoantigen clonality (higher NITH) characterized the tumor as more heterogeneous which correlates with a shorter survival (**Figure 1A**). More homogeneous tumors showed genetic signatures of an inflamed or hot tumor microenvironment with upregulated genes for antigen presentation, T cell migration and effector functions next to inhibitory molecules such as programmed death receptor ligand 1 (PD-L1). As a consequence to this environment, active interferon γ, granzyme B, H and A producing, PD-L1 and lymphocyte activation gene 3 (LAG-3) expressing $CD8^+$ T cells specific to clonal neoantigens could be identified within the tumors by MHC multimer staining and flowcytometric analysis. Whether earlier in the development of the tumor it was more heterogeneous, homogenized by initial neoantigen specific T cell infiltration, leading to attraction of more immune cells and an inflamed tumor microenvironment, is however difficult to investigate due to the lack of samples

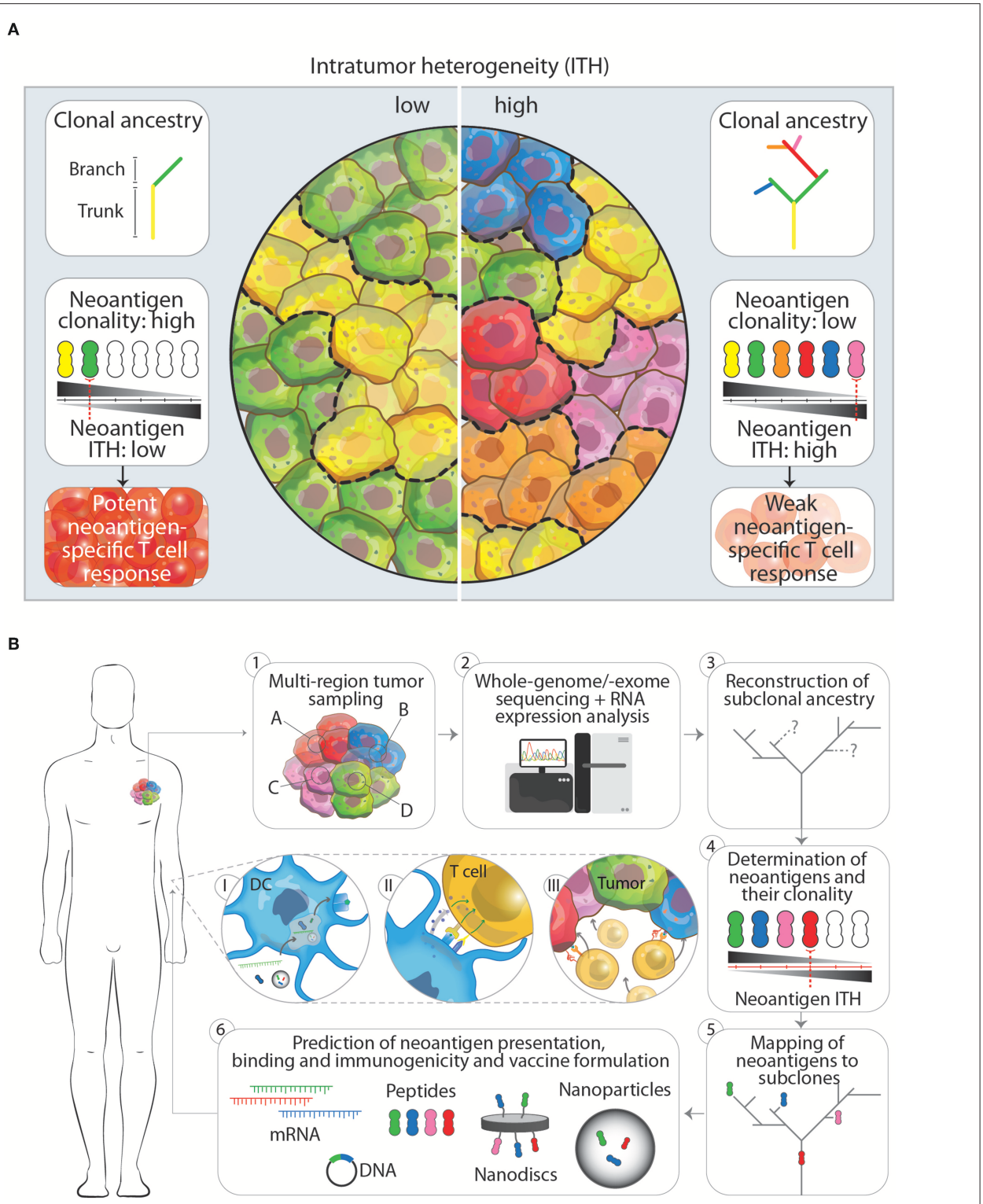

FIGURE 1 | (A) The impact of low and high intratumor heterogeneity (ITH) on clonal ancestry, neoantigen clonality and T cell responses. Tumors that show low ITH (left panel) typically have few branching mutations as indicated in the clonal ancestry panel. In turn, more cells in the tumor harbor the same mutation, which is potentially translated and presented on the cell as a neoantigen. The overall neoantigen clonality (the number of cells that express one specific neoantigen, indicated by black-gray triangle) is therefore higher, leading to a lower neoantigen ITH and subsequently in a better neoantigen-specific T cell response. Tumors that have a high ITH in contrast (right panel), show more branching mutations leading to an increased amount of neoantigens expressed. Having more subclones with specific neoantigens however decreased the neoantigen clonality and increases neoantigen ITH. This will result in a weaker neoantigen-specific T cell response. **(B)** Workflow

(Continued)

FIGURE 1 | for the designing of next generation multiplex neoantigen vaccines addressing ITH (1–6). (1) Ideally, the generation of multiplex neoantigen vaccines starts with multi-region tumor sampling by preferentially, non-invasive techniques. (2) Acquired data will then be analyzed by whole-genome/-exome sequencing for detection of mutations and RNA expression analysis to infer whether these mutations are located within transcribed regions. (3, 4) From this the subclonal ancestry can be inferred to determine the overall neoantigen clonality and ITH. (5) By mapping found neoantigens to subclones in the tumor and the ancestral tree, target neoantigen can be chosen that are located in the trunk and/or branching regions. (6) Finally, state-off the art prediction algorithms can supplement the aforementioned workflow to cross-validate found neoantigen vaccine candidates that will be incorporated in the final vaccine or vaccine carrier. Panels I-III depict the *in vivo* processing of multiplex neoantigen vaccines leading to a multi-angled anti-tumor T cell response. (I) After injection of multiplex neoantigen vaccines dendritic cells (DCs) will take up and process the vaccine and present antigenic epitopes on the cell surface complexed with MHC molecules. (II) Subsequently, T cells will interact with DCs via T cell receptor-MHC interaction and co-stimulatory molecules and will be further activated under the influence of cytokines. (III) Effector T cells will finally perform cytotoxic effector functions targeting several subclones in the heterogenous tumor.

from these earlier developmental stages of the tumor. The observation that these tumors harbored an inflamed, PD-1/L1 expressing microenvironment was the rationale to inhibit PD-1 by checkpoint immunotherapy, which resulted in a clinical benefit for patients with these inflamed tumors. In the same study, similar results have been obtained in a melanoma patient cohort treated with PD-1 checkpoint immunotherapy, where patients with high clonal neoantigen burden and low NITH showed prolonged survival (50). Another example supporting combination of multiplex neoantigen vaccines and checkpoint immunotherapy is provided by two clinical studies of Ott et al. (43) and Sahin et al. (41). In these studies, stage III-IV melanoma patients are initially treated with RNA -or long peptide-based multiplex neoantigen vaccines (**Table 1**). While most of the patients experienced progression free survival as consequence of neoantigen specific T cell infiltration into the tumor, some showed recurrent disease during multiplex neoantigen vaccination. In these cases, combinatorial treatment with PD-1 blocking antibodies was able to remove tumor mediated immunosuppression and unleash neoantigen-specific T cells that were generated by the vaccine (41, 43).

The discovery that checkpoint immunotherapy results in prolonged survival once neoantigen-specific cytotoxic T cells have infiltrated these tumors, presents a rationale to combine multiplex neoantigen vaccination with checkpoint immunotherapy for tumors with low NITH.

Based on the aforementioned clinical examples a sequential treatment with first multiplex neoantigen vaccines and then, if needed, checkpoint therapy can be suggested to reduce the amount of patients that are unnecessarily treated with checkpoint inhibitors.

As already briefly touched upon above, to what extent neoantigen-specific immune responses shape the heterogeneity of a tumor throughout tumorigenesis by targeting dominant subclones and whether this can lead to tumor escape of untargeted clones remains to be determined.

By applying multi-color barcoding of male Eμ-myc lymphoma cells, Milo et al. studied tumorigenesis and subclonal distribution in a metastatic mouse model (51). When injected in male recipient mice, the differentially colored tumor cells seeded in different proximal niches, ultimately resulting in equally heterogeneous tumors, demonstrating equal survival and outgrowth of the injected barcoded tumor cells. When these cells were injected in female recipient mice, homogeneous tumors with one or two dominant colors established in a $CD8^+$ T cell dependent manner. Part of the explanation for this observation can be found in the expression of Y-chromosome derived H-Y antigens, which induced an antigen-specific T cell-mediated immune response. However, the injected mix of color coded tumor cells contained up to 25% non-immunogenic cells due to a loss of the Y-chromosome, suggesting additional clonal reduction as a result of epitope spreading, ultimately resulting in homogeneous Y chromosome deficient tumors. Additionally, whole genome exome-sequencing was applied in this system to infer whether the neoantigen repertoire is narrowed as a consequence of the anti-tumor immune response. In line with the reduction in color-coded subclones in female recipients also NITH was reduced, underlining that the immune system actively shapes subclone diversity and NITH during immunosurveillance, resulting in the evolution of one or few escaping subclones (51).

In a first attempt to study the contribution of neoantigen immunogenicity in the emergence of dominant tumor cell subclones Gejman et al. developed an artificial antigen-presentation system allowing the construction of heterogeneous tumors, expressing up to five thousand defined artificial MHCI neoepitopes (52). Looking at the clearance of immunogenic subclones within a largely heterogeneous tumor in mice, it was revealed that the immune system was incapable of elimintating small clonal fractions of immunogenic subclones. It appeared that the percentage of neoepitope subclonal tumor cell representation is an important determinant for its clearance. This critical subclonal percentage seemed to differ between individual neoepitopes (52). The exact mechanisms behind the persistence of tumor cell subclones, although partly assigned to absence of antigenicity or clonality, remains to be elucidated in more detail. These two studies emphasize the need for controlled systems to investigate the dynamic process of immunosurveillance in the context of heterogeneous tumors with known mutations or neoepitopes to determine how the immune system can be used to reduce subclone diversity and ultimately enable the total clearance of the tumor.

CONCLUSION

The discovery of neoantigens and their use as tumor vaccines generated a lot of momentum in the tumor vaccination field. Personalized neoantigen vaccines hold promise in generating specific anti-tumor immune responses and durable survival benefits as emphasized by several pre-clinical and clinical studies. Especially in the last 2 years, vaccines comprising of not one,

but several neoepitopes, so called multiplex neoantigen vaccines, have been developed in order to successfully increase the breadth of the anti-tumor immune response.

A rationale that supports this development is obtained from recent insights into the dynamic evolution of tumors. This evolution is characterized by the time-dependent acquisition of region-specific mutations and leads to the emergence of genetically distinct tumor cell subclones within one tumor, as shown by multi-region sampling and massive parallel sequencing. These subclone-specific mutations define the neoantigen clonality, burden and therefore the total NITH, which in turn affects the potency of immunosurveillance. Tumors with a high neoantigen clonality and a low NITH show a better tumor clearance. More heterogeneous tumors with lower neoantigen clonality are more difficult to eradicate (**Figure 1A**). Depending on this balance between neoantigen clonality and NITH, T cells are able to reduce the diversity of subclones within a tumor and thereby actively shape the ITH. Two important factors which influence the efficacy with which T cells can clear a specific subclone within a heterogeneous tumor seem to be the antigen itself and the percentage of tumor cells expressing this antigen. The exact underlying mechanism has still to be uncovered and can possibly aid us in choosing the right antigens for preventing the emergence of dominant subclones. In the meanwhile, targeting more neoantigens by multiplex neoantigen vaccines is a feasible approach to induce a specific immune response against several subclones in the tumor and thereby address ITH.

FUTURE PERSPECTIVES

Although current studies with multiplex neoantigen vaccines (**Table 1**) seem to tackle ITH by including more neoepitopes, they are limited by the snapshot of the mutanome acquired by a single biopsy. We believe that the lack of multi-dimensional tumor information in these neoantigen vaccine studies impairs the power of inducing a multi-angled immune response against all the subclones in a tumor. Challenging pre-clinical longitudinal studies of tumorigenesis are needed, taking into account samples from different locations in the tumor at different time points, both before and after treatment. These studies would gain insight in the dynamics of tumor evolution in the context of multiplex neoantigen vaccination. Ultimately, this knowledge could be integrated into the process of designing the next generation of multiplex neoantigen vaccines. Currently, driving neoepitope selection criteria are predicted MHCI binding as well as T cell receptor affinities. We propose a workflow (**Figure 1B**) starting with the multi-regional NITH acquisition of available biopsies, to obtain multi-dimensional tumor biopsy information, from which dominant clonal neoepitope vaccine candidates could potentially be extrapolated. Attractive, less invasive and practically more feasible alternatives, such as circulating tumor DNA or tumor exosome DNA sequencing, deserve special attention. Varying reports exist about the success of these two techniques in comprehending ITH, which underlines the need for further development of these tools (55–57). Next-generation sequencing and bioinformatic tools that have been developed in the recent years (48, 58–62) will then be an essential asset to acquire genomic sequence information of high, subclonal resolution. With this information the clonal architecture and mutational ancestry of subclones can be reconstructed. Subsequently, neoantigen clonality and burden can be inferred to predict total NITH and map neoantigens to the subclone architecture of the tumor. This dynamic genomic blueprint of the tumor will aid in determining optimal neoantigen candidates for vaccination purposes and can be complemented with bioinformatic prediction algorithms and novel tools to assay T cell reactivity on a large scale (63). Current vaccine production platforms as described earlier, can then facilitate the efficient formulation and delivery of the vaccine to the patient *in vivo*, where DCs will process vaccine content and activate neoantigen specific T cells that will infiltrate and eradicate the tumor (**Figure 1B**, p. 1–3).

We are convinced that the development of more refined techniques to sample and predict the right neoantigens for vaccination can address ITH, and will be essential to fuel the progress that is currently made with regard to time efficient formulation, design and delivery of multiplex neoantigen vaccines [as extensively reviewed by others (64, 65)]. With these techniques in mind, we could create a more detailed map of the neoantigen clonality in primary tumors and metastasis to determine shared mutations within these regions as shown earlier (48). Along this line, combining multiplex neoantigen vaccines with TAA or CTA epitopes could present a handle to increase the chances of epitope spreading and sensitize the immune system for a priori low abundant neoantigens. A full comprehension of tumor evolution and neoantigen distribution will be the fundament for counteracting the survival of the fittest tumor clone and will pave the way for powerful next-generation multiplex neoantigen vaccines.

AUTHOR CONTRIBUTIONS

FF, IdV, CF, and MV contributed to researching the data for the article, discussing the content and to reviewing and editing the manuscript before submission. FF and MV were responsible for writing the article. FF was responsible for figure design.

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Increased Akt-Driven Glycolysis Is the Basis for the Higher Potency of CD137L-DCs

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CD137 ligand-induced dendritic cells (CD137L-DCs) are a new type of dendritic cells (DCs) that induce strong cytotoxic T cell responses. Investigating the metabolic activity as a potential contributing factor for their potency, we find a significantly higher rate of glycolysis in CD137L-DCs than in granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 4 induced monocyte-derived DCs (moDCs). Using unbiased screening, Akt-mTORC1 activity was found to be significantly higher throughout the differentiation and maturation of CD137L-DCs than that of moDCs. Furthermore, this higher activity of the Akt-mTORC1 pathway is responsible for the significantly higher glycolysis rate in CD137L-DCs than in moDCs. Inhibition of Akt during maturation or inhibition of glycolysis during and after maturation resulted in suppression of inflammatory DCs, with mature CD137L-DCs being the most affected ones. mTORC1, instead, was indispensable for the differentiation of both CD137L-DCs and moDCs. In contrast to its role in supporting lipid synthesis in murine bone marrow-derived DCs (BMDCs), the higher glycolysis rate in CD137L-DCs does not lead to a higher lipid content but rather to an accumulation of succinate and serine. These data demonstrate that the increased Akt-driven glycolysis underlies the higher activity of CD137L-DCs.

Keywords: CD137L-DC, metabolism, glycolysis, Akt, mTOR, lipid synthesis, succinate

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INTRODUCTION

With the recent success of immune checkpoint inhibitors and chimeric antigen receptor T cells (CAR-T), tumor immunotherapy finally had its long-awaited breakthroughs. However, there are many cancer types where these two approaches have low to no efficacy (1–3). Examples would be solid cancers that lack a cell surface tumor associated antigen (TAA) that can be targeted by CAR-T, and cancers that failed to induce an immune response (2, 3).

Dendritic Cells (DCs) as the pivotal link between the innate immune and the adaptive immune system have been the focus of immunological researches for the last several decades. DC-based immunotherapy for cancer has been proven safe and to prolong survival but the clinical response and efficacy are disappointing (4). To date most of the DCs for cancer immunotherapy are generated by treating patients' monocytes with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) (4, 5), which are generally referred to as monocyte-derived DCs (moDCs). We have discovered a new type of DCs, CD137L-DCs, which are derived from monocytes by CD137 ligand (CD137L) reverse signaling (6). CD137L-DCs are only found in human but not in mouse because of the difference in human and mouse CD137L (7).

Nevertheless, CD137L-DCs are more potent than moDCs in stimulating cytotoxic T cells in an antigen-specific manner and driving a T helper 1 type response (8). T cells activated by CD137L-DCs are less exhausted and metabolically more active (9). CD137L-DCs are promising candidates for the still unmet need of an effective immunotherapy for many types of cancer. A clinical trial testing the safety and optimal dose of CD137L-DCs for the treatment of nasopharyngeal carcinoma is currently ongoing (NCT03282617).

There is accumulating evidence that metabolic reprograming underpins the transition of immune cells between the quiescent and the activated state. The same cells activated by different stimuli usually induce distinctive metabolic programs and the metabolism in turn influences the fate of the cell development. This mutual regulation is particularly evident during T cell differentiation (10) and macrophage polarization (11). DCs are a heterogeneous population consisting of different subsets (12). However, because of the rarity of DCs in peripheral blood, the knowledge of DC metabolism is mainly gained from murine bone marrow-derived DCs (BMDCs). During the activation of BMDCs by toll like receptor (TLR) ligands, especially the TLR4 ligand LPS, BMDCs switch from oxidative phosphorylation (OXPHOS) to glycolysis. This shift is executed in two different stages: The early increase of glycolysis is inducible nitric oxide synthase (iNOS)-independent and mediated by TBK1-IKKε-Akt, while the latter long-term commitment to glycolysis is PI3K-Akt-mTOR-mediated and dependent on iNOS, which generates NO to suppress OXPHOS (13). Glycogenolysis also contributes to the early glycolytic burst in both LPS-activated human moDCs and in murine BMDCs (14). Unlike tumor cells and T cells that rely on glycolysis to provide intermediates as building blocks for proliferation, non-proliferative BMDCs utilize glycolysis mainly to provide acetyl-CoA and nicotinamide adenine dinucleotide phosphate (NADPH) for the synthesis of lipids, leading to an expansion of endoplasmic reticulum (ER) and the Golgi apparatus and increased synthesis and transport of proteins for DC activation (15).

Nevertheless, one should be cautious in applying findings obtained in murine BMDCs to the other types of DCs, as notable differences in metabolism have been found between different subsets of DCs (16). For example, iNOS is induced in LPS-activated murine BMDCs but not the murine classical DCs isolated from the spleen (17). Furthermore, most clinical trials on moDCs to date use a cocktail of cytokines instead of LPS to mature moDCs (4). Whether CD137L-DCs and moDCs matured by cytokine cocktails share similar metabolism as murine BMDCs is unknown. In this study, we have compared the metabolism of CD137L-DCs with that of moDCs, characterized the metabolism-regulating signaling pathways, and explained the high potency of CD137L-DCs from a metabolic perspective. We find that CD137L-DCs are characterized by high Akt-driven glycolysis that is important for both the activation of CD137L-DCs and the persistence of their activated state.

Abbreviations: ACC, acetyl-CoA carboxylase; BMDCs, bone marrow-derived DCs; CD137L-DC, CD137 ligand-induced DC; DC, Dendritic cell; FASN, fatty acid synthase; moDC, monocyte-derived DC; OXPHOS, oxidative phosphorylation.

MATERIALS AND METHODS

Antibodies and Inhibitors

Antibodies to the following proteins were purchased from the indicated vendors: mouse IgG1 Kappa (clone MOPC21) Sigma-Aldrich (St. Louis, MO, USA). CD137L (clone 5F4) Biolegend (San Diego, CA, USA). CD3 (clone OKT3), CD40 (clone 5C3) and PD-L1 (clone M1H1) Affymetrix eBioscience (San Diego, CA, USA). CD80 (clone 2D10), CD86 (clone IT2.2) and CD70 (clone 113-16) Biolegend. Phospho-Akt (Ser473) (clone D9E), Pan-Akt (clone 40D4), Phospho-S6 Ribosomal Protein (Ser235/236), S6 Ribosomal Protein (clone 54D2), Phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204), p44/42 MAPK (Erk1/2, clone L34F12), phospho-AMPKα (Thr172, clone 40H9), AMPKα (clone F6), phospho-GSK-3β (Ser9, clone D85E12), GSK-3β (clone 3D10), rabbit IgG-HRP, mouse IgG-HRP, beta-actin (clone 13E5), and PathScan® Intracellular Signaling Array Kit from Cell Signaling Technology (Danvers, MA, USA). GAPDH (clone 6C5) Abcam (Cambridge, UK). LY294002 and Rapamycin from Cell Signaling Technology. DMSO, 2-DG, C75, and TOFA from Sigma-Aldrich.

Differentiation of DCs

Human peripheral blood mononuclear cells (PBMCs) were isolated from human blood by Ficoll-Paque (GE Healthcare, Chi, IL, US) density gradient centrifugation. Monocytes were isolated from PBMCs by using the EasySep Human Monocyte Isolation Kit (#19359, StemCell technologies, Vancouver, Canada). Isolated monocytes were cultured in RPMI-1640 supplemented by 10% FBS, 50 μg/ml streptomycin and 50 IU/ml penicillin (R10 PS medium). CD137L-DCs were differentiated by seeding monocytes on anti-CD137L antibody pre-coated plate (5 μg/ml, 4°C overnight) at 1 million/ml for 7 d. moDCs were differentiated by treating monocytes with 100 ng/ml IL-4 and 80 ng/ml GM-CSF (ImmunoTools, Friesoythe, Germany) for 7 d. CD137L-DCs were matured by 1 μg/ml Resiquimod (R848, InvivoGen, San Diego, CA, USA) and 50 ng/ml IFN-γ (#285-IF-100, R&D, Minneapolis, MN, USA) and moDCs were matured by 10 ng/ml IL-6, IL-1β, TNFα (ImmunoTools) and PGE2 (#P0409, Sigma-Aldrich) in the last 18 h of differentiation.

For experiments involving inhibitors, cells were incubated with inhibitors 1 h prior to inducing differentiation or maturation. During differentiation, 2 μM LY294002 and 10 nM Rapamycin were used. After 1 day of DC differentiation, inhibitors were washed out and developing DCs were supplemented again with differentiation cytokines. During maturation, 50 μM 2-DG, 10 μM LY294002, 50 nM Rapamycin, 20 μM C75 and 20 μM TOFA were used.

Mixed Lymphocyte Reaction

T cells were isolated from PBMCs using the EasySep Human T cell Isolation Kit (#17951, StemCell technologies), and labeled by CellTrace™ Violet dye (#C34557, ThermoFisher Scientific).

Allogenic mixed lymphocyte reaction (MLR) was done by co-culturing 2×10^4 DCs generated from one donor with 2×10^5 T cells isolated from another donor in AIM V™ medium (#12055091, ThermoFisher Scientific) supplemented with 2% human AB serum (#H3667, Sigma-Aldrich) for 5 d in 96-well plates. The supernatants were collected for cytokine measurements. The proliferation of T cells was quantified by the dilution of CellTrace™ Violet dye which was measured by flow cytometry after gating for $CD3^+$ cells.

Seahorse Metabolic Assays

Seahorse XFe24 FluxPaks, XF Base Medium Minimal DMEM (0 mM Glucose), Seahorse XF Glycolysis Stress Test Kit, XF Mito Fuel Flex Test kit and XF Cell Mito Stress Test Kit were purchased from Agilent (Santa Clara, California, USA). The characterization of DC metabolism was done as described previously (18). Briefly, harvested DCs were washed with PBS once, resuspended in assay medium to make 2 million/ml (1∼4 million/ml), and 0.1 ml DCs were seeded per well in poly-D-lysine (#P6407, Sigma-Aldrich) coated plate. DCs were equilibrated in CO_2-free incubator at 37°C for 30 min. After the medium was topped up to 0.5 ml, the plate was equilibrated in CO_2-free incubator at 37°C for another 30 min before being loaded into the machine. The final concentrations of drugs were: 10 mM Glucose, 1 μM Oligomycin, 50 mM 2-DG, 3 μM FCCP, 1 μM Rotenone+ 1 μM Antimycin A, 3 μM BPTES, 4 μM Etomoxir, and 2 μM UK5099.

Western Blot

For PathScan® Intracellular Signaling Array experiments, cell lysates were prepared and incubated according to the protocol. The signal were measured by a ChemiDoc (Biorad, CA, USA) machine. For LY294002 or Rapamycin treatment, cells were pretreated with the inhibitors for 1 h before maturation or differentiation. 1 or 2 h afterwards, cells were washed with ice-cold PBS twice and lysed by RIPA buffer (#9806, CST) supplemented with protease and phosphatase inhibitor cocktail (#78440, ThermoFisher Scientific) on ice for 10 min. Cell lysates were collected, sonicated by a water bath sonicator, and pelleted at maximum speed at 4°C for 15 min on a bench top centrifuge. The concentrations of cell supernatants were quantified by Bradford assay. Equal amount of proteins was run on a SDS-PAGE gel, transferred to a PVDF membrane and blocked by 5% non-fat milk at room temperature for 1 h. The PVDF membrane was probed with primary antibodies at 4°C overnight, washed with 1% TBST three times, and probed with secondary antibodies at rt. for 1 h. The PVDF membrane was washed again with 1% TBST three times before development. The developed X-ray films were scanned and the bands were semi-quantified by ImageJ.

qPCR

Total DNA were extracted by organic solvents (19). The mitochondrial DNA copy number per cell was quantified by the ratio of the copy number of mitochondrial tRNA to the copy number of β-2-microglobulin (β2M) (quantified by qPCR) as previously described (20).

TMRE Staining

CD137L-DCs were grown on cell-culture treated coverslips (#174985, ThermoFisher Scientific). DCs were generated and loaded with TMRE ($\Delta\psi_m$ indicator; 100 nM) in the dark for 20 min at 37°C. Cells were then washed and resuspended in Hank's buffered salt solution (HBSS), pH 7.2. Images were acquired using an Olympus IX73 fluorescent imaging system with excitation at 561 nm. Twenty images were collected randomly for each sample, and the fluorescence was quantified using Image J software.

ELISA

IL-8, IL-10, TNFα, and IL-1β in the supernatant were measured by respective Ready-SET-Go!® Set (eBioscience) ELISA kits according to the protocol. IL-12 and IFN-γ in the supernatant were measured by respective DuoSet ELISA kit according to the protocol (R&D Systems, Minneapolis, USA). All cytokines are in pg/ml.

Flow Cytometry

CD137L-DCs were washed with cold PBS, incubated in L7™ hPSC Passaging Solution (#FP-5013, Lonza, Basel, Switzerland) at 37°C for 15 min followed by R10 PS medium addition, and harvested by scraping. moDCs were harvested by flushing. For the proper comparison of cell surface markers, moDCs were also incubated with L7™ hPSC Passaging Solution at 37°C for 15 min. Cells were pelleted and washed with cold PBS for once, followed by cell surface Fc receptor blockage by FcR blocking reagent (#130-059-901, Miltenyi Biotec, Bergisch Gladbach, Germany). Cell surface markers were stained at 4°C for 30 min. Cells were spun down and washed with cold FACS buffer twice before the analysis on LSR Fottessa or X20 (BD, NJ, USA) or Attune NxT Flow Cytometer (ThermoFisher Scientific, Carlsbad, CA, USA). For live / dead cell staining, 1 μg/ml 7-AAD (Biolegend) was added 5 min before the measurement by flow cytometry. Data were analyzed with FlowJo 10.

Lipid Staining

HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit (#H34158, ThermoFisher Scientific) or BODIPY (#790389, Sigma-Aldrich) was used to stain the lipid. LipidTOX Red phospholipid stain was added to the cell culture 18 h before harvesting. Harvested DCs were washed with PBS and stained with LipidTOX Green neutral lipid stain at room temperature for 30 min. DCs were spun down and washed with PBS once before acquisition on flow cytometer. If BODIPY was used, harvested DCs were stained in the same way as LipidTOX Green neutral lipid stain.

Metabolomics

The metabolic profiling of organic acids, amino acids, and glycolysis intermediates was done in collaboration with the Duke-NUS metabolomics facility. DCs were washed with ice-cold PBS thrice and resuspended in 50% acetonitrile, 0.3% formic acid. The extraction and measurement of metabolites by LC-MS was done as described previously (21, 22). The concentration of metabolites was normalized by the protein contents of DCs.

Gene Set Enrichment Analysis (GSEA)

The dataset is obtained from Gene Expression Omnibus, accession number GSE60199 that was deposited by Harfuddin et al. (23). The GSEA analysis was performed by using the javaGSEA Desktop Application (24, 25). For all gene sets, 1,000 permutations with "phenotype" algorithm were used.

Statistical Analysis

Statistical significance was determined by two-tailed unpaired Student's *t*-test unless specified otherwise. If the sample was normalized by the control, statistical significance was determined by one-sample *t*-test against one. The scatter dot plots and bar charts were plotted by GraphPad Prism 6.

RESULTS

CD137L-DCs Have Higher Glycolysis Rates and Akt-mTOR1 Activity

As the activation of DCs is accompanied by metabolic reprograming to a higher rate of glycolysis (15, 26), we compared the glycolysis rates of CD137L-DCs and moDCs at baseline and under metabolic stress induced by Oligomycin. As expected, all DCs had higher extracellular acidification rate (ECAR) due to higher basal glycolysis rates and glycolytic capacities than the undifferentiated monocytes (**Figure 1A**). Maturation of both CD137L-DCs and moDCs further elevated basal glycolysis rates. Notably, immature CD137L-DCs have significantly higher basal glycolysis than both immature and mature moDCs, while mature CD137L-DCs have the highest basal glycolysis and glycolytic capacity (**Figure 1A**). In agreement with the higher glycolysis in CD137L-DCs, GSEA also showed an enrichment in enzymes involved in glycolysis in immature CD137L-DCs (**Figure 1B**), such as hexokinase 2 (HK2), which is a key enzyme in promoting aerobic glycolysis (27).

As the main source of ATP, mitochondrial respiration has also been studied by measuring the oxygen consumption rate (OCR). After their differentiation from monocytes, all DCs had a higher basal respiration rate and a higher maximal respiration than the starting monocytes, though not all comparisons were statistically significant (**Figure 1C**), indicating a biogenesis of mitochondria during DC differentiation (28). In line with previous observations (15, 26), moDCs had a lower maximal respiration after maturation. Though the basal respiration in moDCs was higher than in CD137L-DCs, there was no significant difference in maximal respiration between the two types of DCs (**Figure 1C**), suggesting that the mitochondria in CD137L-DCs are still healthy and that their function is not significantly compromised. In line with their higher basal respiration rate, immature moDCs have a higher enrichment in enzymes involved in the TCA cycle than immature CD137L-DCs and mature moDCs (**Figure 1D**). The lower basal respiration in CD137L-DCs could be a result of fewer mitochondria than in moDCs (**Figure 1E**). The average mitochondrial membrane potential, which is controlled by respiration, did not differ significantly among the four types of DCs (**Supplementary Figures 1A,B**). In fact, the responsiveness of moDCs but not CD137L-DCs to the mitochondrial pyruvate carrier blocker, UK5099, implied that moDCs had a mixed glycolytic and aerobic energy phenotype for glucose utilization while CD137L-DCs were mostly glycolytic (**Supplementary Figure 1C**).

Signaling pathways mediate and regulate the diverse activities of cells. We utilized the CST PathScan® Intracellular Signaling Array Kit to unbiasedly screen the main signaling pathways for an involvement in CD137L-DC differentiation and maturation. Among the 18 targets screened, the Akt-mTORC1 pathway but not the MAPK or Stat pathways consistently showed a stronger activation in CD137L-DCs than moDCs differentiated from monocytes from two healthy donors (data not shown). This result was further confirmed by Western blot analysis. 24 h after the differentiation was initiated, the nascent CD137L-DCs showed a robust Akt activation that could not be detected in nascent moDCs. Although Akt activation was present in moDCs at later time points, this stronger activation of Akt in CD137L-DCs persisted during the entire period of differentiation and maturation (**Figure 1F**). Ribosomal protein S6, which is a downstream target of mTORC1, was comparably activated in immature CD137L-DCs and immature moDCs but showed higher activation in mature CD137L-DCs than in mature moDCs (**Figure 1F**). The result is reproducible with the pooled semi-quantified results shown in **Figure 1G**. Some comparisons are not statistically significant due to the large donor variation and relatively small sample size of three donors. Other molecules related to mTORC1, such as PRAS40, p70S6, and mTOR itself, also displayed stronger activation in mature CD137L-DCs than in mature moDCs (**Supplementary Figure 2**).

Glycolysis Is Essential for Sustaining the Activated State of Mature CD137L-DCs

It has been previously reported that glycolysis is indispensable for the activation of murine BMDCs and human moDCs (15, 26). Our data are in line with these observations. When glycolysis was inhibited by 2-Deoxy-D-glucose (2-DG) during moDCs maturation, expression of CD70 and CD86 was significantly decreased (**Supplementary Figure 3A**). The maturation of CD137L-DCs was more affected by 2-DG than the maturation of moDCs. For example, CD40, CD70, and IL-12 were downregulated by 2-DG to a much higher extent in mature CD137L-DCs than in mature moDCs (**Supplementary Figures 3A,B**). This could be explained by the higher rate of glycolysis in mature CD137L-DCs than in mature moDCs.

Since DCs used for tumor immunotherapy are always generated in nutrient-rich medium, we investigated how important glycolysis is for the function of different types of *in vitro* generated DCs. Surprisingly, glycolysis remained necessary for the expression of most co-stimulatory molecules examined and for the secretion of inflammatory cytokines even after DC differentiation and maturation had been completed. Representative sets of histogram are shown in **Supplementary Figure 4A**. Mature CD137L-DCs, which had the highest glycolysis rate, were the DC type most inhibited by 2-DG. For example, the MFI of CD80 decreased in mature CD137L-DC after 2-DG treatment but increased in the other three types

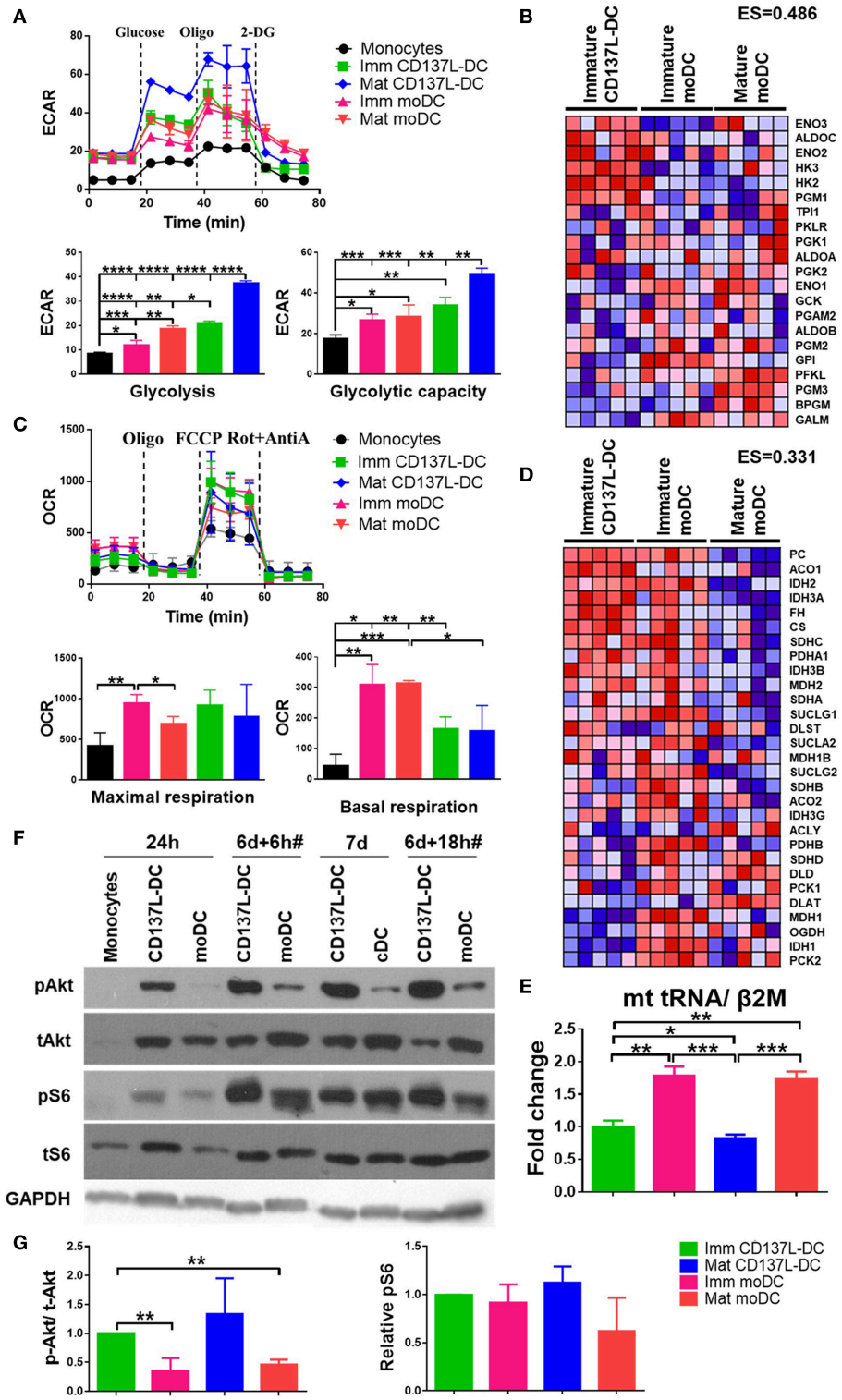

FIGURE 1 | CD137L-DCs have a higher glycolysis rate and higher Akt and mTORC activity than moDCs. **(A)** Glycolysis stress assay and **(C)** Mitostress assay were done by Seahorse XFe24 Analyzer. ECAR (pmol/min/Norm. Unit) and OCR (mpH/min/Norm. Unit) were normalized by the cell protein content. The basal glycolysis, glycolytic capacity, basal respiration, and maximal respiration were calculated according to the instructions provided by the kit's manufacturer. The heatmaps of the glycolysis gene signature **(B)** and the TCA gene signature **(D)** were drawn by comparing the levels of RNAs between immature CD137L-DCs and the other DC types (immature moDCs and mature moDCs) by GSEA. Shown are results from five different donors. Red: relatively enriched. Blue: relatively decreased. The enrichment score (ES) of the gene set in immature CD137L-DC relative to immature moDC and mature moDC as a whole is stated above the heatmap. **(E)** Relative mitochondrial

(Continued)

FIGURE 1 | counts in different DC types were measured by the mitochondrial DNA copy number. Shown are means ± standard deviations of triplicate measurements. **(F)** Monocytes were differentiated by GM-CSF + IL-4 or anti-CD137L antibody (clone 5F4) or the isotype antibody (clone MOPC-21) for 7 d. Cells were lysed at indicated time points. CD137L-DCs were matured by 1 μg/ml R848 + 50 ng/ml IFN-γ and moDCs were matured by 10 ng/ml IL-6, IL-1β, TNFα, and PGE2 during the last 18 h of differentiation, which is indicated by #. The activation of Akt and ribosome protein S6 were measured by Western blot analysis. These data are representative of three independent experiments. **(G)** The activation of Akt (p-Akt/ t-Akt) and mTORC1 (p-S6/ loading controls) was semi-quantified by ImageJ and normalized by the protein level in immature CD137L-DCs. Data from three different donors were statistically analyzed. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (two-tailed, two sample student *t* test).

of DCs. CD70, CD86, and CD137L also significantly decreased when glycolysis was suppressed by 2-DG (**Figures 2A,B**). However, this inhibition by 2-DG was not permanent. After 2-DG was washed out and the DCs were cultured in normal medium, all the co-stimulatory molecules increased to the level of control cells (**Supplementary Figure 5**), indicating that DCs are plastic and responsive to the changes in the environment.

Interestingly, CD137L-DCs treated with 2-DG were more resistant to cell death than moDCs. The inhibition of glycolysis altered the forward scatter and side scatter of mature moDCs but not of mature CD137L-DCs, indicating an increased percentage of cell death in mature moDCs (**Supplementary Figure 4B**). This vulnerability of mature moDCs to 2-DG induced cell death was further supported by in-plate trypan blue staining (**Supplementary Figure 4C**), which did not require cell scraping and thereby avoided potential damage to cells. These data tally with the data from the PathScan Intracellular Signaling Array showing that there is more extensive phosphorylation of the Bcl-2-associated death promoter (Bad) and less cleavage of caspase 3 in CD137L-DCs than in moDCs (**Supplementary Figure 4D**), indicating a lower degree of apoptosis. This dependence of moDCs on glycolysis for cell survival confirms previous findings in murine BMDC (17). In contrast, CD137L-DCs were more viable and not as dependent on glycolysis for cell survival.

Akt Drives the Increased Glycolysis and Activation of CD137L-DCs

As demonstrated above, the activation of CD137L-DCs was accompanied by an elevated glycolysis rate and an increased Akt-mTORC1 activity. An increased Akt-mTORC1 activity is the cause of an elevated glycolysis rate in LPS-activated murine BMDCs (26). In order to test whether such a causal relationship is also the case for human DCs, LY294002, an inhibitor of PI3K-Akt, and Rapamycin, an inhibitor of mTORC1, were used. The efficacy and specificity of the inhibitors were first confirmed (**Supplementary Figures 6A,B**). Inhibition of the Akt-mTORC1 pathway by LY294002 or Rapamycin slightly reduced the increase in glycolysis in mature moDCs (**Figure 3A**) but completely blocked it in mature CD137L-DCs (**Figure 3B**). Similarly as the inhibition of glycolysis by 2-DG, inhibition of glycolysis by LY294002 significantly impaired the expression of most co-stimulatory molecules and the secretion of pro-inflammatory cytokines by mature CD137L-DCs, while mature moDCs were not much affected (**Figures 3C,D**). In contrast, Rapamycin generally increased the expression of co-stimulatory molecules and IL-12 secretion, of which the reason is currently not known.

However, once the DCs were matured, the inhibition of Akt or mTORC1 had little effect on the expression of costimulatory molecules and cytokines by mature CD137L-DCs or mature moDCs (**Supplementary Figure 7**). The reason for this non-responsiveness may be that the signaling pathways are usually upstream of an activation decision point, and are only active for a short period after encountering a stimulus, such as the TLR ligands or maturation cocktails, while the metabolism is fundamental and active for an extended period.

mTORC1 Is Indispensable for the Differentiation of CD137L-DCs

Since the PI3K - Akt - mTORC1 pathway was activated early on upon the induction of DC differentiation (**Figure 1F**), we were wondering whether the PI3K - Akt - mTORC1 pathway could affect the differentiation of monocytes to DCs in addition to its effect on maturation. For that the concentration of inhibitors was first optimized (**Supplementary Figures 8A,B**). The most striking effect was that inhibition of mTORC1 from 1 h before to 24 h after induction of immature moDCs or CD137L-DCs differentiation always blocked the differentiation of DCs, as evidenced by the absence of the typical morphology of immature moDCs or CD137L-DCs (**Figure 4A**). After 7 d, fewer live DCs were present. The increased cell death after Rapamycin treatment is mainly a result of differentiation blockade but not of acute cytotoxicity of Rapamycin, since the viabilities of monocytes and nascent moDCs on day 1 were comparable between Rapamycin treatment and the control sample (**Supplementary Figure 8C**). Analysis of costimulatory molecule expression confirmed that mTORC1 inhibition during differentiation impaired the differentiation of DCs, with immature CD137L-DCs being more affected than immature moDCs (**Figure 4B**). The effect of Akt inhibition during differentiation was more variable among different donors. Expression of costimulatory molecules and cytokines by immature CD137L-DCs was reduced although the difference was not always statistically significant due to large donor to donor variation (**Figure 4C**).

The inhibition of Akt or mTORC1 during the first day of differentiation had a long-term influence on DC maturation. IL-12 is usually secreted by activated DCs, especially by mature CD137L-DCs. Even though the inhibitors were washed out by the end of the first day, LY294002-treated mature CD137L-DCs still secreted much less IL-12 while Rapamycin-treated mature CD137L-DCs secreted more IL-12 than the control cells, which is reminiscent of the IL-12 secreted by mature CD137L-DCs treated by LY29002 or Rapamycin during maturation (**Figure 3D**).

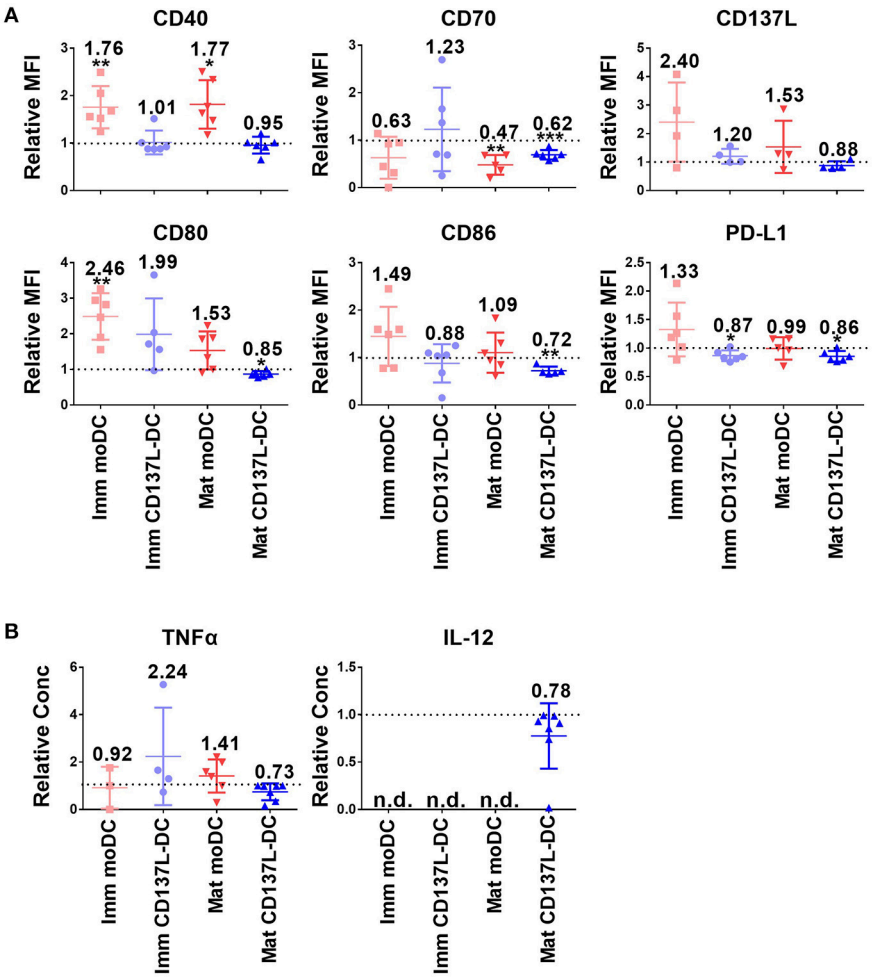

FIGURE 2 | The inhibition of glycolysis after maturation significantly impairs the function of mature CD137L-DCs. DCs were generated and on day 8 treated with 50 mM 2-DG for 24 h. **(A)** Cell surface expression of co-stimulatory and inhibitory molecules was measured by flow cytometry. **(B)** The secretion of cytokines by DCs was measured by ELISA. Depicted are the means ± standard deviations of relative changes upon addition of 2-DG to respective medium controls (values set at 1) from up to six independent experiments with DCs from different donors. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed, one sample student *t* test). MFI, geometric mean fluorescence intensity. n.d., not detected.

The Increased Glycolysis During DC Maturation Does Not Fuel Lipid Synthesis

Glycolysis can favor the function of DCs in many different ways, such as providing carbons and reducing power for lipid synthesis (15). However, there are conflicting data concerning the effect of fatty acid synthesis blockade on DC function (15, 29). Our previous data showed an enrichment in gene expression related to the lipid metabolism in immature CD137L-DCs compared to moDCs (23). But neither had mature CD137L-DCs more phospholipids or neutral lipids than mature moDCs, nor did the lipid content in CD137L-DCs increase upon maturation (**Figure 5A**), indicating that the synthesis of fatty acids is not the main output of the increased glycolysis in CD137L-DCs.

Since acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) are key enzymes for lipid metabolism, we inhibited them with TOFA and C75, respectively. Both inhibitors did not lead to a decrease of the lipid content in treated DCs (data not shown). Nevertheless, C75 significantly suppressed the maturation of CD137L-DCs and moDCs as evidenced by the lower expression of most co-stimulatory molecules (**Figure 5B**) and the almost complete block of IL-12 secretion (**Figure 5C**). In the allogenic mixed leukocyte reaction (MLR), T cells activated by C75-treated DCs secreted less IFN-γ and proliferated less than T cells activated by the control DCs (**Figure 5D**). However, the expression of co-stimulatory molecules and IL-12 were not suppressed by TOFA (**Supplementary Figures 9A,B**). TOFA-treated DCs did not have a defect in stimulating the T cells (**Supplementary Figure 9C**). A representative set of histogram of T cell proliferation is shown in **Supplementary Figure 9D**.

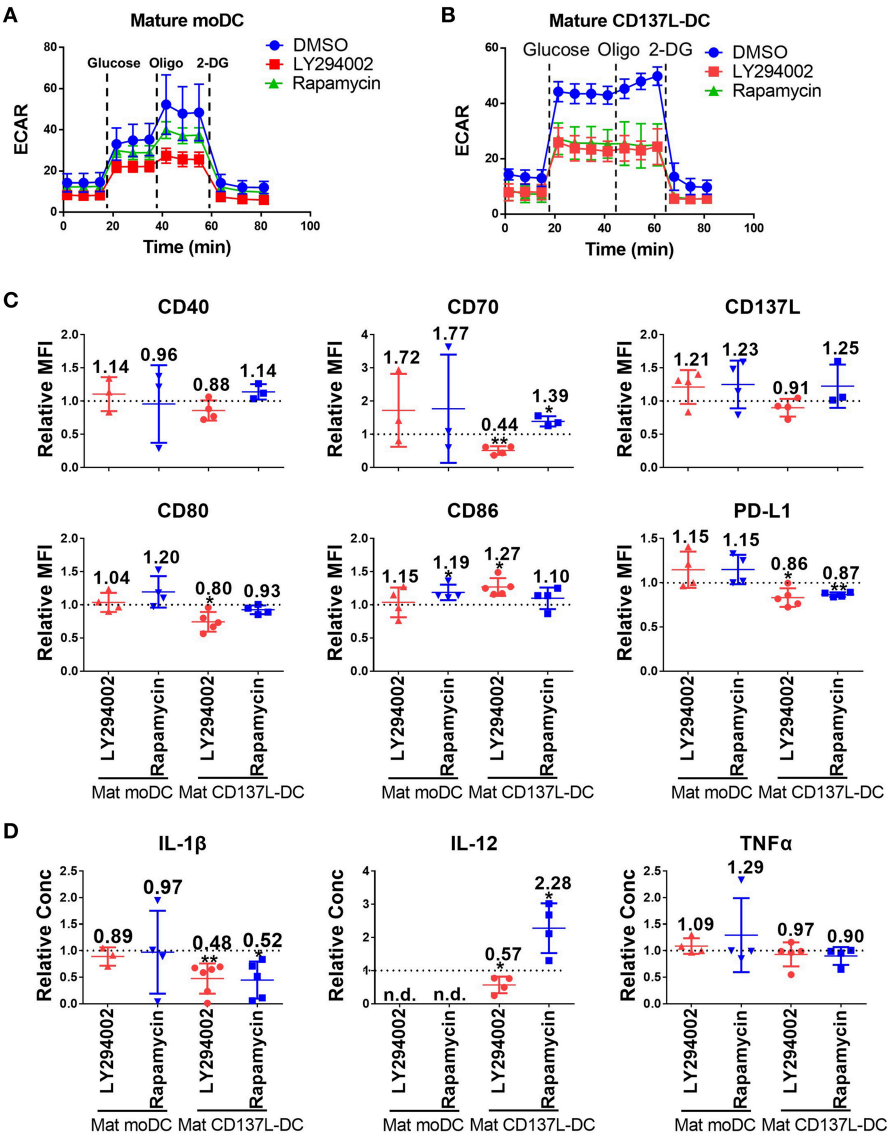

FIGURE 3 | The activation of Akt and mTORC1 is important for the commitment to glycolysis and maturation of CD137L-DCs. DCs were differentiated and pre-treated with DMSO or 10 μM LY294002 or 50 nM Rapamycin for 1 h before maturation. Glycolysis stress assays of **(A)** mature moDCs and **(B)** mature CD137L-DCs were done with a Seahorse XFe24 Analyzer. **(C)** Cell surface expression of co-stimulatory and inhibitory molecules was measured by flow cytometry. **(D)** The secretion of cytokines by DCs was measured by ELISA. Depicted are means ± standard deviations of changes upon addition of 10 μM LY294002 or 50 nM Rapamycin relative to respective DMSO controls (values set at 1) from up to 5 independent experiments with DCs from different donors. $*p < 0.05$, $**p < 0.01$ (two-tailed, one sample student *t* test). n.d., not detected.

Succinate and Serine Are Enriched in CD137L-DCs

To determine the consequence of the higher glycolysis rate in CD137L-DCs, an unbiased metabolomics experiment, covering amino acids and intermediates from glycolysis and the TCA cycle, was performed. Unexpectedly, citrate, a TCA intermediate that has been reported to accumulate in activated BMDCs (15), was not elevated in mature moDCs, and was lower in CD137L-DCs than moDCs (**Figure 6A**). However, succinate, another intermediate in TCA cycle, was found to be highly enriched in CD137L-DCs (**Figure 6B**). A further highly enriched metabolite in CD137L-DCs was serine (**Figure 6C**), which can be derived from glycolysis. Both succinate and serine play a role in DNA and histone methylation (30, 31).

DISCUSSION

It is increasingly appreciated that metabolic reprograming accompanies the activation of leukocytes. We found that CD137L-DCs have a higher basal glycolysis rate than moDCs

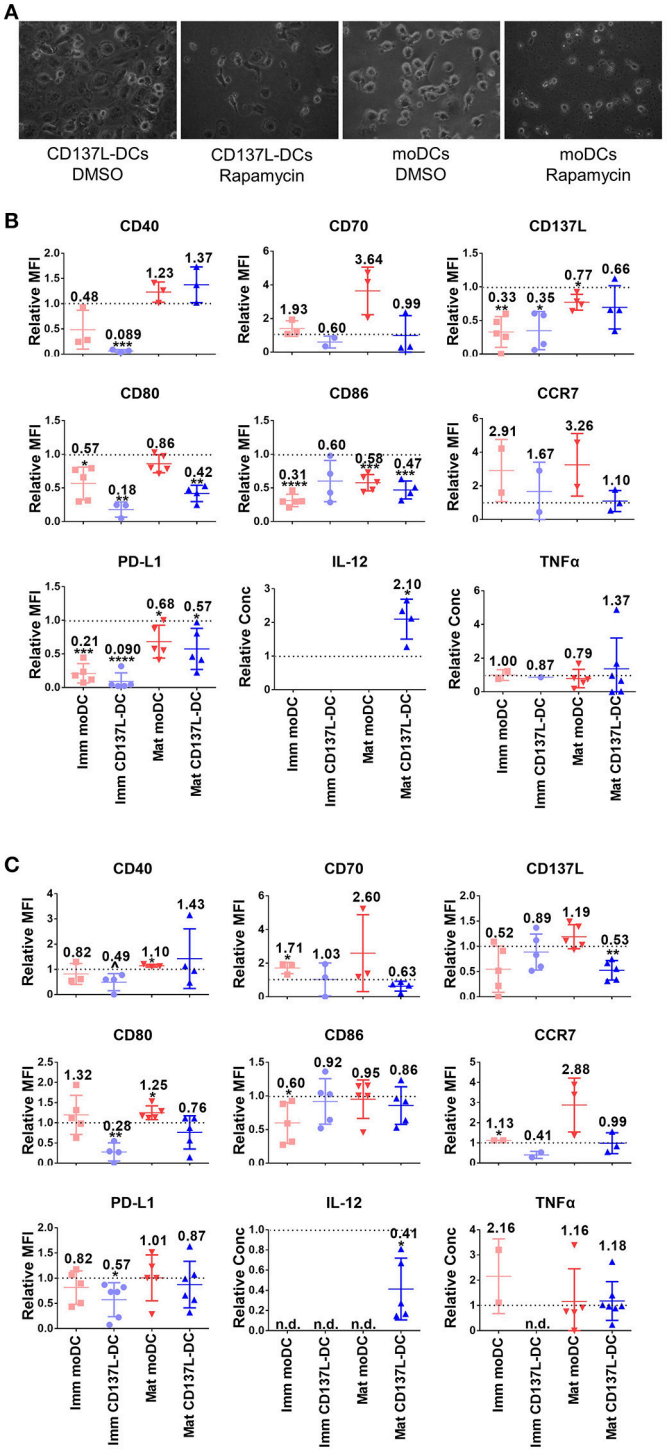

FIGURE 4 | The activation of mTORC1 is indispensable for the differentiation and function of CD137L-DCs and moDCs. Primary monocytes were pre-treated with DMSO or 2 μM LY294002 or 10 nM Rapamycin for 1 h before the differentiation to moDC or CD137L-DCs was induced. 24 h after the initiation of differentiation, inhibitors were washed out and the moDCs were re-supplemented with GM-CSF + IL-4. Where indicated, DCs were matured during the last 18 h of the 7-day culture. **(A)** mTORC1 inhibition by Rapamycin blocks differentiation. Shown are representative photos of DCs, taken before the drug wash-out. Cell surface expression of co-stimulatory and inhibitory molecules and the secretion of cytokines after **(B)** Rapamycin and **(C)** LY294002 treatment were measured. Depicted are means ± standard deviations of changes upon addition of 2 μM LY294002 or 10 nM Rapamycin relative to respective DMSO controls (values set at 1) from up to 7 independent experiments with DCs from different donors. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, $p = 0.0584$ (two-tailed, one sample student *t* test). n.d., not detected.

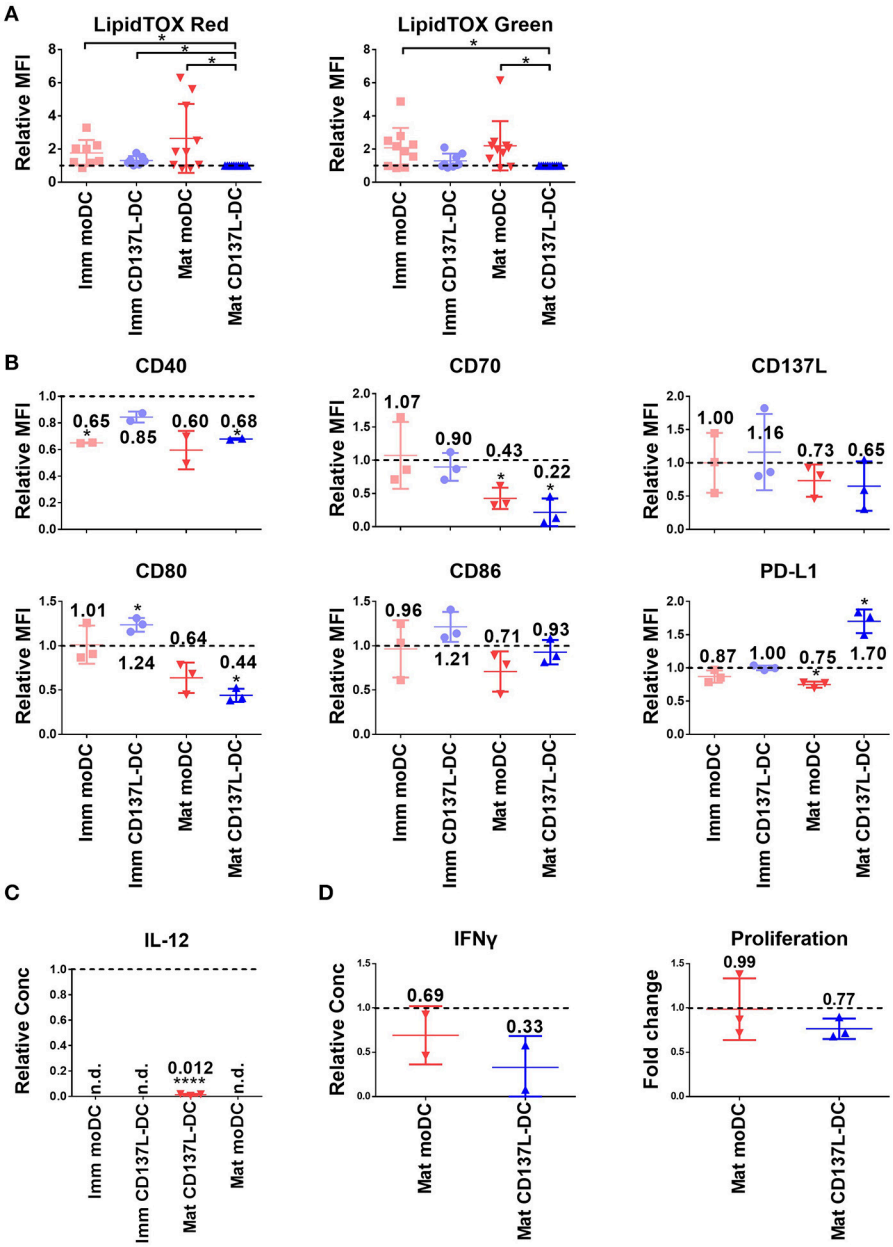

FIGURE 5 | C75 suppresses the maturation of moDCs and CD137L-DCs. **(A)** The phospholipid (left) and neutral lipid (right) contents were measured by LipidTOX reagents. The MFIs of other DCs were normalized by the MFIs of the respective mature CD137L-DCs from up to 11 different donors. DCs were pretreated with 20 μM C75 for 1 h before the last 18 h of maturation/culture. **(B)** Cell surface markers and **(C)** IL-12 in the supernatant were measured. The values were normalized by the respective DMSO-treated controls, and results from three donors were pooled. **(D)** C75-treated DCs were co-cultured with CellTrace Violet labeled allogenic T cells at 1:10 ratio for 5 days. The proliferation of T cells and secretion of IFN-γ by T cells were measured. *$p < 0.05$, ****$p < 0.0001$ (two-tailed, one sample student t test). n.d., not detected.

because of a higher activity of Akt. After maturation by IFN-γ and the TLR7/8 ligand R848, mature CD137L-DCs have an even higher activity of the Akt-mTORC1 pathway, leading to a further increase in the basal glycolysis rate and the glycolytic capacity. We demonstrated that glycolysis is not only important for the increased expression of co-stimulatory molecules and the increased secretion of inflammatory cytokines during the maturation of CD137L-DCs, but is also important for the preservation of their activated state after maturation. The inhibition of Akt nicely recapitulates the suppressive effects of inhibition of glycolysis on CD137L-DC activity. It is therefore the high Akt-driven glycolysis rate that is the basis for the higher potency of CD137L-DCs compared to moDCs. The essence of our findings is graphically depicted in **Figure 7**.

Fast growing tumor cells often deplete glucose in the microenvironment (32, 33), leading to a dampened immune

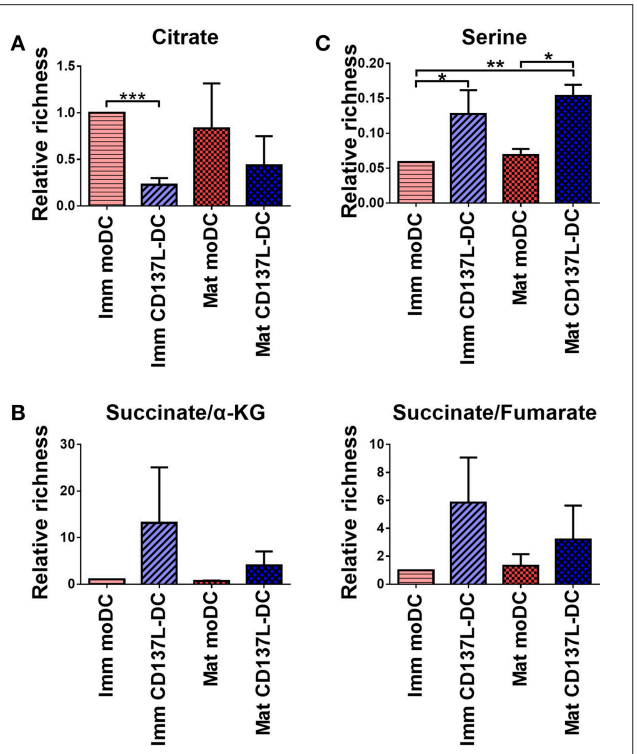

FIGURE 6 | Succinate and serine are enriched in CD137L-DCs. Results from three different donors were normalized based on protein concentrations and statistically analyzed. **(A)** Relative citrate concentrations. **(B)** Relative succinate/α-ketoglutarate and succinate/fumarate ratios. **(C)** Relative abundance of serine. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed, paired *t* test).

response (34). Similarly as tumor cells, T cells upon activation also switch to aerobic glycolysis to support their proliferation (10). In the lymph node, where T cells become primed and activated, glucose level may be low. It is possible that T cells, after being activated by tumor associated antigen (TAA)-loaded DCs, proliferate for some time before the low glucose level in the lymph node suppresses DCs and limits T cell activation. Therefore, multiple injections of *in vitro*-generated DCs are needed to achieve sufficient T cell activation against tumors (35). One advantage of CD137L-DCs is that they are more resistant to spontaneous apoptosis and 2-DG-induced cell death. It is possible that CD137L-DCs survive longer in the lymph node, and therefore deliver stronger and longer-lasting activation to T cells. The plasticity of CD137L-DCs allows them to adapt to the changing environment, and may make it possible to fine-tune the tumor microenvironment and lymph node microenvironment with drugs in order to augment DC-based immunotherapy (36).

We have proven that both Akt and its downstream target mTORC1 mediate the increase of glycolysis in mature CD137L-DCs. However, only the inhibition of Akt during maturation suppresses inflammatory mature CD137L-DCs. The inhibition of mTORC1 by Rapamycin generally enhances the inflammatory features of mature moDCs and mature CD137L-DCs. This discrepancy suggests there are other regulating factors downstream of Akt and mTORC1 besides glycolysis that are involved in the activation of DCs. On top of that, the inhibition of mTORC1 by Rapamycin can be both pro-inflammatory and anti-inflammatory. Sukhbaatar et al. proposed a model where the effect of mTORC1 inhibition on DC function is spatiotemporal: mTORC1 inhibition during early DC activation in the periphery suppresses inflammatory DCs while mTORC1 inhibition during late DC activation in the lymph node enhances the T cell activating ability (37). Our results support this model. For example, the early cytokine IL-1β secreted by mature CD137L-DCs is inhibited, whereas the late cytokine IL-12 is enhanced by Rapamycin.

During the differentiation of DCs, mTORC1 rather than Akt plays the more important role. mTORC1 inhibition blocks the DC differentiation and leads to massive cell death. This blockade of Akt or mTORC1 during differentiation has long-lasting consequences on the generated cells. Even when the monocytes were treated with the PI3K inhibitor LY294002 only during the first 24 h of differentiation, IL-12 secretion was still suppressed in the resulting matured DC on day 7. But the opposite, i.e., and enhancement of IL-12 secretion in resulting DC, was obtained when monocytes had been treated with the mTOR inhibitor Rapamycin. This long-term effect resembles the reported innate memory where monocytes are more inflammatory to a second stimulus (38). The molecular basis for this long-term effect may be the Akt-mTORC1-mediated glycolysis which has been reported to be involved in the epigenetic regulation of monocyte memory (39).

Everts and colleagues suggested that the increased glycolysis during activation results in an accumulation of citrate for the synthesis of lipids, which expands the ER and Golgi apparatus (15). However, we could not find an accumulation of citrate. Neither did we observe a higher lipid content in moDCs or CD137L-DCs after maturation, nor could we measure more lipids in CD137L-DCs than in moDCs. It has been shown that in monocytes different TLR ligands induce very different metabolic changes and transcriptomes (40). It is possible that the increased lipid synthesis is specific to LPS-activated moDCs but not to CD137L-DCs or cytokine-activated moDCs. The two fatty acid synthesis inhibitors, C75 and TOFA, did not decrease the lipid contents in any of the four types of DCs. However, the inhibition of FASN by C75 inhibited the maturation of both, CD137L-DCs and moDCs. It is possible that C75 reduces the level of Acetyl-CoA for acetylation, which plays an important role in the regulation of inflammation-related gene expression (41). Another possibility is that C75 alters the ratio of pro-inflammatory lipids to anti-inflammatory lipids (42).

Succinate and α-ketoglutarate have been reported to be involved in the polarization of M1 and M2 macrophages (43). Succinate accumulates in M1 macrophages and promotes inflammation (44). A higher succinate to α-ketoglutarate ratio preferentially induces pro-inflammatory macrophage differentiation while a lower succinate / α-ketoglutarate ratio promotes anti-inflammatory macrophage differentiation (31). It is very likely that the higher succinate/α-ketoglutarate ratio contributes to the pro-inflammatory features of CD137L-DC.

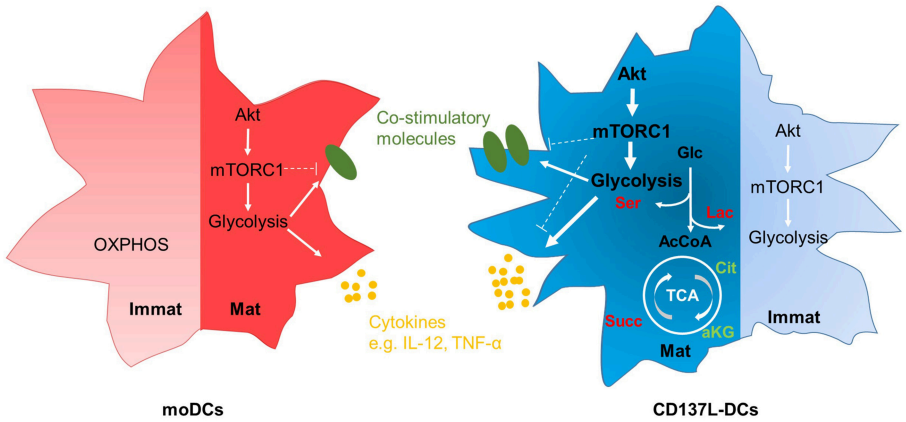

FIGURE 7 | Graphic abstract of the main findings. Immature moDCs have minimal activation of the Akt-mTORC1 pathway, and rely mainly on OXPHOS at the resting stage. In contrast, immature CD137L-DCs have a high activation of the Akt-mTORC1 pathway at the resting stage, leading to an increased glycolysis. After maturation, both mature moDCs and mature CD137L-DCs display an elevated activity of Akt-mTORC1, leading to higher glycolysis and the increased expression of co-stimulatory molecules and pro-inflammatory cytokines. Compared with mature moDCs, mature CD137L-DCs have a significantly higher Akt-driven glycolysis, and secrete more pro-inflammatory cytokines. This higher glycolysis leads to a relative accumulation of succinate and serine rather than citrate or lipids. Red: relative accumulation. Green: relative depletion.

Succinate and α-ketoglutarate are also involved in the epigenetic regulation of cancer cells and macrophages (31, 45). Serine as an indispensable substrate for the synthesis of S-adenosylmethionine (SAM), a methyl group donor, plays a role in the epigenetic regulation of gene expression (30). The accumulation of succinate and serine in CD137L-DC might not be a coincidence, but may have a synergistic effect on epigenetic upregulation of pro-inflammatory gene expression.

Inflammatory DCs, M1 macrophages and effector T cells all reprogram their metabolism and increase glycolysis rates upon activation (46), and the function of these cells can be dampened if glycolysis is inhibited. However, it is unknown at present if the functions of these cells can be enhanced by simply increasing their (1) glycolysis, (2) activation of the Akt-mTORC1 pathway, or (3) their ability to compete for glucose in the tumor microenvironment. We have tried to achieve this by using the Akt agonist SC-79 (47) and the mTORC agonist MHY1485 (48), but to no avail. It is also not clear by what mechanisms glycolysis supports the functions of these immune cells. Our data argue for further in-depth investigation of the already increasingly appreciated interplay between metabolism and epigenetics.

It would have been informative to demonstrate the enhanced Akt-driven glycolysis as the basis of the higher potency *in vivo*, e.g., in a murine tumor model, by genetic manipulation of key glycolytic enzymes (e.g., HK2) specifically in CD137L-DCs. Unfortunately, CD137L-DCs do not exist in mouse (7), and the reason may be the large difference in CD137L between human and mouse. While for most members of the TNF and TNF receptor families the human—mouse homology is 60–80%, it is only 36% for human and murine CD137L (49).

In summary, we have demonstrated (1) that the Akt-driven glycolysis is crucial for the sustained activation of CD137L-DCs, (2) that the higher Akt-driven glycolysis is part of the reason why CD137L-DCs are more potent than the conventional moDCs, and (3) that Akt-driven glycolysis leads to an accumulation of succinate and serine instead of lipids in CD137L-DCs. Our finding suggests that the Akt-driven glycolysis could be a therapeutic target to manipulate the function of CD137L-DCs for better clinical efficacy.

ETHICS STATEMENT

Blood was obtained from healthy volunteers who provided written and informed consent. The protocol was approved by the National University of Singapore (NUS) IRB number B15-320E.

AUTHOR CONTRIBUTIONS

QZ and HS: conceptualization and writing—original draft; QZ and KM: methodology; QZ: investigation and data curation; HS, QZ, and KM: writing—review and editing; HS: supervision and funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00868/full#supplementary-material

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Combining the Specific Anti-MUC1 Antibody TAB004 and Lip-MSA-IL-2 Limits Pancreatic Cancer Progression in Immune Competent Murine Models of Pancreatic Ductal Adenocarcinoma

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Immunotherapy regimens have shown success in subsets of cancer patients; however, their efficacy against pancreatic ductal adenocarcinoma (PDA) remain unclear. Previously, we demonstrated the potential of TAB004, a monoclonal antibody targeting the unique tumor-associated form of MUC1 (tMUC1) in the early detection of PDA. In this study, we evaluated the therapeutic benefit of combining the TAB004 antibody with Liposomal-MSA-IL-2 in immune competent and human MUC1 transgenic (MUC1.Tg) mouse models of PDA and investigated the associated immune responses. Treatment with TAB004 + Lip-MSA-IL-2 resulted in significantly improved survival and slower tumor growth compared to controls in MUC1.Tg mice bearing an orthotopic PDA.MUC1 tumor. Similarly, in the spontaneous model of PDA that expresses human MUC1, the combination treatment stalled the progression of pancreatic intraepithelial pre-neoplastic (PanIN) lesion to adenocarcinoma. Treatment with the combination elicited a robust systemic and tumor-specific immune response with (a) increased percentages of systemic and tumor infiltrated CD45+CD11b+ cells, (b) increased levels of myeloperoxidase (MPO), (c) increased antibody-dependent cellular cytotoxicity/phagocytosis (ADCC/ADCP), (d) decreased percentage of immune regulatory cells (CD8+CD69+ cells), and (e) reduced circulating levels of immunosuppressive tMUC1. We report that treatment with a novel antibody against tMUC1 in combination with a unique formulation of IL-2 can improve survival and lead to stable disease in appropriate models of PDA by reducing tumor-induced immune regulation and promoting recruitment of CD45+CD11b+ cells, thereby enhancing ADCC/ADCP.

Keywords: ADCC, Antibody, IL-2, Immunocompetent, Immunotherapy, MUC1, PDA, TAB004

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) has the poorest prognosis of all malignancies with more than 260,000 deaths annually worldwide, a 5% 5-year survival rate, a mean life expectancy of <6 months, and a high degree of resistance to standard therapy (1–4). Radiotherapy and chemotherapy remain largely ineffective. While surgery is an option, only 20% of PDA patients have resectable tumors at the time of diagnosis and the recurrence rate remains high in these patients. In addition to surgery, PDA is treated with adjuvant therapies including gemcitabine to reduce the incidence of local recurrences and distant metastases (3, 5). Combination treatments such as rosiglitazone and gemcitabine, FOLFIRINOX (5-FU, leucovorin, irinotecan, and oxaliplatin), monoclonal antibody (mAb), and 5-fluorouracil, or gemcitabine and nab-paclitaxel have been shown to significantly reduce tumor progression and metastases and significantly extend overall patient survival (1, 6–8). While those treatments led to some improvements and extended overall survival in small subsets of patients (from 6–7 months to ∼25 months) (8, 9), improved approaches to treat patients with pancreatic cancer remain urgent (3, 10).

Cancer immunotherapies that target tumor associated antigens present attractive alternatives as these approaches are expected to cause fewer side effects while preventing metastasis and recurrence better than standard therapies. Antibody-based immunotherapy for cancer was established within the past 15 years, and is now one of the most successful strategies for treating patients with hematological and solid tumors (11). The fundamental basis of antibody-based therapy of tumors relies on the presence of cell surface antigens that are overexpressed, mutated or selectively expressed compared with normal tissues (11). A key challenge has been to identify antigens that are suitable for antibody-based therapeutics. There are approximately 460 active clinical trials with 38 antibody-based drugs and several new products under development. Some examples of FDA approved antibodies for solid tumors include Herceptin®, Avastin®, Erbitux®, Vectibix®, and Ipilimumab®. However, none is approved for pancreatic cancer.

There is clinical evidence for mAb driven T cell immunity. For instance, the therapeutic effect of rituximab was augmented by eliciting a T cell response (12). Further, administration of cetuximab triggered expansion of EGFR-specific T cells (13); and trastuzumab elicited a Her-2/neu-specific cellular response (14). Since interleukin-2 (IL-2) through enhancement of NK ADCC greatly improved the therapeutic efficacy of mAbs (15, 16), trials with trastuzumab and rituximab in combination with IL-2 were conducted. The results were disappointing, with little to no objective clinical response observed with the combination (17, 18). This is most likely because IL-2 in its native form is short lived *in vivo* and increasing the dose is toxic. Indeed, combining antibodies with a form of IL-2 with extended circulation provided surprisingly robust control of B16 melanoma tumor growth, in the absence of any marked toxicity (19). Administration of IL-2, which supports the survival and function of tumor-reactive T cells (20), has been shown to benefit some patients with melanoma (21). However, the vascular leak syndrome associated with the high-dose IL-2 treatment regimen has limited its use in tumor immunotherapy (21). More recently, Lip-MSA-IL-2, a formulation stabilizing IL-2, was associated with the generation of an immune response that prevented melanoma progression in a murine model (22).

Mucin-1 (i.e., MUC1, CD227) is a membrane-tethered mucin overexpressed and aberrantly glycosylated in many epithelial malignancies, including >90% of human PDA (23–29). The hypo-glycosylated MUC1 expressed on malignant cells renders normally cryptic MUC1 epitopes open to detection and is hereto forth referred to as tMUC1. MUC1 has long been an interesting target molecule for immunotherapy development, given its highly increased cell surface expression and altered glycosylation in tumors [reviewed in (30)]. Many antibodies have been developed that recognize epitopes of those tumor-associated hypo-glycosylated MUC1 regions, including PankoMab, Pemtumomab (also known as HMFG1) and TAB004 (26, 27, 31–33). TAB004 (patent #8,518,405, and 9845362 B2) was initially developed using pancreatic tumors expressing the altered form of MUC1 (34). TAB004 targets the epitope area (AA950-958) which is only accessible for antigenic detection in cells expressing the hypo-glycosylated form of MUC1 (35–38). In contrast to most other MUC1 antibodies, TAB004 distinguishes between normal and tumor-associated forms of MUC1 by relying solely on the expression of hypo-glycosylated MUC1. Further, TAB004 was effective in identifying primary PDA and pancreatic cancer stem cells in PDA patients, while sparing recognition of normal tissue (27, 39).

Previously, we have demonstrated the effectiveness of MUC1-directed tumor vaccines in colorectal, pancreatic, and breast cancer models (38, 40, 41); however, immunosuppression within the tumor microenvironment hindered the effectiveness of the vaccine (41). We have recently shown that an anti-MUC1 antibody can be used as a therapeutic antibody when conjugated to the immune modulating agent CpG ODN via enhanced NK cell anti-tumor activity against PDA tumors (42).

Here we sought for the first time to determine whether the combination of TAB004 and stabilized Lip-MSA-IL-2 elicits an immune response and confers a survival benefit in orthotopic and spontaneous immunocompetent murine models of PDA. Our results indicate that, with minimal toxicity, the combination of TAB004 + Lip-MSA-IL-2 was associated with improved survival in the orthotopic murine model of PDA, as well as a lower cancer burden in the PDA.MUC1 mouse spontaneous model of PDA.

Abbreviations: ADCC, Antibody Dependent Cell Cytotoxicity; ADCP, Antibody Dependent Cell Phagocytosis; APC, Allophycocyanin; CBC, Cell Blood Counts; EGFR, Epidermal Growth Factor Receptor; FITC, Fluorescein Isothiocyanate; IFN, Interferon; IL, Interleukin; MPO, Myeloperoxidase; PDA, Pancreatic Ductal Adenocarcinoma; PE, Phycoerythrin; RBC, Red Blood Cells; TAB004, Anti-tMuc1 antibody; TIL, Tumor Infiltrating Lymphocytes; TNF, Tumor Necrosis Factor; WBC, White Blood Cells.

MATERIALS AND METHODS

TAB004 Antibody and Lip-MSA-IL-2

The antibody TAB004 has been described earlier (27, 43) (OncoTab Inc., Charlotte NC). The stabilized Lip-MSA-IL-2

has been described (22) and was provided by Dr. Wittrup (Massachusetts Institute of Technology, Cambridge, MA). The optimal dose of TAB004 (500 μg/mouse/injection i.e., 25 mg/kg/dose) was determined in preliminary experiments using doses ranging from 62.5 to 1,000 μg/mouse/injection. The dose of Lip-MSA-IL-2 used (25 μg/mouse/injection, i.e., 1.25 mg/kg/dose) was derived from previous experiments (22).

KCM and KCM-LUC+ PDA Cells

KCM cells were generated from spontaneous PDA tumors from PDA.MUC1 triple transgenic mice (LSL-$Kras^{G12D}$ X ^{P48}Cre X human MUC1.Tg mice) (44) and, therefore, express human MUC1 (43, 45). The KCM-Luc cell line was generated by retroviral transduction of KCM cells with the MSCV Luciferase PGK-Hygro plasmid (Addgene plasmid # 18782, a generous gift from Scott Lowe, Memorial Sloan Kettering Cancer Center, New York, NY) (46). Both KCM and KCM-Luc+ cells were cultured and expanded in DMEM (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Gibco), glutamine, penicillin, and streptomycin (Cellgro, Corning, Manassas, VA).

Spontaneous Mouse Model of PDA

Triple transgenic mice (i.e., PDA.MUC1 also designated KCM mice) express human MUC1 as a self-molecule and is the first model of invasive pancreatic cancer that expresses human MUC1 (44). Indeed, KCM mice develop ductal lesions with complete penetrance (100%), very similar to all three stages of human pancreatic intraepithelial neoplasia (PanIN) lesions (PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3) and progress to adenocarcinoma and lung metastasis. As early as 6–16 weeks of age, mice develop PanINs of different stages including PanIN-IA, PanIN-IB, and PanIN-2. By 20–26 weeks of age, early PanIN lesions progress to PanIN-3 and carcinoma *in-situ* and by 30–36 weeks, invasive adenocarcinoma and metastasis are observed. As in human PDA, tumor cells express high levels of tMUC1 (44) that were detectable using the TAB004 antibody (27). KCM mice are characterized by (1) tumors arising spontaneously in the pancreatic ductal epithelial cells due to the $KRAS^{G12D}$ mutation; (2) the normal human MUC1 transformed to tMUC1 with disease progression just as observed in the human disease; (3) tumors arising in fully immune competent host; and (4) tolerance to MUC1 immunization as MUC1 is expressed as a self-molecule driven by its own regulatory sequence (47).

Following an Institutional Animal Care and Use Committee (IACUC) approved protocol, KCM triple transgenic mice were primed to activate the KRAS mutation through a CRE tamoxifen sensitive cassette (41, 44) during week 12–13 of age. All KCM mice treated with tamoxifen (20 mg/ml/mouse 5 day/week for 2 weeks) develop PanIN lesions by 20–23 week of age. At that age, animals ($n = 18$) were randomized and treated with either PBS (vehicle, $n = 4$), TAB004 alone ($n = 4$), Lip-MSA-IL-2 ($n = 4$) alone or the combination of TAB004 + Lip-MSA-IL-2 ($n = 6$; for treatment schedule and dose, see **Figure 1A**). Two animals died or were removed from the study per IACUC guidelines: one in the PBS group and one in the TAB004 alone group, respectively. Animals were treated for 5 weeks and monitored daily for health concerns. Body weight was recorded weekly and all mice were euthanized at 36–40 week of age. At euthanasia, pancreata were collected free of fat and surrounding tissue, fixed in 10% buffered formalin and embedded in paraffin. Pancreas sections (4–6 μm) were stained with hematoxylin and eosin (H&E), and the presence of PanIN lesions and/or adenocarcinoma was determined following a microscopic assessment of 5 sections per pancreas. For each animal, pancreatic lesions were counted and scored in 10 microscope fields (100x) and for each pancreas, the most advanced stage was reported (41, 44).

Orthotopic Mouse Model of PDA

Surgeries were performed in a sterile environment under the supervision of the UNCC attending veterinarian and IACUC approved protocols. The resident veterinarian (Dr. Williams, DVM) orthotopically injected 20,000 KCM-Luc+ cells (∼50 μl) in the pancreas of both male and female human MUC1.Tg mice (originally received from Dr. Gendler, Mayo Clinic, Arizona and bred in-house at UNC Charlotte) (47). When implanted orthotopically in those mice, KCM cells generate tumors (45). The optimal number of cells to be injected was determined in a preliminary experiment using 10,000–200,000 KCM-Luc+ cells. Following surgical healing (i.e., on day 7 post-surgery), the presence of KCM-Luc+ tumors in mice was assessed using an IVIS system (Perkin Elmer). Treatment started on day 8 post-surgery and included four groups: vehicle (PBS), TAB004, Lip-MSA-IL-2, or the combination TAB004 + Lip-MSA-IL-2. Treatments were administered IP once weekly and tumor progression was monitored by chemiluminescence through weekly IVIS imaging. Detailed schedules and dose are provided in **Figures 2A**, **3A** for survival and mechanistic studies, respectively.

Mice were weighed weekly and monitored for activity level and adverse health events daily. Loss of more than 20 percent of body weight led to the euthanasia of the animal at which time tumor and serum were collected. For the survival studies, animals ($n = 6$–7 per treatment group) were treated for up to 5 weeks. For the mechanistic study, animals ($n = 3$ per treatment group) were euthanized at day 18 based on preliminary studies in which control animals (vehicle-injected group) had to be removed from the study by day 19–22 due to high tumor burden and morbidity. No TILs were available for one animal from the TAB004 group. For all animals, tumors and surrounding pancreas, spleen, and blood were collected. For the mechanistic studies, cells from spleen, tumor, and blood were used for ADCC/ADCP assays, flow-cytometry analyses of tumor infiltrating lymphocytes, and of systemic immune cells. In addition, sera were assayed for cytokines, myeloperoxidase (MPO) and the presence of tMUC1. Collected whole blood was used to determine cell blood counts (CBC; IDEXX, Columbia, MO).

Flow Cytometry Analyses of Blood and Intra-Tumoral Immune Cells

Blood, spleen and tumors, obtained on day 18 post-tumor implantation from animals orthotopically implanted with KCM-Luc+ cells and treated as detailed above, were assessed by flow cytometry for specific subsets of lymphocytes. Tumor cell suspensions were obtained following mechanical disruption

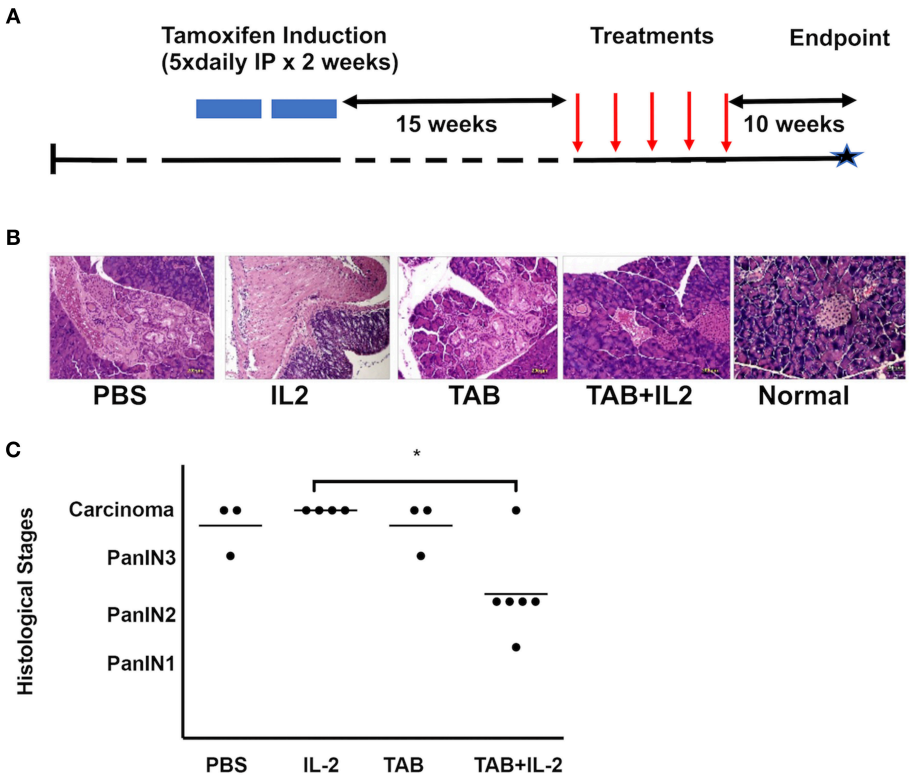

FIGURE 1 | TAB004 + Lip-MSA-IL-2 treatment significantly slowed tumor progression in a spontaneous pancreatic cancer mouse model. KrasG12Dmut; P-48Cre; MUC1.TG (KCM) triple-transgenic 8–16 week-old mice were induced with Tamoxifen (75 mg/kg, IP, for 5 consecutive days for 2 weeks). Following tamoxifen induction, all KCM mice develop pancreatic cancer lesions around 30–40 week of age (41, 44). **(A)** Tamoxifen-induced 23–31 week-old mice were administered once weekly either PBS ($n = 3$), Lip-MSA-IL-2 ($n = 4$), TAB004 ($n = 3$), or the combination TAB004 + Lip-MSA-IL-2 ($n = 6$). Ten weeks later, pancreata were collected and processed for histology. Pancreas slides (5–6 μm thick) from each mouse were stained using hematoxylin and eosin and the presence of Pan lesions and/or carcinoma blindly assessed and recorded. **(B)** Representative micro-photographs of H&E stained pancreas sections from normal and tamoxifen-induced KCM mice treated with PBS, Lip-MSA-IL-2, TAB004, and TAB004 + Lip-MSA-IL-2. Note the presence of carcinoma in all pancreases except those of normal and TAB004 + Lip-MSA-IL-2 treated mice. **(C)** Each pancreas was evaluated using the pancreatic cancer histological stages, i.e., PanIN1, PanIN2, PanIN3, and carcinoma. *$p < 0.05$. TAB, TAB004; IL2, Lip-MSA-IL-2; IP, intraperitoneal.

of the tumor mass and filtration through 70 μm strainers (BD Biosciences San Jose, CA). Non-necrotic areas were used to generate cell suspensions, and cell suspensions were further treated 15 min with DNAse1 (10 μg/ml). Tumor cell suspensions were washed, counted and resuspended in PBS (1×10^7 cells/ml). For each sample, 10^6 cells were stained for CD45 (anti-CD45-APC), CD4 (anti-CD4-FITC), CD8 (anti-CD8-PE), CD69 (anti-CD69-PE-cy7), NK (anti-NK-1.1-PE), CD107 (anti-CD107-FITC), anti-ly6G-PE, and/or CD11b-FITC. Ly6G and CD11b have been used to identify and deplete neutrophils (48) and macrophages (49) predominantly, respectively. Of note, CD11b in particular is also expressed on the surface of other immune cells (50). Corresponding isotype controls for APC, PE, FITC, PE-Cy7 were run concurrently. All antibodies were purchased from BD-Biosciences. Additionally, cells were stained to exclude dead cells using Fixable Viability Dye (FVD, eBioscience, CA) (51). Samples were run on a Fortessa flow-cytometer (BD Biosciences) and the data analyzed using FlowJo software (BD Biosciences).

Systemic Cytokines

Sera obtained on day 18 post-tumor implantation from animals orthotopically implanted with KCM-Luc+ cells and treated as detailed above, were assessed for the following 20 cytokines: GMCSF, IL-1α, IL-2, IL-4, IL-6, IL-10, IL-13, CXCL1, M-CSF, TNF-α, IL-1β, IL-3, IL-5, IL-9, IL-12, IL-17, MCP1, RANTES, and VEGF (Quantibody® Mouse Cytokine Array 1; Raybiotech) according to the manufacturer's recommendations. Following incubations, washing, and detection steps, fluorescent signal was detected using a Tecan LS300 Series Scanner (Tecan, San Jose, CA).

Systemic Myeloperoxidase

The concentrations of MPO, a phagocyte hemoprotein (that primarily mediates host defense reactions) abundantly expressed in neutrophils and moderately expressed in macrophages and secreted during their activation (52, 53), was determined by ELISA (Boster Biological Tech, CA). MPO levels were determined in sera obtained on day 18 post-tumor implantation. MPO concentrations expressed in pg/ml of sera were

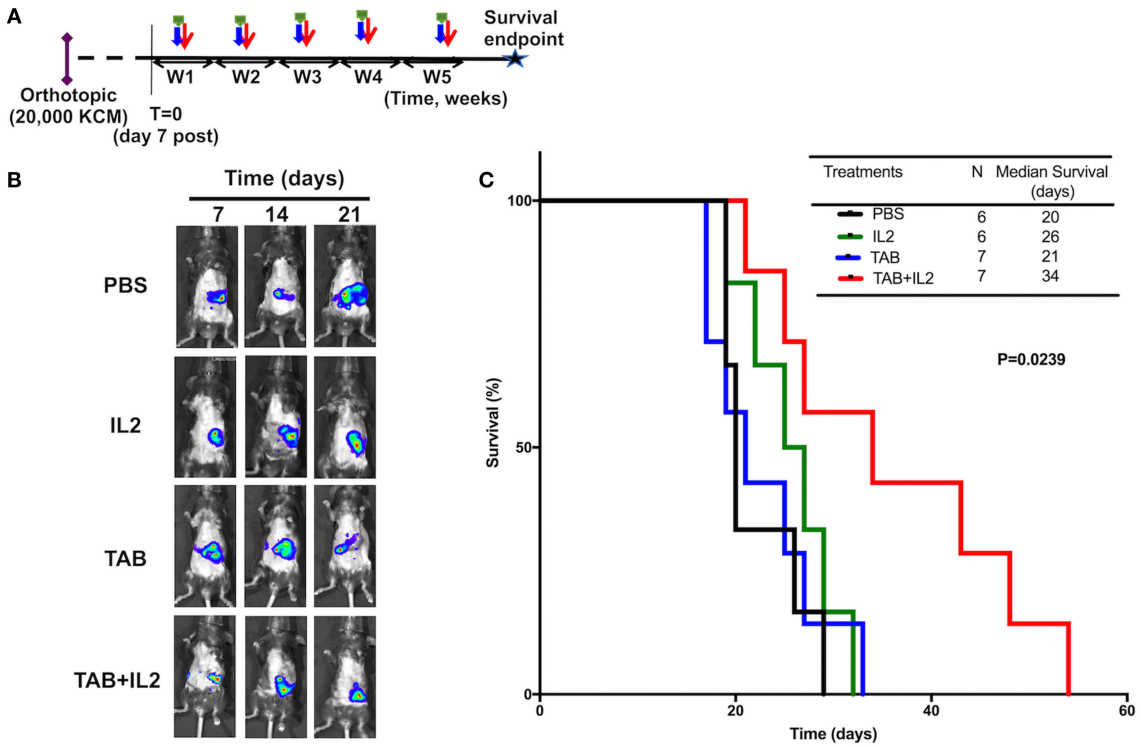

FIGURE 2 | TAB004 + Lip-MSA-IL-2 treatment significantly slowed tumor progression in the KCM orthotopic pancreatic cancer mouse model. MUC1.Tg mice were implanted orthotopically with KCM-Luc+ pancreatic cancer cells (20,000 cells) and treated [see schedule **(A)**; details in Materials and Methods section]. Mice implanted orthotopically with KCM-Luc+ cells were treated with either PBS, Lip-MSA-IL-2, TAB004, or the combination TAB004 + Lip-MSA-IL-2. The tumor growth was monitored *in vivo* through luciferase bioluminescence imaging. **(B)** Representative IVIS bioluminescent images of mice treated with PBS, Lip-MSA-IL-2, TAB004, or the combination TAB004 + Lip-MSA-IL-2 at days 7, 14, and 21 post-tumor implantation, respectively (One mouse per treatment group shown from baseline i.e., day 7–day 21). **(C)** Kaplan Meier survival curves of mice ($n \geq 6$ mice/group) implanted orthotopically with KCM-luc+ pancreatic tumor cells (20,000 cells) treated with PBS, TAB004, Lip-MSA-IL-2, or the combination TAB004 + Lip-MSA-IL-2 (Log-rank test, $p = 0.0239$). TAB, TAB004; IL2, Lip-MSA-IL-2; W, week.

derived from a standard curve that was run along with the samples tested.

Blood Cell Counts

White blood cells counts, RBC counts and features were determined by IDEXX Bioresearch (Columbia, MO) on whole blood, obtained on day 18 post-tumor implantation from animals orthotopically implanted with KCM-Luc+ cells and treated as detailed above.

Antibody-Dependent Cell Cytotoxicity (ADCC)/Antibody-Dependent Cell Phagocytosis (ADCP)

ADCC/ADCP was evaluated by flow cytometry as detailed previously (54). Briefly, target cells (KCM) were labeled with carboxyfluorescein succinimidyl ester (CFSE, BioLegend 488 nm) dye for ∼5 min, seeded in 24 well tissue culture plates and incubated overnight (37°C, 5% CO_2, humidity >80%) (55, 56). Splenocytes were added to target cells at a 1:5 tumor:splenocyte ratio in the presence of TAB004 (0.1–1.0 μg/ml). Maximum lysis was obtained following incubation with saponin 0.1%. After a 24 h incubation, cells were harvested and stained with the viability dye Vital fluorophore (Fixable Viability Dye ∼360–405 nm, BioLegend) (51). Cells were then run on a Fortessa flow cytometer (BD Biosciences), and gating on CFSE+ cells, percent of dead KCM cells were determined.

tMUC1 Concentrations

Concentrations of tMUC1 in the serum obtained on day 18 post-tumor implantation from animals orthotopically implanted with KCM-Luc+ cells and treated as detailed above, were determined by ELISA as previously described (27, 43, 57).

Statistical Analyses

Data are presented as mean ± SEM. Survival differences between treatments were represented using Kaplan-Meier and tested using Log-rank tests. Other parameters measured (e.g., % stained cells, tumor weight, % of lysis, number and type of pancreatic lesions, MPO, and MUC1 concentrations) were assessed for normality using the Shapiro-Wilk normality test. For parameters with normal distribution, differences between treatment groups were tested using ANOVA and *post-hoc* tests. Correlations between tumor weight and parameters measured was assessed by Pearson r correlations. All analyses were completed using Prism 7 (GraphPad software Inc.). *A priori* $p < 0.05$ was defined as significant.

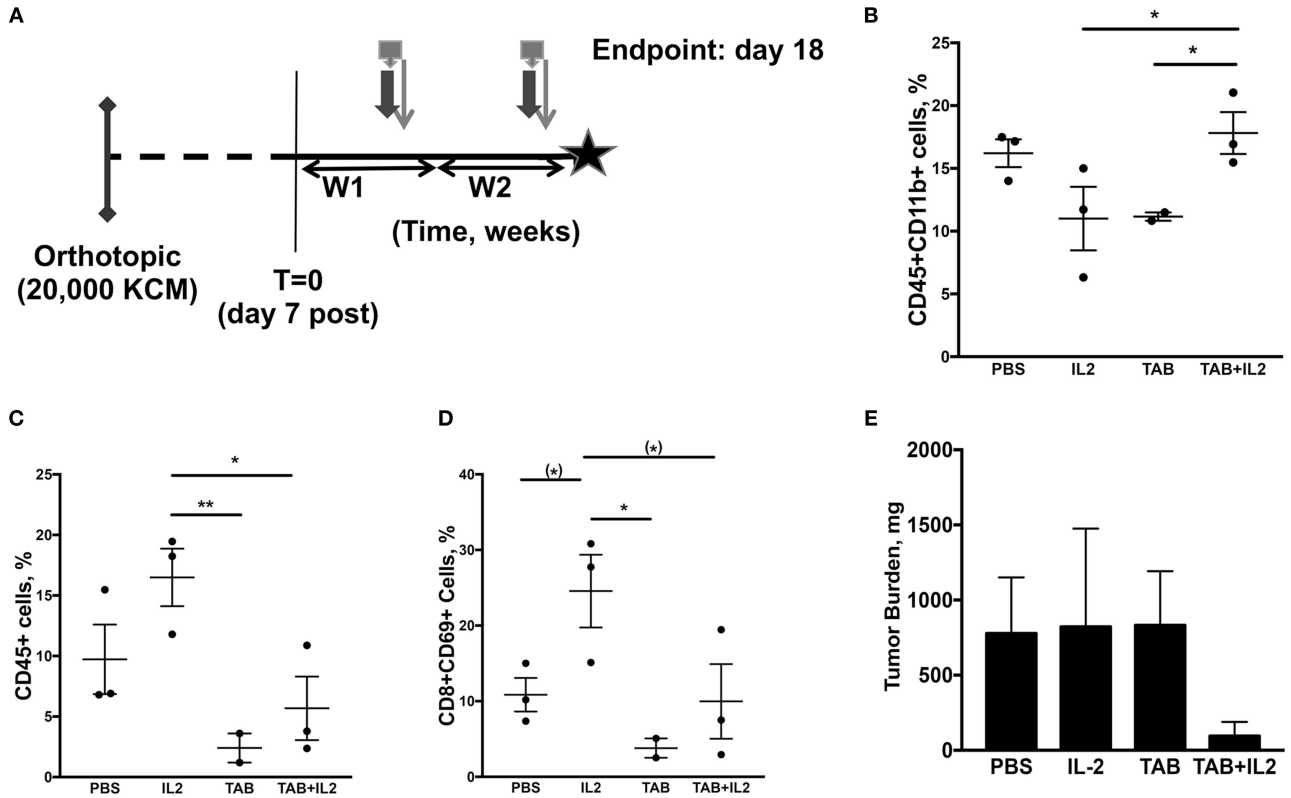

FIGURE 3 | Tumor-infiltrating immune cell populations, particularly macrophages, were altered in mice treated with the combination TAB004 + Lip-MSA-IL-2. MUC1.Tg mice were implanted orthotopically with KCM-Luc+ pancreatic cancer cells (20,000 cells) and treated with either PBS, Lip-MSA-IL-2, TAB004, or the combination TAB004 + Lip-MSA-IL-2 [see schedule **(A)**; details in Materials and Methods section] for 18 days. On day 18, tumor cell suspensions were obtained and the presence (%) of CD45+ immune cells **(C)** CD45+CD11b+ cells **(B)**, and of activated effector T cells (CD45+CD8+CD69+) **(D)** was determined. Additionally, tumor burden **(E)** was recorded [$n = 3$ per treatment group except for TILs evaluations **(B–D)** in TAB group, $n = 2$]. For **(B–E)**, data are presented as mean ± SEM. (*)$p < 0.08$, *$p < 0.05$, **$p < 0.01$. TAB, TAB004; IL2, Lip-MSA-IL-2; W, week.

RESULTS

Treatment With TAB004 + Lip-MSA-IL-2 Limited Pancreatic Cancer Progression in the KCM Triple Transgenic Mice That Develop Spontaneous PDA

The effects of TAB004 with or without Lip-MSA-IL-2 were assessed in the KCM mice, a model of human MUC1-expressing spontaneous PDA. KCM mice carry the human MUC1 transgene (driven by its own promoter) and the $KRAS^{G12D}$ transgene (driven by tamoxifen-inducible P48 promoter) (41, 44). When KCM mice are injected with tamoxifen for 2 weeks starting at 11 weeks of age, all (100%) mice develop early stage PanIN lesions by 20–24 weeks of age (∼12 weeks post tamoxifen) (41, 44).

KCM mice were treated with vehicle (PBS: $n = 3$), TAB004 (500 μgs/mouse. $n = 3$), Lip-MSA-IL-2 (25 μgs/mouse. $n = 4$), or TAB004 + Lip-MSA-IL-2 ($n = 6$) for 5 weeks starting 15 weeks post-tamoxifen treatment (or 26 weeks of age) (**Figure 1A**). At 40 weeks of age, pancreata were harvested and processed for histology. Tumor grade was determined following hematoxylin and eosin (H&E) staining. Representative microphotographs of H&E stained pancreas sections from each treatment group compared to normal pancreas highlight the presence of tumor lesions (**Figure 1B**). Results show that in 5 out of 6 mice treated with the combination TAB004 + Lip-MSA-IL-2, the PanIN lesions did not progress beyond the PanIN2 grade. One mouse in the combination TAB004 + Lip-MSA-IL-2 group progressed to adenocarcinoma. In sharp contrast, all 4 mice treated with Lip-MSA-IL-2 alone progressed from PanIN lesions to adenocarcinoma. Similarly, 2 out of 3 mice in the PBS group and in the TAB004 group progressed to adenocarcinoma while one mouse from each group progressed to high-grade PanIN3/CIS grade (**Figure 1C**). Of note, we did not observe any adverse effect of the treatment on the health of the KCM mice.

Treatment With TAB004 and Lip-MSA-IL-2 Also Significantly Improved Survival of MUC1.Tg Mice Bearing Orthotopic KCM Tumors

MUC1.Tg mice bearing orthotopic KCM.Luc+ pancreatic tumors were treated with either vehicle (PBS), TAB004, or Lip-MSA-IL-2 alone or the combination of TAB004 + Lip-MSA-IL-2 weekly for up to 5 weeks (**Figure 2A**, $n \geq 6$ mice per treatment

group). The *in vivo* growth of KCM cells expressing luciferase was monitored post-luciferin injection as shown in representative IVIS images of orthotopic tumor (one mouse per treatment over time from day 7 to day 21, **Figure 2B**). By day 21, while tumors grew in all mice, reduced bioluminescence indicative of smaller tumors was detected in treated mice groups, suggesting that those tumors grew more slowly compared to tumors in mice treated with PBS (**Figure 2B**). More importantly, TAB004 + Lip-MSA-IL-2 treatment was associated with a significantly improved survival ($p = 0.02$, Log rank test, **Figure 2C**) compared to mice that received any other treatments. Notably, TAB004 alone or Lip-MSA-IL-2 alone did not improve mouse survival.

No significant toxicity was associated with the treatments except for mild to severe skin dermatitis observed in a third of the mice treated with Lip-MSA-IL-2 alone and half of the mice treated with the TAB004 + Lip-MSA-IL-2 combination. Complete blood cell count (WBC and RBC) analyses were conducted on whole blood. No difference was observed in RBC measured parameters (**Supplemental Figure 1S**) and WBC populations (**Supplemental Figure 2S**) when comparing tumor bearing and treated mice with control non-tumor bearing MUC1.Tg mice.

Treatment With TAB004 + Lip-MSA-IL-2 Was Associated With Increases in CD45+CD11b+ Cells and Decreases in Both CD45+ Lymphocytes and CD8+CD69+ T Cells Within the Orthotopic KCM Tumors

In another set of experiments, tumors were collected 18 days post tumor challenge and 2 weeks post treatment (**Figure 3A**) to assess the treatment induced immune responses ($n = 3$ mice per treatment group). Changes in specific immune cell populations including macrophages, neutrophils, NK cells, lymphocytes, and lymphocyte subsets are associated with effective immunotherapy (58, 59). In particular, IL-2 treatment is associated with increases in neutrophils and activated NK (NK1.1+CD107+) cells (19). Moreover, tumor infiltration by specific subsets of macrophages is also associated with improved pre-clinical responses (60, 61). Therefore, we assessed immune cell subpopulations in spleen, blood and tumors from MUC1.Tg mice orthotopically implanted with KCM tumor cells, treated as detailed in **Figure 3A**. In the spleen, there was no significant difference in the populations of CD45+, CD8+, CD4+, CD4+CD69+, CD8+CD69+, NK1.1+, NK1.1+CD107+, CD11b+, or Ly6G+ as determined by flow cytometry (data not shown).

In the tumors, a significant increase in the percent of tumor-associated CD45+CD11b+ cells was observed in the tumors of mice treated with the combination of TAB004 + Lip-MSA-IL-2 compared to tumors from mice in all other treatments ($p < 0.01$; **Figure 3B**). Interestingly, in mice treated with the combination TAB004 + Lip-MSA-IL-2 or TAB004 alone, we observed a significant decrease in the percent of tumor-associated CD45+ lymphocytes and of CD8+CD69+ T lymphocytes compared to tumors from mice treated with PBS or Lip-MSA-IL-2 alone ($P < 0.01$; **Figures 3C,D**). A subset of CD8+CD69+ cells has been identified as activated CD8+ regulatory cells previously (62). Although tumor infiltrating CD45+ cells were lower in the mice treated with the TAB004 + Lip-MSA-IL-2 combination, the CD45+CD11b+ cell population remained high. There was no significant change in any other subpopulation including Tregs, CD4+CD69+, CD8+, NK1.1+, or activated NK cell populations (data not shown). The tumor weight was also assessed at day 18 and was highly variable. Nevertheless, the orthotopic tumor burden in the mice treated with the combination TAB004 + Lip-MSA-IL-2 tended to be smaller compared to the tumor burden observed the other treatment groups (ns, **Figure 3E**).

Of note, the number of monocytes per μl of blood was significantly decreased in mice treated with the combination of TAB004 + Lip-MSA-IL-2 compared to mice treated with Lip-MSA-IL-2 alone (**Supplemental Figure 3AS**). Additionally, the number of CD45+CD11b+ cells per gram of tumor was higher in tumors isolated from animals treated with Lip-MSA-IL-2 alone compared tumors collected from mice treated with PBS or TAB004 (**Supplemental Figure 3BS**).

Together, the survival and mechanistic experiments conducted in the orthotopic KCM tumor model highlight that the tumor progression is slower and the tumor burden is lower in the mice treated with the combination TAB004 + Lip-MSA-IL-2 compared to mice treated with PBS, TAB004 or Lip-MSA-IL-2 alone. Furthermore, a significant survival benefit was observed in the mice treated with the combination TAB004 + Lip-MSA-IL-2 (see **Figure 2C** above).

The Combination TAB004 + Lip-MSA-IL-2 Treatment Markedly Increased the Myeloperoxidase Present in the Serum of MUC1.Tg Mice Bearing Orthotopic KCM Tumors

Multiple immune cells, especially neutrophils and macrophages, have a MPO activity (52, 53). MPO is produced during degranulation of neutrophils and macrophages and produces hypochlorous acid that is generally associated with cellular cytotoxicity. MPO concentrations were determined using ELISA in sera. Significantly higher MPO concentrations were detected in the sera of KCM tumor bearing mice treated with the combination of TAB004 + Lip-MSA-IL-2 than in the sera of the mice that were administered the other treatments ($P < 0.001$; **Figure 4**).

The Combination TAB004 + Lip-MSA-IL-2 Treatment Led to Significant Increases in Circulating Levels of CXCL1 and IL-5 as Well as Decreased IL-6 in the Sera of KCM Tumor Bearing MUC1.Tg Mice

Along with changes in immune cell infiltration, successful immunotherapies are associated with changes in multiple cytokines (63, 64). We assessed the concentrations of 20 Th1, Th2, Th17, and/or macrophage-related cytokines in sera collected 18 days post tumor challenge and 2 weeks post treatment (**Figure 3A**). Mice treated with the combination

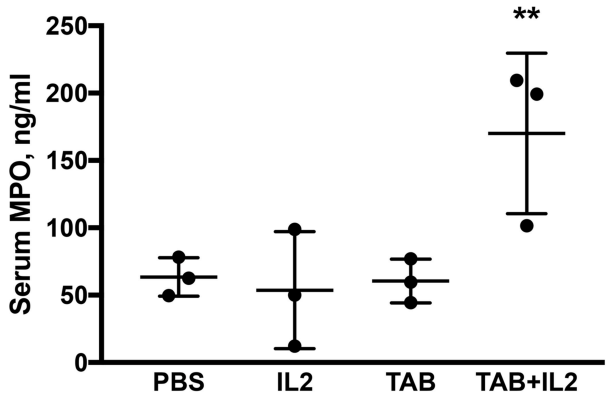

FIGURE 4 | Blood myeloperoxidase (MPO) concentrations were significantly higher in KCM-tumor bearing mice following TAB004 + Lip-MSA-IL-2 treatment. Sera were assessed by ELISA for the presence of MPO and the MPO concentration expressed in pg/ml ($n = 3$ per treatment group). Data are presented as mean ± SEM. $^{**}p < 0.01$. TAB, TAB004; IL2, Lip-MSA-IL-2.

TAB004 + Lip-MSA-IL-2 showed a significant increase in levels of IL5 (**Figure 5B**) and CXCL1 (**Figure 5D**) compared to all other treatment groups. Following Lip MSA IL2 treatment, serum concentrations of RANTES were highly variable (**Figure 5A**, $p < 0.05$; Shapiro–Wilk normality test). Also noteworthy is the decrease in the serum levels of IL-6 in mice treated with Lip-MSA-IL-2 and the combination TAB004 + Lip-MSA-IL-2 compared to PBS and TAB004 treated mice ($p < 0.05$; **Figure 5C**).

Treatment With TAB004 Antibody Markedly Decreased tMUC1 Serum Concentrations in the MUC1.Tg Mice Bearing Orthotopic KCM Tumors

Because tMUC1 is associated with immune suppression (31) and increased aggressiveness of pancreatic tumors (44), we determined the concentrations of serum tMUC1 in the treated mice using a specific ELISA. As was expected, serum tMUC1 concentrations were significantly lower in TAB004 and TAB004 + Lip-MSA-IL-2 treated mice when compared to serum from mice treated with Lip-MSA-IL-2 alone or with PBS ($p < 0.05$; **Figure 6A**). Further, similar observations were made when the serum concentrations were normalized to tumor mass ($p < 0.05$; **Figure 6B**).

Treatment With TAB004 Alone and the Combination of TAB004 + Lip-MSA-IL-2 Led to an Increased Antibody-Dependent Cell Cytotoxicity/Phagocytosis

To further assess the cytotoxic mechanisms induced by the combination of TAB004 + Lip-MSA-IL-2, splenocytes isolated from treated KCM-tumor bearing mice (**Figure 3**) were assayed *in vitro* with TAB004 antibody for antibody-dependent cell cytotoxicity (ADCC)/antibody-dependent cell phagocytosis (ADCP) against KCM tumor cells as detailed previously (54). Splenocytes isolated from KCM tumor bearing mice treated with either TAB004 alone or the combination TAB004 + Lip-MSA-IL-2 had a significantly higher ADCC/ADCP response against KCM tumor cells compared to splenocytes isolated from mice treated with PBS or Lip-MSA-IL-2 alone ($p < 0.05$, **Figure 7**).

Specific Immune Parameters Measured Correlated With Tumor Size and the Treatment With TAB004 Led to Lower Neutrophil/Lymphocyte Ratios

To shed light on the robustness of the association between immune variables and tumor progression in the treated mice, the immune parameters measured were correlated with tumor size regardless of treatment (**Table 1**). Interestingly, the immune parameters measured in the mechanistic study had significant correlations with tumor size. Specifically, a smaller tumor size was correlated with increases in CD45+CD11b+ cells present in the tumors ($r = -0.956$; $p = 0.04$), in serum MPO ($r = -0.969$; $p = 0.03$), in serum IL-5 ($r = -0.948$; $p = 0.051$), and in serum CXCL1 ($r = -0.938$; $p = 0.06$) concentrations. Additionally, a smaller tumor size was correlated with a decrease in blood neutrophil numbers ($r = 0.969$; $p = 0.03$).

As the blood neutrophil/lymphocyte ratio has been demonstrated to have prognostic value in monitoring tumor progression (with a lower ratio associated with improved outcomes (65, 66)), we compared the blood neutrophil/lymphocyte ratio between mouse treatment groups (**Figure 8A**). Treatments with either TAB004 or the combination of TAB004 + Lip-MSA-IL-2 led to significant decreases in the blood neutrophil/lymphocyte ratio compared to the blood of mice treated with PBS or Lip-MSA-IL-2 alone ($p < 0.05$, **Figure 8A**). Furthermore, the ratio of neutrophils (i.e., CD45+Ly6G+ cells per gram of tumor) to T lymphocytes (defined as the sum of CD45+CD4+ and CD45+CD8+ per gram of tumor) tended to be lower in the tumors of mice treated with the combination of TAB004 + Lip-MSA-IL-2 compared to tumors from mice treated with the vehicle (PBS), and was lower than the neutrophil/T lymphocyte ratio in tumors from mice treated with Lip-MSA-IL-2 ($p < 0.05$; **Figure 8B**).

DISCUSSION

The most effective pre-clinical studies are conducted in immune competent spontaneous tumor models (67). Here, using both orthotopic (KCM implanted tumor cells in the pancreas) and spontaneous PDA models, we demonstrate for the first time a significant slowdown of tumor growth and improved survival following combination treatment with TAB004 and Lip-MSA-IL-2. The beneficial therapeutic effect was associated with specific immune changes, including increases in CD45+CD11b+ cells and decreases in immune regulatory lymphocytes within the tumor mass. Treatments also led to increases in the serum concentrations of MPO and of specific cytokines as well as ADCC/ADCP activities of splenocytes. The immunocompetent orthotopic pancreatic model was informative on the effects of treatments on immune responses. Indeed, notwithstanding the number of animals per group [$n = 3$ except TAB004 group

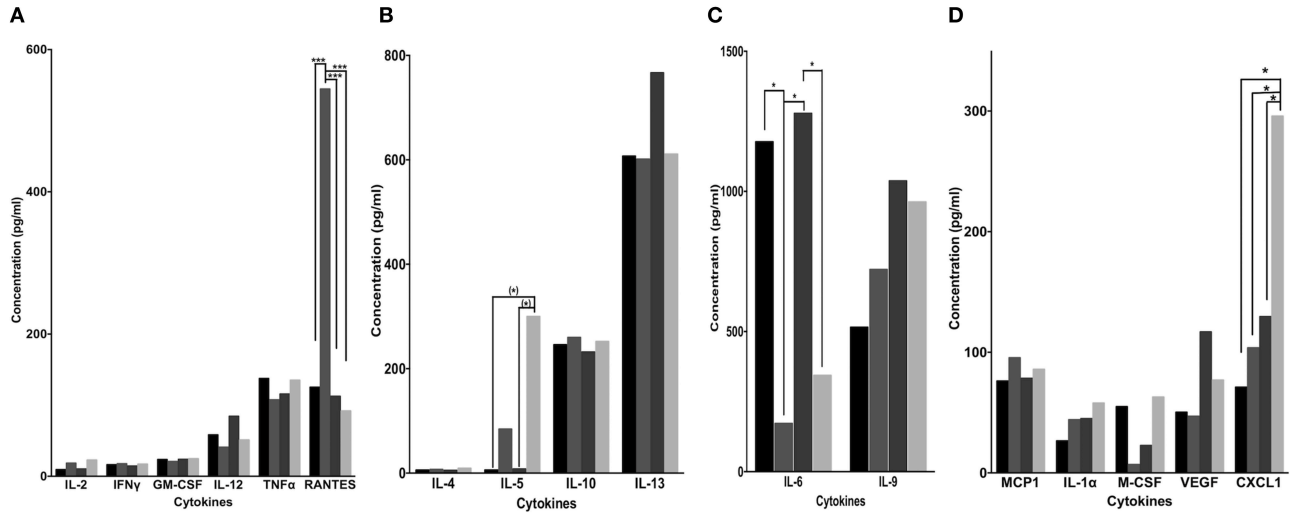

FIGURE 5 | Blood cytokine concentrations following PBS, TAB004, Lip-MSA-IL-2, and TAB004 + Lip-MSA-IL-2 treatments. The presence of multiple cytokines in sera (**A–D**, pg/ml) collected from mice implanted with 20,000 KCM-luc cells and treated with PBS (black), TAB004 (dark gray), Lip-MSA-IL-2 (darker gray), and TAB004 + Lip-MSA-IL-2 (light gray) were determined using multiplex quantitative cytokine arrays ($n = 3$ per treatment group). (*)$p < 0.08$; *$p < 0.05$; ***$p < 0.001$. [Values and variation (Average ± SEM) are provided in the **Supplemental Table 1S**].

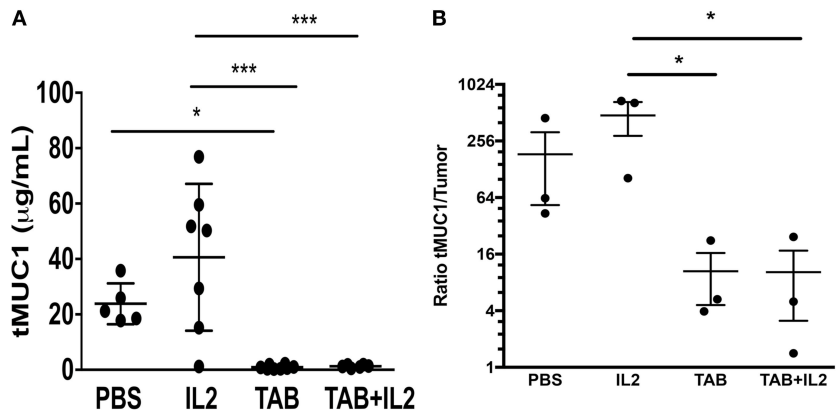

FIGURE 6 | tMUC1 serum concentrations were significantly lower in TAB004 and TAB + Lip-MSA-IL-2 treated mice. tMUC1 was measured by ELISA in the serum of KCM tumor-bearing mice (**A**, $n \geq 5$) treated with PBS, Lip-MSA-IL-2, TAB004, or the TAB004 + Lip-MSA-IL-2 combination and normalized to tumor size (**B**, $n = 3$). Data are presented as mean ± SEM. *$p < 0.05$; $p < 0.01$; ***$p < 0.001$. TAB, TAB004; IL2, Lip-MSA-IL-2.

($n = 2$)] that requires a cautious interpretation of the data, significant changes in immune parameters were observed, and most were correlated to tumor size. Of note, there was minimal toxicity associated with this treatment.

Both mouse models used here mimic the development of the human tumor, including similarities in MUC1 expression, the native immune responses against MUC1 as tumors progress, and the immune suppressive microenvironment within the developing tumor (28). In these clinically-relevant models, tumors arise in an appropriate tissue background and in a host conditioned by the physiological events of neoplastic progression and tumorigenesis and in the context of a viable immune system (67). Regardless of treatment, multiple factors likely modulate the immune responses observed here including the desmoplasia and immune evasion. For example, desmoplasia is routinely observed in pancreatic tumors and hinders chemotherapy (68, 69). Indeed, in our model, collagen accumulation was observed in all treatments (**Supplemental Figure 4S**) and may have affected the immunotherapy tested. Numerous mechanisms of immune evasion have been identified and reviewed elsewhere (70–72). As our tumor model is not highly immunogenic, evasion mechanisms were not investigated here and will be assessed in future studies. Moreover, both mouse models express human MUC1 as a self-molecule and thus are tolerant to MUC1. Additionally, KCM mice develop spontaneous tumors of the pancreas, with tumor cells expressing large amounts of

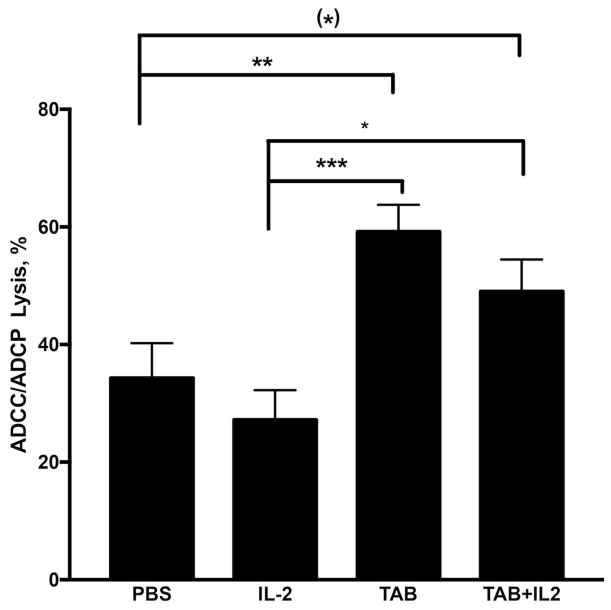

FIGURE 7 | ADCC/ADCP lysis was significantly increased in mice treated with TAB004 or TAB + Lip-MSA-IL-2. Splenocytes isolated from KCM tumor-bearing mice were assayed *in vitro* for antibody-dependent cell cytotoxicity (ADCC)/antibody-dependent cell phagocytosis (ADCP) as detailed previously (54). KCM tumor cells were seeded and stained with the dye (CSFE) and incubated for 24 h with isolated splenocytes at a 1:5 tumor:splenocyte ratio in the presence of TAB004 with gating on non-CD45+ cells. The KCM cells CSFE+ dead cells (%) stained with vital fluorophore were evaluated by flow-cytometry ($n = 3$ per treatment group). Data are presented as mean ± SEM. (*)$p < 0.08$;*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. TAB, TAB004; IL2, Lip-MSA-IL-2.

TABLE 1 | Pearson *r* correlation coefficients between and tumor size and immune parameters.

Tumor size (mg) vs.	Pearson *r* coefficient	Significance
Immune cells within tumors		
CD45+, %	0.355	n.s.
CD8+CD69+, %	0.360	n.s.
CD11b+, %	−0.956	0.04
Immune cells within blood		
WBC (Number/ul)	0.632	n.s.
Lymphocytes (Number/ul)	0.244	n.s.
Monocytes (Number/ul)	0.734	n.s.
Neutrophils (Number/ul)	0.970	0.03
Cytokines within serum		
IL-2 (pg/ml)	−0.736	n.s.
IL-4 (pg/ml)	−0.911	n.s
IL-5 (pg/ml)	−0.948	0.05
IL-9 (pg/ml)	−0.335	n.s.
CXCL1 (pg/ml)	−0.939	0.06
RANTES (pg/ml)	0.417	n.s.
Serum Muc1 (pg/ml)	0.562	n.s.
Serum MPO (pg/ml)	−0.969	0.03

hypo-glycosylated MUC1 as observed in human PDAs (29). Therefore, the tumor growth reduction along with a significantly higher survival observed here support the potential of these therapeutic approaches in humans.

Clinically, combination treatments such as rosiglitazone and gemcitabine, FOLFIRINOX (5-FU, leucovorin, irinotecan, and oxaliplatin), monoclonal antibody and 5-fluorouracil, adriamycin, and mitomycin chemotherapy, or gemcitabine and nab-paclitaxel have been shown to significantly reduce tumor progression and metastases and significantly extend overall patient survival (1, 6–8). While those treatments led to some improvements and extended overall survival in small subsets of patients (8, 9), improved approaches to treat patients with pancreatic cancer are required (3, 10).

Clinical trials with combinations of antibodies to specific tumor antigens along with IL-2 treatment have not shown significant efficacy (22, 73). Modified IL-2 formulations alone led to longer IL-2 half-life, but without significant clinical benefit when used as a monotherapy (74). We have previously demonstrated that treatment with an anti-tumor antigen antibody and a fusion protein bestowing prolonged IL-2 signaling (i.e., Lip-MSA-IL-2 used here) led to significantly improved survival in a melanoma mouse model (22). Furthermore, in a murine model, the sustained persistence of IL-2 signaling enhanced the antitumor effects of peptide vaccines (75), highlighting the key role of sustained IL-2 signaling activation in successful immunotherapy. Although PDA is classically resistant to immunotherapy and lacks baseline T cell infiltration (76), higher clinical benefits were observed when immunotherapy/chemotherapy/chemoprevention combinations were used (41, 77). Indeed, our data support the benefits of sustained IL-2 signaling when combined with the specific tumor targeting antibody TAB004, as Lip-MSA-IL-2, or TAB004 treatments alone had no effects on survival, whereas the combination was associated with clearly improved survival.

Immunological responses observed in the PDA models following treatment in part mimic those observed and summarized earlier (22) in a murine melanoma model, including critical interactions between various effectors during administration of cancer immunotherapy. In particular, the administration of anti-MUC1 antibodies leads to effective tumor cell killing by antibody-dependent cell-mediated cytotoxicity/phagocytosis (ADCC/ADCP) in part through NK cell and/or macrophage-mediated killing activities (24, 32, 42). Remarkably, populations of CD45+CD11b+ cells (including macrophages), but not NK cells, were increased in tumors treated with Lip-MSA-IL-2 and TAB004, suggesting a key role for CD45+CD11b+ cells (including macrophages) in the limitation of tumor progression *in vivo*. Our observation is supported by the required role of macrophages in the anti-MUC1 tumor response *in vivo* (42). Notably, overall survival of PDA patients who had alterations in the genes for CD45 (*PTPRC*) and CD11b (*ITGAM*) was significantly lower than the survival of all patients with PDA (CBioportal.org query, **Supplemental Figure 5S**) (78, 79). Future depletion studies will be required to confirm that the CD45+CD11b+ cells involved here are macrophages.

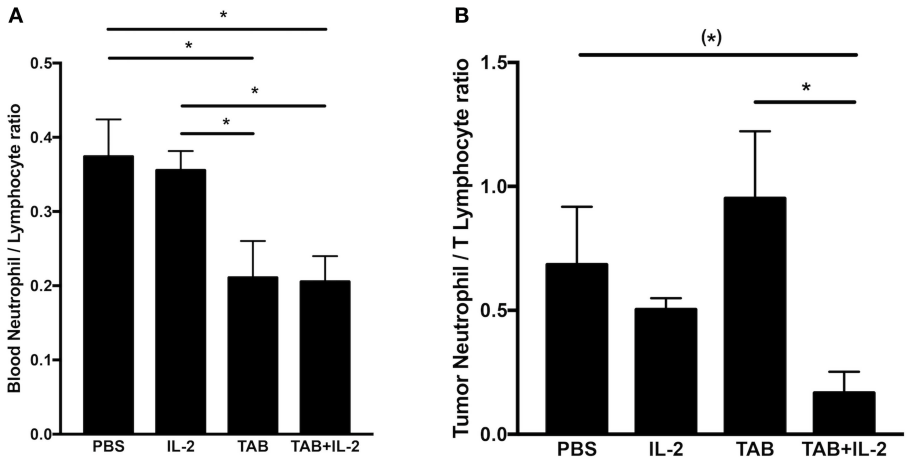

FIGURE 8 | The combination TAB004 + Lip-MSA-IL-2 treatment led to lower neutrophil/lymphocyte ratio in blood **(A)** and Tumors **(B)**. Blood and tumor cells were characterized by blood count and flow-cytometry, respectively (see section Materials and Methods for details). For blood, ratios were derived using number (#) of neutrophils and lymphocytes per μl of blood. For each tumor mass, the cell suspensions obtained were stained for CD45+and ly6G+ (neutrophils) and CD45+ and either CD4+ or CD8+(T lymphocytes) evaluated by flow-cytometry and normalized to gram of tumor ($n = 3$ per treatment group). Data are presented as mean ± SEM. (*)$p < 0.08$;*$p < 0.05$; TAB, TAB004; IL2, Lip-MSA-IL-2.

This immune response was complemented with a significant decrease in immune regulatory cells (CD8+CD69+ cells). Interestingly, enhanced anti-tumor immunity against MHC class I tumors (RMA-S ad RM-1) was reported in CD69 knockout mice and mice treated with an anti-CD69 antibody (80). Indeed, CD8+CD69+ T cells are immunoregulatory cells that are known to promote tumor progression by inducing the production of indoleamine 2,3, dioxygenase (IDO) (62). Our previous studies indicated that IDO, one of the major players in immune tolerance but also in tumor progression, metastasis, and angiogenesis, is overexpressed in MUC1-expressing PDA (44). Thus, tMUC1 expression may contribute toward a highly tolerogenic tumor microenvironment by influencing the IDO/tryptophan pathways.

Our data suggest that the increased percent of tumor infiltrating CD45+CD11b+ cells and serum MPO concentrations are associated with the increased survival observed in the mice treated with the combination. MPO is produced especially during degranulation of neutrophils and macrophages, leading to the generation of hypochlorous acid that is commonly indicative of cellular cytotoxicity. In contrast with previous increases in neutrophils associated with Lip-MSA-IL-2 treatment in the melanoma model (22), no significant changes in neutrophil populations were observed in this study. Nevertheless, we do report that tumor progression is correlated with an increase in the number of blood neutrophils. Interestingly, the blood neutrophil/lymphocyte ratio, an independent prognostic marker of tumor progression (i.e., the lower the blood neutrophil/lymphocyte ratio, the better the outcome (65, 66), was determined to be lower in mice treated with TAB004 alone or with the combination of TAB004 + Lip-MSA-IL-2. Furthermore, the ratio of neutrophils/T lymphocytes per gram of tumor (approximated using the sum of CD45+CD4+ cells and CD45+CD8+ cells) was also lower in the tumors from mice treated with the combination of TAB004 + Lip-MSA-IL-2.

We also detected increases in serum IL-5 and CXCL1 concentrations and decreases in serum IL-6 concentrations in mice treated with the combination vs. control mice. In particular, the significant increase in circulating CXCL1, along with the correlation of the number of blood neutrophils with the tumor size, may be related to the recruitment of tumor entrained neutrophils (TENs) from the bone marrow into possibly other organs. TENs are associated with inhibiting seeding in the metastatic niche (81) by generating H_2O_2 and tumor secreted MCP1 (also noted in our treatment group) which are both critical mediators of anti-metastatic entrainment of stimulated neutrophils. IL-6 is a critical pleiotropic cytokine associated with innate immunity and cancer; it is known to inhibit expression of CXCL1, and is a prominent target for clinical intervention (82). Together, these data hint that the combination treatment may be associated with wound healing and macrophage/monocyte recruitment.

The presence of plasma IgG antibodies specific to tMUC1 has been associated with survival benefits in patients with breast, lung, pancreatic, ovarian and gastric carcinomas (24). Interestingly, circulating shed tMUC1 accurately detected tumor stage progression in PDA patients (27). Possible mechanisms by which anti-tMUC1 antibodies prevent tumor progression include enhanced NK cell anti-tumor activity (42), restoration of cell-cell interactions altered by tumor-associated MUC1 (24), and prevention of tMUC1-associated reduction of T cell proliferation and anergy of cytotoxic T cells (23, 31). Interestingly, the inhibition of human T cell responses by cancer-associated MUC1 was abrogated by IL-2 (31). Moreover, when conjugated to tMUC1 antibody, IL-2 stimulated the proliferation of activated human lymphocytes *in vitro* and triggered resting NK cells to lyse tumor cells (23). Furthermore, the IL-2-antibody complex

promoted antitumor immunity in mice by activating tumor-reactive CD8+ T cells (20). Previous imaging analyses clearly indicated strong co-localization of TAB004 and tumor cells (43, 57), and our data highlight clear tumor responses to combined TAB004 + Lip-MSA-IL-2 immunotherapy. The neutralization of tMUC1 in circulation is likely due to TAB004 complexing with circulating tMUC1, which in turn dampens the tMUC1-induced immune suppression. This enables immune effector cells (in this case, the macrophages) to elicit an anti-tumor immune response and enhance survival. Although TAB004 alone did not improve survival, since Lip-MSA-IL-2 has been shown to activate macrophage cytotoxicity against cancer cells (83), it is possible that Lip-MSA-IL-2 likely enhances the recruitment and activation of macrophages once TAB004 is bound to tMUC1-expressing tumor cells.

Taken together, our data, for the first time, indicate that treatment with Lip-MSA-IL-2 + TAB004 significantly improved survival in an orthotopic model, and resulted in retardation of tumor progression in a spontaneous model of PDA. Remarkably, these results are the first to demonstrate improved PDA outcomes in immunocompetent mouse models. In contrast, the use of Lip-MSA-IL-2 alone or TAB004 alone were not associated with any significant improvement in tumor burden or survival in the *in vivo* PDA models tested. Beside the benefits of TAB004 as an early monitoring approach to detect cancers earlier and monitor their progression, these data indicate that TAB004 may also have clear therapeutic benefits when combined with IL-2 to stimulate a targeted immune response. Success in developing FDA-approved TAB004-based treatments of patients with non-resectable PDA would have enormous long-term clinical impact. Furthermore, TAB004 antibody therapy may usher a new area of immunotherapy for other malignancies.

ETHICS STATEMENT

All animal experiments were conducted following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Charlotte. All experiments were conducted following the Guide for the Care and Use of Laboratory Animals guidelines under the supervision of Dr. Chandra Williams DVM, in an AAALAC accredited facility.

DISCLOSURE

PM, RP, and MW are Oncotab Inc. employees. Oncotab Inc. had no involvement in data analyses, data presentation and manuscript writing.

AUTHOR CONTRIBUTIONS

DD conceived, performed, and analyzed the experiments and wrote and reviewed the manuscript. LM, MW, LDR, LD, and TP performed and analyzed the experiments and reviewed the manuscript. RP, NM, and KW participated in the analysis the data, and reviewed the manuscript. PM participated in conception of the experiments and the analysis the data and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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Biomaterial-Based Activation and Expansion of Tumor-Specific T Cells

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Traditional tumor vaccination approaches mostly focus on activating dendritic cells (DCs) by providing them with a source of tumor antigens and/or adjuvants, which in turn activate tumor-reactive T cells. Novel biomaterial-based cancer immunotherapeutic strategies focus on directly activating and stimulating T cells through molecular cues presented on synthetic constructs with the aim of improving T cell survival, more precisely steer T cell activation and direct T cell differentiation. Synthetic artificial antigen presenting cells (aAPCs) decorated with T cell-activating ligands are being developed to induce robust tumor-specific T cell responses, essentially bypassing DCs. In this perspective, we approach these promising new technologies from an immunological angle, first by identifying the $CD4^+$ and $CD8^+$ T cell subtypes that are imperative for robust anti-cancer immunity and subsequently discussing the molecular cues needed to induce these cells types. We will elaborate on how biomaterials can be applied to stimulate T cells *in vitro* and *in vivo* to improve their survival, activation and function. Scaffold-based methods can also be used as delivery vehicles for adoptive transfer of T cells, including tumor-infiltrating lymphocytes (TILs) and chimeric antigen receptor expressing (CAR) T cells, while simultaneously stimulating these cells. Finally, we provide suggestions on how these insights could advance the field of biomaterial-based activation and expansion of tumor-specific T cells in the future.

Keywords: cancer immunotherapy, biomaterials, T cells, artificial antigen-presenting cells, scaffold, anti-tumor immune response, synthetic immune niche, molecular cues

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INTRODUCTION

Immunotherapy provides a revolutionary treatment modality for cancer. A variety of strategies have been developed to improve the clinical outcome of patients by generating long-term anti-tumor immune responses. The development of therapeutic monoclonal antibodies that block co-inhibitory receptors on T cells, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1), has shown exceptional clinical benefit in cancer patients and is seen as a crucial breakthrough in the cancer immunotherapy field (1).

Apart from relieving suppression in pre-existing T cells, other immunotherapeutic strategies focus on increasing the number of tumor-reactive T lymphocytes that recognize either tumor-specific antigens, tumor-associated antigens, cancer-testis antigens or neo-antigens. Dendritic cell (DC) vaccination targets antigen-presenting DCs, which are capable of priming T cells by capturing, processing and presenting antigens to naïve T cells together with

co-stimulatory cues (2). To generate DC vaccines, patient-derived DCs are cultured *ex vivo*, maturated and loaded with antigens, after which they are infused back into the patient where they activate tumor-reactive T cells (3). Increased overall survival, functional tumor-specific immune responses and low toxicities have been observed with this strategy (4, 5). Other cell-based therapies attempt to increase the number of circulating tumor-specific T cells by reinfusing autologous *ex vivo*-expanded T cells derived from tumors (tumor-infiltrating lymphocytes, TIL) or genetically engineering them to confer tumor reactivity using high affinity T-cell receptors (TCR) or chimeric antigen receptors (CAR) (6). Promising preclinical data have been obtained with these adoptive T-cell therapies (1, 7, 8). The prominent immunotherapeutic strategies described above all focus on generating robust tumor-directed T cell responses, which is crucial to inducing effective and long-lasting anti-tumor immunity, as there is a strong correlation between tumor-infiltrating $CD8^+$ T cells and patient survival in virtually all cancer types (9). In addition, antigen-specific $CD4^+$ T helper cells are believed to be critically involved in the induction of optimal anti-tumor responses (10, 11). It is therefore evident that T cells play a central role in cancer immunotherapy.

Although current cancer immune therapies have shown promising preclinical and clinical results, challenges remain that may limit therapeutic benefit. To improve on this and to design new therapeutic strategies, interest in the field of biomaterial engineering has grown. Biomaterials have proven valuable in reducing systemic toxicities, enhancing accumulation in tumors, improving pharmacokinetics and ensuring sustained release by controlled (targeted) drug delivery (12, 13). Biomaterial-based immunotherapeutic strategies led to the development of nanoparticles for the targeted delivery of cargo to immune cells *in vivo*, such as cytokines, DC-activating agents or small inhibitors (13–15). Careful design can be applied to tune the delivery of DC-targeted vaccines using materials responsive to temperature (16) or pH (17, 18). Other biomaterial-based approaches focus on improving *ex vivo* immune cell expansion or on supporting immune cells after adoptive transfer (13, 14). Furthermore, there has been a rise in the development of synthetic, acellular artificial antigen presenting cells (aAPCs) that can target and activate T cells directly (19, 20), thereby bypassing the need for DC activation. By presenting molecular cues on synthetic constructs based on biomaterials, specific signals are transmitted to T cells in a well-defined context and controlled manner to support T cell viability, activation and differentiation.

In this perspective, we will detail what T cell subtypes are imperative for robust anti-cancer immunity and which molecular cues are needed to induce these T cells. Next, we will elaborate on how these molecular cues can be presented by biomaterials for direct activation and expansion of T cells. The use of biomaterials to aid the adoptive transfer of T cells will also be discussed. Finally, we will illustrate in which direction the field of biomaterial engineering for cancer immunotherapy should go for the next generation of biomaterial-based cancer immunotherapies.

T CELL SUBSETS IN CANCER IMMUNOTHERAPY

To generate durable anti-tumor immune responses that have a beneficial impact on the clinical outcome of cancer patients, potent $CD8^+$ and $CD4^+$ T cell responses are crucial (9–11). Here, we will discuss the roles of different T cell subtypes in cancer-specific immune responses and we will highlight the cellular and molecular characteristics of these T cells (**Figure 1**).

Upon interaction with their cognate antigen in the context of major histocompatibility complex class I (MHC I) and co-stimulatory cues, $CD8^+$ T cells will undergo extensive proliferative expansion to create a large population of short-lived effector cytotoxic T lymphocytes (CTLs) that have tumor-killing capacities. The CTL population comprises functionally distinct subsets (21). For instance, expression of CX3CR1 on CTLs is associated with their ability to generate memory subsets and serves as a predictor for CX3CR1 expression on the generated memory cells, which is associated with robust cytotoxic effector functions (22, 23). CXCR5-expressing CTLs are involved in chronic viral infections and show reduced susceptibility to exhaustion (24). Additional heterogeneity may exist regarding cytokine production and the (co-)expression of perforin and various granzymes (25). In addition to these short-lived CTLs, the formation of $CD8^+$ memory T cells is required to support long-term anti-tumor immunity. Following a progressive differentiation model, primed naive $CD8^+$ T cells (Tn) will progress into different memory T cell populations [T stem cell memory (Tscm), T central memory (Tcm), T effector memory (Tem)] (21, 22, 25–27). The Tscm subset displays increased anti-tumor activity, enhanced proliferation, increased survival capacities and multipotency (27, 28). The Tcm generally have higher proliferative abilities while Tem are more cytotoxic (22). In contrast to circulating memory T cells, there is also a population of non-circulating memory T cells, tissue resident memory T cells (Trm). These Trm cells were shown to be superior in providing rapid long-term protection against recurrent infections (29). Inducing a broad repertoire of potent CTLs together with $CD8^+$ memory T cells will be highly beneficial for robust anti-tumor immunity (**Figure 1A**).

$CD4^+$ T cell help is imperative for potent $CD8^+$ T cell activation by supplying cytokines and co-stimulation, by enhancing persistence and migration, and by reactivating memory $CD8^+$ T cells (30–32). Recently, it has been reported that $CD4^+$ T cells are also dependent on $CD8^+$ T cells, underlining the mutual dependence of $CD4^+$ and $CD8^+$ T cell responses (10). Furthermore, a RNA vaccination study clearly showed the importance of $CD4^+$ T cell neo-epitopes in controlling murine tumors (11). The $CD4^+$ T cell population can be subdivided into specific subsets, each having their own signature cytokine repertoire (33, 34). The T helper 1 (Th1) subset is strongly associated with better prognosis, improved survival, low incidence of tumor recurrence and prolonged disease-free survival in cancer immunology (35). This is in part due to their supportive role in cellular immunity, the cytokines they produce [including interferon-γ (IFN-γ)] (35), and their role in inducing immunological memory (36). In addition, notable

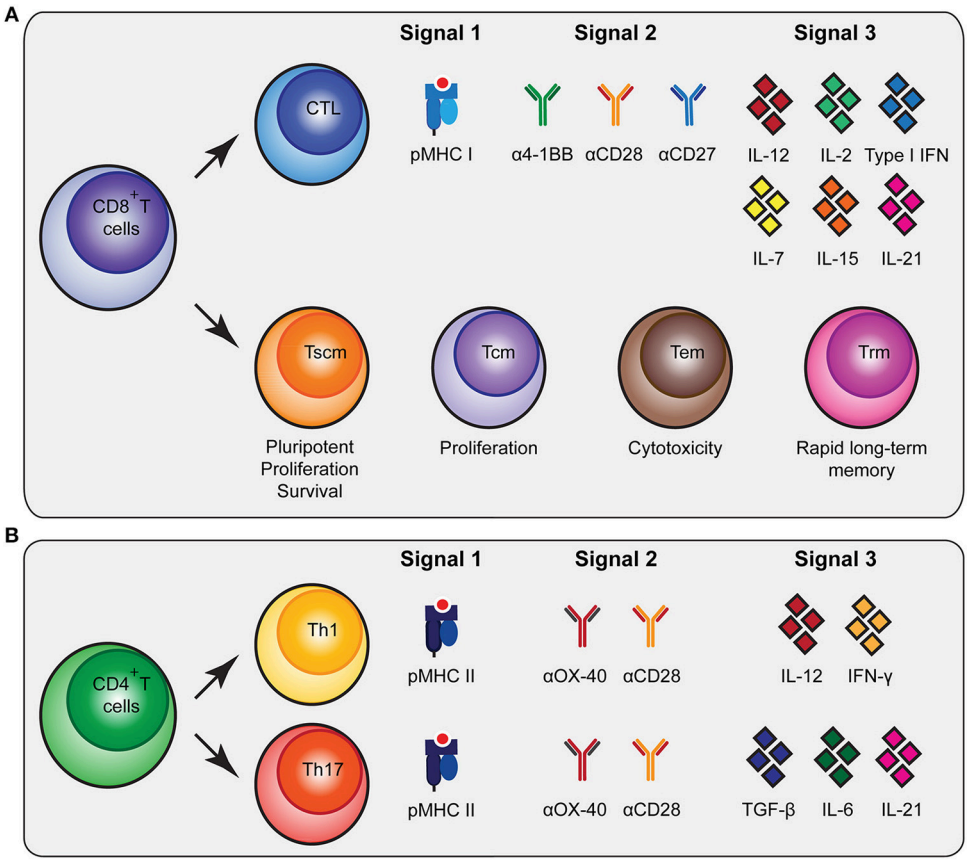

FIGURE 1 | Molecular cues involved in $CD8^+$ and $CD4^+$ T cell activation and differentiation. **(A)** $CD8^+$ T cells can be subdivided in cytoxic T lymphocytes (CTLs) and memory subsets [memory stem cells (Tscm), central memory (Tcm), effector memory (Tem) and tissue-resident memory (Trm)] that all have specific functionalities. To stimulate antigen-specific CTLs, biomaterials should present peptide MHC (pMHC) class I, agonistic antibodies that trigger co-stimulatory receptors for signal 2 and cytokines as signal 3 as depicted. **(B)** To trigger differentiation of $CD4^+$ T cells into T helper 1 (Th1) and Th17 cells, biomaterials need to present pMHC class II together with co-stimulatory signals and different combinations of cytokines. As an alternative to agonistic antibodies to trigger co-stimulatory signaling pathways, natural ligands of co-stimulatory receptors can be used.

results of a mouse melanoma study showed that the T helper 17 (Th17) subset producing IL-17 was involved in B16 tumor rejection (37). Other studies have also indicated a positive role for Th17 cells in the development of long-term anti-tumor immunity and their help in CTL activation and recruitment to the tumor (38, 39). Besides providing support to CTLs, $CD4^+$ T cells can also contribute to the anti-tumor immune response independent of $CD8^+$ T cells (30, 40, 41) by acquiring cytotoxic activity and executing a direct anti-tumor effect (36, 40). Finally, $CD4^+$ T regulatory cells (Tregs) mainly encompass the immune inhibitory subset, which is important in physiological settings to prevent autoimmunity (35). Due to their immune inhibitory profile, Tregs can prevent tumor clearance by inhibiting CTL functions (42).

The generation of the various $CD4^+$ T cell subsets *in vitro* is mainly determined by the cytokine profile present during T cell receptor-mediated activation (34). The presence of interleukin-12 (IL-12) and IFN-γ will skew $CD4^+$ T cells toward a Th1 profile, while the presence of TGF-β, IL-6 and IL-21 will drive the differentiation toward a Th17 profile (34) (**Figure 1B**). Besides inducing Th1 differentiation, IL-12 also enhances the proliferation of activated T cells and induces cell-mediated immunity (43). In addition, IL-1 is able to directly act on $CD4^+$ T cells, especially IL-17 producing cells, increasing antigen-specific T cell expansion and enhancing survival (**Figure 1B**) (44).

Taken together, these studies emphasize the importance of inducing potent CTL and stimulatory $CD4^+$ T helper cell subsets (in particular Th1 and Th17 cells) to induce potent anti-tumor responses, but they also highlight the need for differentiation of the effector subsets into memory cells to help prevent relapse. Insight into the molecular cues that can optimize the design of biomaterial strategies to gain control over the repertoire of T cells that is induced is therefore pivotal.

MOLECULAR CUES TO ACTIVATE AND EXPAND T CELLS

To provide T cells with the signals that are required for activation and differentiation, inspiration could be sought in the

mechanism of action of natural antigen-presenting cells (APCs) for T cell priming, thereby creating aAPCs. Three fundamental signals for T cell activation are (i) triggering TCR signaling; (ii) adequate co-stimulation, e.g., through the CD28 signaling axis; and (iii) the availability of cytokines to direct T cell differentiation (45, 46). TCR engagement can be mimicked by agonistic αCD3 antibodies for polyclonal T cell expansion or recombinant MHC peptide complexes (pMHC) for antigen-specific T cell expansion. Artificial co-stimulation can be provided using agonistic αCD28 antibodies or recombinant natural ligands CD80 (B7.1) and CD86 (B7.2). Recombinant cytokines that provide signal 3 are widely available and are typically presented in soluble form.

Various biomaterial designs have been synthesized to mimic DCs which vary in their shape, the signals they present and the method of administration (19, 47) (**Figure 2A**). Traditionally, soluble polymers or polymeric beads presenting agonistic αCD3/αCD28 antibodies to T cells are used to induce vigorous polyclonal expansion. Besides CD28, co-stimulatory signals belonging to the tumor necrosis factor receptor superfamily (TNFRSF), such as OX-40, 4-1BB, CD27, and LIGHT, are also potentially interesting to steer T cell activation (56, 57). Though OX-40, 4-1BB and CD27 can perform co-stimulation for both $CD4^+$ and $CD8^+$ T cells, OX-40 was shown to predominantly act as a co-stimulatory molecule for $CD4^+$ T cells (58–61). Engagement of 4-1BB and CD27 were more prone to induce potent $CD8^+$ activation. An aAPC design presenting αCD3 antibodies with α4-1BB antibodies as the co-stimulatory cue was reported to preferentially expand memory cells and induce enhanced cytolytic activity compared to aAPCs presenting αCD3 and αCD28 (62). CD27 co-stimulation enhances activation and survival of $CD8^+$ T cells (60, 61), prevents activation-induced cell death (60) and supports the presence of tumor-specific $CD8^+$ T cells residing within established melanoma (63). Expression of LIGHT in the tumor microenvironment of patients increases T cell expansion, activation and infiltration and correlates with improved clinical outcome (64, 65). Furthermore, LIGHT signaling enhanced T cell proliferation, IFN-γ production, tumor infiltration and regression of established tumors in a P815 mastocytoma tumor model and a CT26 colon cancer model (57, 64). These studies imply that careful tuning of co-stimulatory cues presented by biomaterials can steer T cell priming and functionality.

The third signal consisting of cytokines is especially important for naïve $CD8^+$ T cells to differentiate, develop their effector functions, and form potent memory populations (66). Absence of this third signal can result in deletion or anergy of the activated cells (67, 68). In a normal immunological setting, $CD4^+$ T helper cells promote IL-12 or type I IFN production by DCs in a CD40-dependent manner to ensure potent CTL development (67, 68). Apart from supplying T cells with recombinant cytokines presented by biomaterials, the adaptor molecule Stimulator of IFN Gene (STING) could be used to induce type I IFN production (69, 70). Besides IL-12 and type I IFN, there are various other cytokines involved in T cell activation and differentiation such as IL-2, IL-7, IL-15 and IL-21 (**Figure 1A**) (66). IL-7, IL-15 and IL-21 are important for $CD8^+$ T cell memory formation and maintenance (71), while IL-2 promotes the expansion of both $CD4^+$ and $CD8^+$ T cells, thus augmenting the effector T-cell response (72). Moreover, cytokines steer $CD4^+$ T cell development into the different subsets (34), which emphasizes the necessity to include these signals into a biomaterial design.

MOLECULAR CUES TO GENERATE T CELLS OF HIGH QUALITY

Not only the quantity of the generated T cells is important, as the quality needs also to be considered. When T cells reach a more differentiated state, the cell effector functions increase while the memory functions and proliferation capacity decrease (26). Experimental studies in mice and patients have shown a superior role for less differentiated cells (Tscm and Tcm) in adoptive cell transfer, as was demonstrated by enhanced engraftment, expansion, persistence and anti-tumor responses of these minimally differentiated T cells *in vivo* (28, 73–77).

The low numbers of circulating Tscm (28) cells have resulted in the development of *in vitro* culture practices where the differentiation of naïve T cells is controlled by supplementing culture media with IL-7 plus IL-15 or IL-21 and/or small molecules to activate the Wnt/β-catenin pathway (71, 78). The cells generated with these culture protocols showed increased engraftment, expansion and higher tumor reactivity (71, 78). In addition, longer expansion time of T cells *ex vivo* can also drive T cell differentiation and negatively affect cytolytic activity, proliferation, tumor control and T cell persistence *in vivo* (79).

T cell differentiation may also be influenced by T cell metabolism. TILs cultured with a small inhibitory drug for protein kinase B (AKT) (80), naïve T cells cultured with an inhibitor for mammalian target of rapamycin (mTOR) (81) and $CD8^+$ T cells exposed to 2-hydroxyglutarate (82) induces T cells with transcriptional and metabolic properties characteristic of memory T cells that show increased persistence and anti-tumor response *in vivo* after adoptive transfer. These studies indicate the importance of T cell quality and how this may affect persistence, proliferation, survival and effector functions *in vivo*, and demonstrate possibilities for improving *ex vivo* T cell cultures that could also be highly relevant in designing biomaterial-based systems for T cell expansion.

The multifunctionality of the effector T cells also influences the quality of the generated T cells (72). T cells are considered to be multifunctional when having two or more functions including, but not limited to, the production of cytokines, chemokines and/or degranulation (72). Multifunctional $CD4^+$ and $CD8^+$ T cells are able to secrete more IFNγ, and T cells producing both IFNγ and TNF can mediate more efficient killing compared to single cytokine-producing cells (72). Even though these are illustrations from the field of infectious diseases, multifunctional effector T cell responses could also benefit anti-tumor immunity.

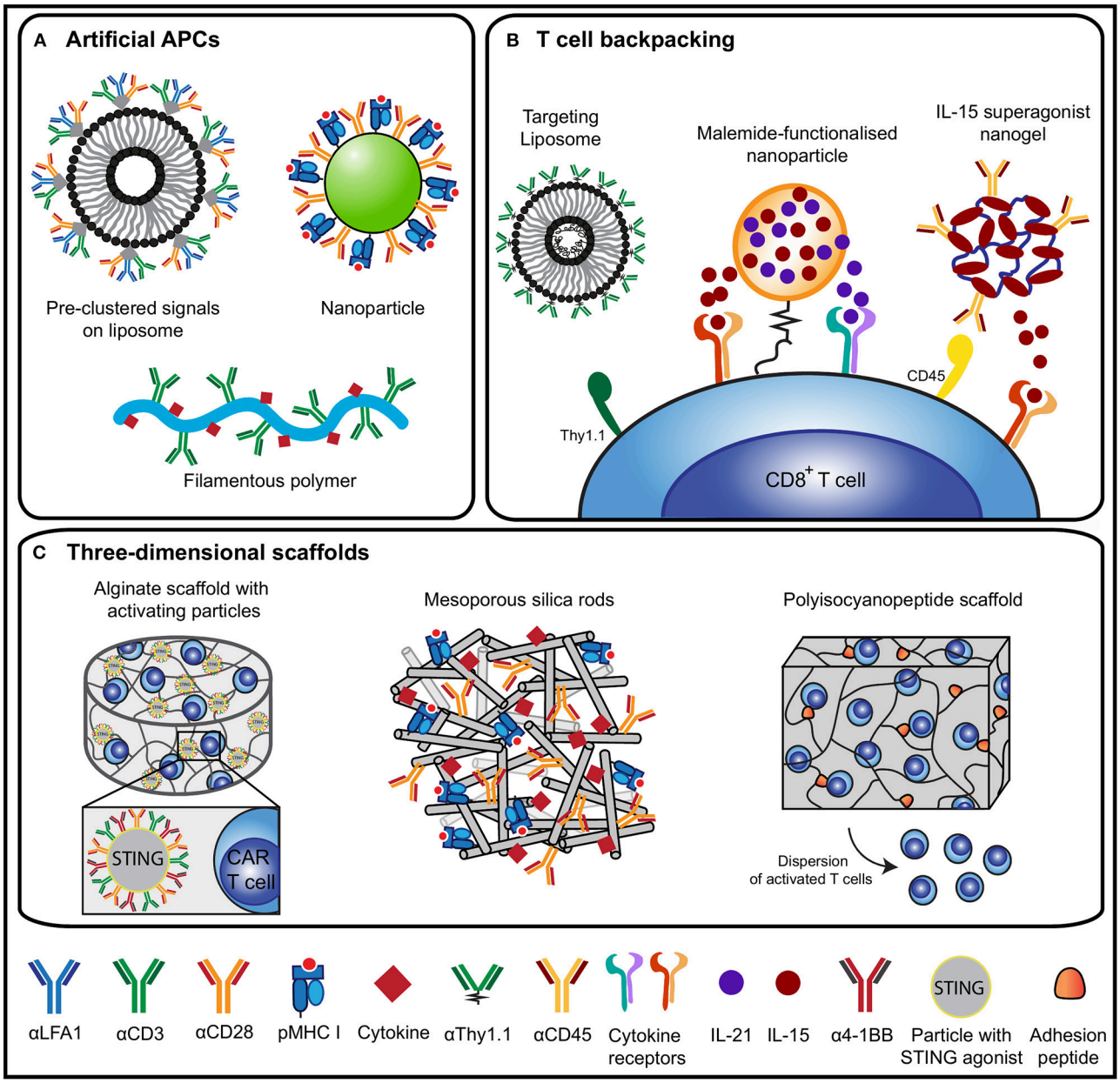

FIGURE 2 | Overview of biomaterial design strategies for T cell activation and expansion. **(A)** An overview of design strategies of artificial antigen-presenting cells (aAPCs) based on liposomes (48), nanoparticles (19) or filamentous polymers (49). aAPC designs present various molecular cues to induce T cell activation, including pMHC or αCD3 antibodies as signal 1, αCD28 antibodies to mimic signal 2 and cytokines as signal 3. **(B)** Different T cell backpacking strategies for the *ex vivo* or *in vivo* targeting of cytokine-loaded particles to T cells using antibody as targets [liposome (50) and nanogel (51)] or through chemical [binding (52) (nanoparticle)]. These strategies ensure targeted delivery of cytokines to support persistence of adoptively transferred cells *in vivo*. **(C)** 3D scaffold-based strategies to expand T cells and to support adoptively transferred (CAR) T cells. Designs include alginate scaffolds with stimulatory microparticles for CAR T cell expansion (53), mesoporous silica rods for T cell activation (54) and a synthetic polyisocyanopeptide-based scaffold that disperses T cells (55).

DESIGNING BIOMATERIALS FOR OPTIMAL TUMOR-SPECIFIC T CELL PRIMING

Biomaterials can be used to present the three imperative signals to T cells to support activation, expansion and differentiation in a spatiotemporally well-defined and sustained manner (83). The defined structural nature of biomaterials enables chemical modification to introduce desired functionalities through standard conjugation or bio-orthogonal chemistries such as "click chemistry" (84, 85). The characteristics of the biomaterial, such as biodegradability, biocompatibility, half-life and the implementation of biological targeting moieties, but also physical properties such as shape, surface topology and mechanical properties, can shape the interaction

with T cells and thus alter the immune response that is provoked (86).

The context in which molecular cues are provided can also influence T cell responses. Immobilizing signal 1 on a surface was reported to promote robust T cell activation (87), because physical forces play an important role in TCR signal transduction (88). The relevance of force upon the TCR was interrogated in more detail by making use of materials with different stiffness (89, 90). MHC class I molecules presented on a softer poly(dimethylsioxane) surface led to enhanced T cell proliferation, improved IL-2 production and increased Th1 differentiation compared to TCR engagement using rigid polystyrene beads (89, 90). In addition to the effect of material stiffness, polymeric aAPC presenting T cell-stimulating cues in a multivalent context demonstrated the importance of multivalency for long-lasting T cell activation (91, 92). Furthermore, superior $CD8^+$ T cell activation was observed for biomaterials displaying signals 1 and 2 in a pre-clustered manner (19, 47, 93, 94), for instance on liposomes (48). Signal density also affects T cell responses, as $CD4^+$ T cells were reported to remain unresponsive when signal 1 is presented in a density that is too low (95–97). In addition to signal density, the quantity of signals 1 and 2 can also influence the effector Th1 $CD4^+$ and effector $CD8^+$ T cell responses (72). Limited amounts of signal 1 or 2 were shown to induce Th1 cells and $CD8^+$ T cells that only secrete IFNγ, while in the presence of increased concentrations of signal 1 or 2 cells both IFNγ and IL-2 were secreted (98, 99).

Most biomaterial designs implement cytokines (signal 3) either in release vesicles or via adsorption (47, 54, 100), creating a local high concentration of soluble cytokines. Robust T cell activation can also be obtained with immobilized cytokines and might preferentially deliver these to T cells through co-presentation of T cell-specific antibodies (49), providing a new range of possibilities for the addition of cytokines in biomaterial-based immune therapies.

When using biomaterials, care must be taken to prevent the induction of exhausted T cells due to persisting T cell stimulation, leading to diminished cytokine production, reduced proliferative capacity, decreased killing abilities, and high expression of co-inhibitory molecules, such as PD-1 and CLTA-4 (101). Therefore, one should not only focus on trying to induce a strong activation signal, but special care should be taken to achieve appropriate stimulation levels to ensure desired T cell activation and prevent T cell exhaustion. To prevent or counteract the exhausted state of T cells, the biomaterial might need to be equipped with PD-1 and CTLA-4 blocking antibodies. Several biomaterial designs have been tested to improve the delivery and sustained release of these antibodies, displaying improved anti-tumor efficacy (14, 102). An alternative strategy to prevent T cell exhaustion could be to provide co-stimulation with an αCD2 antibody (103).

BIOMATERIALS FOR ADOPTIVE T CELL TRANSFER

Apart from applying biomaterials to prime and expand T cells with stimulatory cues, biomaterials are also excellent tools to support the adoptive transfer of T cells for cancer immunotherapeutic purposes (**Figures 2B,C**). Adoptive T cell therapy (ACT) has shown promising results in inducing durable anti-tumor immune responses (1, 7, 8). However, efficacy generally depends on the co-administration of lymphodepleting chemotherapy and/or high doses of IL-2 to support the persistence of these cells *in vivo* (1, 104, 105). Biomaterials have been designed to reduce toxicities seen with the systemic administration of these adjuvants and to enhance the response of adoptively transferred cells (51, 52) (**Figure 2B**). In an elegant design of liposome-like synthetic nanoparticles that encapsulate IL-15 superagonist and IL-21, reduced thiol groups on the T cell surface were used to covalently bind nanoparticles onto the cells before ACT (52). These "backpacks" resulted in considerably more proliferation and persistence of the transferred cells *in vivo* and led to complete tumor clearance in mice bearing metastasized B16F10 melanoma. Further development led to stimuli-responsive particles that release IL-15 superagonist upon increased redox activity at the T cell surface upon TCR signaling (51). To enable repeated *in vivo* stimulation of adoptively transferred T cells, particles were designed that target T cells through an αThy1.1 antibody or IL-2 (50). To circumvent the need to culture cells *ex vivo*, αCD3 antibody fragments can be used to target biodegradable poly(β-amino ester)-based nanoparticles to T cells *in vivo*. These nanoparticles contained a DNA plasmid encoding a leukemia-specific CAR gene combined with 4-1BB and CD3ζ cytoplasmic signaling domains. This strategy resulted in the *in vivo* generation of CAR-T cells that perform comparable to CAR-T cells generated using the conventional *ex vivo* culture method (106).

Most biomaterials for T cell activation are designed to function in soluble form or in suspension as two-dimensional systems. Limited work has been performed using three-dimensional (3D) scaffold-based designs for T cell activation, whereas within the field of DC activation there are multiple examples of 3D scaffolds to create local DC-recruiting and activating niches. 3D scaffolds have proven advantageous as they present DCs with activating cues in a sustained manner at a localized site (107–109). We believe that designing such scaffolds and thereby creating synthetic immune niches for localized *in vivo* T cell activation could contribute significantly to the current T cell mediated anti-cancer therapies. A synthetic immune niche as a site of T cell priming and dispersion could replace or augment the function of tumor-draining lymph nodes, which were shown to be key regulators in the anti-tumor immune response (110).

One area in which 3D scaffolds have been explored for T cell activation is in the field of ACT (**Figure 2C**). An alginate implant equipped with T cell-stimulating signals (αCD3, αCD28, α4-1BB, and IL-15 superagonist) and migration-promoting peptides induced a substantial increase in the proliferation of adoptively transferred T cells at the tumor resection site in a 4T1 mouse breast tumor model (100). Moreover, these cells did not acquire an exhausted phenotype, but migrated toward the tumor-draining lymph nodes where they differentiated into

central memory T cells. In another alginate-based scaffold approach, a combination of adoptive transfer of CAR-T cells and the incorporation of a STING agonist was used to trigger anti-tumor host immunity (53). A chitosan thermogel has also been tested for ACT and resulted in a supportive environment where antigen-specific T cells could proliferate and subsequently migrate toward target cells, which were effectively killed *in vitro* (111). Mesoporous silica microrods with supported lipid bilayers were used to provide T cells with either polyclonal cues (αCD3) or antigen-specific cues (pMHC) in combination with αCD28 for co-stimulation and adsorbed IL-2 to provide paracrine delivery of cytokines (54). Alternatively, a fully-synthetic hydrogel composed of triethylene glycol-substituted polyisocyanopeptides functionalized with integrin-binding motifs supported the *ex vivo* expansion and survival of T cells (55). The *ex vivo*-stimulated T cells could successfully egress from the hydrogel over time when administered *in vivo*, identifying these hydrogels as effective cellular delivery vehicles. Together, these studies demonstrate proof of the concept that 3D scaffolds can be used as a multifunctional platform to enhance polyclonal and antigen-specific T cell expansion and cell persistence *in vivo*.

The use of biomaterials for the adoptive transfer of T cells might make ACT more efficient, as *ex vivo* culture time could be reduced and be potentially superfluous. This would make ACT more feasible and at the same time benefit T cell functionality (105). Moreover, implementing molecular cues like IL-7, IL-15, or IL-21 in 3D biomaterial-based scaffolds could provide an *in vivo* immune niche for the generation and support of adoptively transferred Tscm, which in turn could improve *in vivo* T cell persistence, proliferation and anti-tumor response (71, 78), underlining the promise of biomaterial-based 3D scaffolds for T cell activation *in vivo*. Careful investigation of the behavior of T cells in response to different combinations of molecular cues is required to create the most desirable T cell-activating synthetic immune niche.

CONCLUDING REMARKS

Biomaterials are highly promising tools to present molecular cues to T cells to evoke robust immune responses both *in vitro* and *in vivo*. In this perspective, we presented an overview of molecular cues that could be used to selectively expand T cell subsets that are beneficial for strong anti-tumor immune responses. Biomaterials can be exploited to control the presentation of specific combinations of these molecular cues to T cells and can thus be used to regulate the stimulation level and the induction of specific T cell phenotypes. Careful consideration of how to combine the insights on important T cell-activating molecular cues with material-intrinsic factors is highly important for the design of biomaterials for the expansion and activation of tumor-specific T cells. In this respect, biomaterials could be used as tools to delineate the molecular cues and scaffold design parameters that dictate T cells' responses. When designing biomaterials for controlled activation of the immune system, it is important to take into account the intrinsic immunogenicity of materials and the potential change in immunomodulatory properties after biodegradation (112, 113). In our opinion, one of the major factors that needs to be considered is implementing potent $CD4^+$ T helper cues alongside $CD8^+$ T cell signals on biomaterials. In particular, tuning the T helper response toward a more Th1 and/or Th17 response might have considerable effects on clinical outcomes. Moreover, an improved understanding of the cues essential for memory cell formation is needed in order to develop biomaterial designs that can elicit long-term memory and thus better protect against tumor recurrence. It will be imperative to pursue a balanced and controlled system in terms of number and the release kinetics of molecular cues and the biodegradability of the biomaterial of choice (114).

As is evident from the studies discussed above, biomaterial-based cancer vaccines constitute a very promising field, but a number of challenges remain, especially those related to clinical translation. The ultimate goal is clinical application of biomaterial-based systems to induce long-term and systemic anti-cancer immunity in cancer patients. To ensure smooth transition to clinical translation, it is important to recognize key design parameters from the beginning of the design process, such as biomaterial composition, reproducible and large-scale production under good manufacturing practice (GMP), *in vivo* behavior, degradation, toxicities and safety. This can contribute to decreasing the time and cost of the regulatory pathway. Cell-free biomaterials for local administration, like 3D scaffolds, typically need less extensive testing to get approval, due to limited risks of systemic toxicities (115). However, local toxicity and inflammation may still arise and need to be carefully tested. The soluble biomaterial strategies, such as the particle-based aAPCs, might be considered as biologicals which would indicate a longer and more expensive regulatory pathway. Moreover, modifying already existing and approved therapeutic designs, including materials such as poly(lactic-co-glycolic acid) (PLGA) and hyaluronic acid, will allow for a clearer regulatory pathway (115). The field of regenerative medicine has already contributed a range of clinically-approved biomaterial products from which biomaterial-based cancer immunotherapies may benefit (115, 116). Besides meeting the safety criteria, the biomaterial-based cancer immunotherapies will need to demonstrate efficacy in appropriate preclinical animal tumor models when compared to current therapies (116).

The backpacking of adoptively transferred cells using particle-based biomaterials is considered to have great clinical promise (51, 52). In the beginning of this year, a phase 1 clinical trial with these biomaterial-based T cell backpacks started in patients with solid tumors and lymphomas (117). This example, together with the developments and future directions described in this perspective, illustrates that innovative designs of biomaterials for the direct activation of T cells will bring clinical implementation of biomaterial-based expansion and differentiation of tumor-reactive T cells closer.

AUTHOR CONTRIBUTIONS

MS, RH, CF, MV, and JW contributed to researching the data for the article, discussing the content and to reviewing and editing the manuscript before submission. MS, MV, and JW were responsible for writing the article.

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REVIEW
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Determinants for Neoantigen Identification

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All tumors accumulate genetic alterations, some of which can give rise to mutated, non-self peptides presented by human leukocyte antigen (HLA) molecules and elicit T-cell responses. These immunogenic mutated peptides, or neoantigens, are foreign in nature and display exquisite tumor specificity. The correlative evidence suggesting they play an important role in the effectiveness of various cancer immunotherapies has triggered the development of vaccines and adoptive T-cell therapies targeting them. However, the systematic identification of personalized neoantigens in cancer patients, a critical requisite for the success of these therapies, remains challenging. A growing amount of evidence supports that only a small fraction of all tumor somatic non-synonymous mutations (NSM) identified represent *bona fide* neoantigens; mutated peptides that are processed, presented on the cell surface HLA molecules of cancer cells and are capable of triggering immune responses in patients. Here, we provide an overview of the existing strategies to identify candidate neoantigens and to evaluate their immunogenicity, two factors that impact on neoantigen identification. We will focus on their strengths and limitations to allow readers to rationally select and apply the most suitable method for their specific laboratory setting.

Keywords: cancer, immunotherapy, neoantigen, vaccine, T-cell therapy, review

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INTRODUCTION

Cancer arises as a result of the accumulation of DNA damage and genetic alterations. Mutated gene products can be processed and presented in the form of small peptides on major histocompatibility complex (MHC) molecules of tumor cells and some can elicit T-cell responses. Such immunogenic mutated peptides, referred to as neoantigens, are emerging as promising targets to develop personalized clinical interventions.

Awareness that T cells can target cancer neoantigens is not novel. The dissection of the molecular nature of neoantigens derived from tumor variants induced through exposure to chemical carcinogens was first performed in mice in the late 1980s. The coding regions of three tumor-rejection antigens identified all contained mutations that changed one amino acid in proteins that were ubiquitously expressed (1–3). Importantly, the corresponding wild-type peptides were not immunogenic. The first strategy employed to identify human T-cell reactivities to neoantigens involved the laborious screening of cytotoxic tumor-reactive lymphocytes for recognition of tumor cDNA library pools by transfecting them along with the proper human leukocyte antigen (HLA) restriction element into transfectable target cells (4). In addition, neoantigen-specific responses dominated compared to responses targeting shared antigens in a patient with melanoma suggesting a greater contribution of neoantigen-specific T cells to antitumor immunity (5). The

immunotherapeutic potential of targeting neoantigens was already acknowledged at the time. Neoantigens are specifically expressed by tumor cells and immunotherapeutic targeting of these antigens should be safe. In addition, neoantigens elicit T-cell responses that are not subject to central tolerance in the thymus, suggesting that immune responses against these antigens should be more potent. However, the difficulties of identifying such personalized peptides and T cells were daunting.

Recent technological innovations have enabled the systematic dissection of the personalized T-cell response targeting the tumor mutanome. Retrospective studies have shown that patients that exhibited complete tumor regressions following tumor-infiltrating lymphocyte (TIL) therapy have a higher tumor mutation burden (6) and TILs from responders frequently contain neoantigen-specific lymphocytes (7–11). Antibodies targeting the CTLA-4 and PD-1 pathways have shown the greatest clinical activity in tumor histologies with higher mutation load and brisk T-cell infiltrates such as metastatic melanoma, non-small-cell lung carcinoma (NSCLC), bladder cancer, and tumors with DNA-mismatch-repair deficiencies (12). Even within one same tumor histology, patients whose tumors have a higher mutation load display greater clinical benefit following treatment with immune checkpoint inhibitors (13–15), and this association has been observed across multiple cancer types (16). It is worth noting that a few retrospective studies have also reported a lack of correlation between high tumor mutational burden and clinical benefit in some tumor types (17, 18). Overall, the majority of clinical data are consistent with the hypothesis that higher mutation load is associated with higher likelihood to present neoantigens which can facilitate immune recognition of tumors as foreign.

The clinical correlative data coupled with the technological innovations to sequence tumors and to functionally dissect the personalized T-cell responses in cancer patients have spurred the development of immunotherapies targeting neoantigens. Active immunization strategies employed to treat patients rely on the identification of the non-synonymous mutations (NSM) by tumor whole exome sequencing (WES), *in silico* peptide HLA binding affinity prediction and prioritization of 10–20 candidate neoantigens, to manufacture RNA, synthetic long peptide or dendritic cell-based vaccines of unique composition. In one clinical trial, the vaccines also included candidate neoepitopes identified through elution from tumor cell-surface HLA-I molecules. Results reported thus far in patients with melanoma (19–21), and glioblastoma (22, 23) demonstrate that immunization with vaccines targeting neoantigens is feasible, safe and well tolerated. The melanoma trials reported clinical activity in some patients with detectable tumors at the time of vaccination, and some patients who progressed after vaccination and received anti-PD-1 therapy showed complete responses. More recently, two clinical studies of personalized neoantigen vaccines in patients with resected glioblastoma reported that, although vaccines triggered strong systemic T-cell responses, the majority of patients showed tumor recurrence. These first five clinical trials provide proof of principle that these approaches can enhance the frequency of pre-existing or *de novo* neoantigen-responses following immunization. However, induction of T cell responses were previously observed following immunization against shared antigens and this rarely translated into clinical benefit (24). Hence, significant challenges remain to be overcome including improvement of neoantigen selection, identifying the best route and method for immunization and overcoming intrinsic factors in the tumor microenvironment. However, the complete responses observed in post-vaccination melanoma patients receiving immune checkpoint inhibitors open a window of opportunity for the design of combinatorial approaches in the future.

In another approach different to vaccination, the infusion of large numbers of TILs targeting personalized cancer neoantigens have shown antitumor responses in selected cases of patients with cholangiocarcinoma (25), colorectal cancer (26), and breast cancer (27). This together with the prospective analyses of neoantigen reactivity in peripheral blood of melanoma, gastrointestinal (GI) and ovarian cancer patients suggesting that neoantigen-specific lymphocytes can be detected in the vast majority of patients screened (28–31), provide rationale to develop personalize T-cell based therapies targeting neoantigens.

DETERMINANTS FOR NEOANTIGEN IDENTIFICATION

Despite the increasing interest in clinical interventions targeting neoantigens, substantial challenges remain to enable a more precise identification of neoantigens that are relevant for patient treatment. RNA and synthetic peptide-based vaccines targeting neoantigens used to treat patients thus far lack prospective immunological testing of candidate neoantigens. Rather, these are selected largely based on *in silico* HLA-I binding affinity, making the selection of candidate neoantigens crucial for this therapeutic approach. Surprisingly, neoantigen vaccines reported appear to favor $CD4^+$ over $CD8^+$ responses. Moreover, only few of the patients immunized generated T-cell responses targeting the autologous melanoma cell lines (21), manifesting the limitations of *in silico* peptide HLA binding prediction alone to effectively identify neoantigens naturally processed and presented by the tumor.

Evidence arising from available studies is that only a small fraction of all NSM identified by tumor WES are actually processed, presented and recognized by T cells (8, 28, 29, 31–33). Many of these screenings interrogated the immunogenicity of all the candidate NSM identified by tumor WES, without using *in silico* prediction algorithms. Instead, they used a high through-put immunological screening method relying on the expression of all the mutated minigenes in the patient's own antigen presenting cells (APCs), which enables unbiased processing and presentation on the patient's own HLA-I and HLA-II molecules (described in more detail in section Unbiased Screening of All Candidate Neoantigens Identified by Tumor WES). Hence, the paucity of reactivities detected cannot be attributed to the limitations of *in silico* peptide prediction algorithms. Furthermore, the vast majority of selected candidate neoantigens identified in a tumor are also not effective in tumor rejection in mouse models (34, 35). Part of the reason that could

explain this lack of immunogenicity lies in the fact that for a neoepitope to be recognized in a cancer patient, the T-cell receptor (TCR) repertoire of the patient needs to contain a TCR that specifically targets this peptide bound to a specific HLA allele. Although the TCR repertoire diversity in any given individual is thought to be capable of recognizing virtually any pathogen, this may not hold true for neoantigens which frequently differ from their wild-type counterparts only by one residue. Tumor heterogeneity is yet another potential factor that could hinder neoantigen identification.

Estimating the exact number of neoantigen-specificities in a cancer patient is further complicated by the fact that *the absence of evidence is not evidence of absence*. Neoantigen identification is technically challenging and all the steps involved can impact on the outcome. Briefly, as depicted in **Figure 1**, WES from tumor and matched normal DNA is typically used to identify all cancer-specific NSM, all candidate neoantigens. The resulting neoepitope candidates can be further selected based on their likelihood to be processed and presented on the cell surface HLA molecules using *in silico* prediction algorithms or through selection of mutated epitopes bound to tumor cell-surface HLA molecules through immunopeptidomics. Finally, a variety of novel high-throughput immunological screening methods, with enhanced capacity to interrogate large numbers of candidate neoepitopes, are used to screen cancer-derived $CD8^+$ and $CD4^+$ T-cell populations of interest for neoantigen recognition. Given the technical complexity, it is entirely possible that a fraction of neoantigen-reactive lymphocytes are not detected due to limitations arising from the specific computational analysis performed to identify NSM from WES data, from the *in silico* peptide prediction algorithms, from the specific immunological screening assay and read-outs chosen and/or the limited frequency of neoantigen-specific TCR clonotypes within the chosen source of effector T-cell population used for the screening.

Overall, two critical factors can greatly influence the identification of *bona fide* immunogenic neoantigens: (1) the identification of candidate neoantigens, and (2) the evaluation of their immunogenicity. Given the emerging potential of neoantigens as therapeutic targets, and the crucial importance of these factors for neoantigen identification, the technical implications of these steps and advantages and disadvantages will be reviewed in detail.

IDENTIFICATION OF CANDIDATE NEOANTIGENS

The first element that can influence the identification of immunogenic neoantigens is the tumor-derived DNA and RNA sequencing and the computational analysis necessary to identify tumor-specific NSM.

Identification of Tumor-Specific Non-synonymous Mutations

The process for discovering immunogenic neoantigens starts with the identification of all tumor somatic NSM. To date, this is generally done by mapping genetic alterations in the tumor genome using next generation sequencing (NGS). For each patient, Whole genome sequencing (WGS) or WES data from matched tumor and normal DNA is required. Following the alignment of normal and tumor reads to the human reference genome, somatic variants, which include single nucleotide variants (SNV), gene fusions and insertion or deletion variants (indels), can be detected using variant-calling algorithms. Multiple variant callers have been developed to date and each of them differ in their accuracy and sensitivity to detect different somatic variant types (i.e., SNV, gene fusions, or indels) (36). Indeed, several studies have compared distinct variant calling pipelines and reported substantial discrepancies in the detected variants from the same set of raw sequencing data (37, 38). Consequently, computational analysis pipelines commonly use more than one variant caller and select those somatic variants that are identified by several independent variant callers to reduce the number of false positives (39, 40). Integration of these pipelines will however not solve false-negative calls, which are somatic variants that, despite being potential neoantigens, will remain undetected, pointing out the need for improvement of sensitivity of variant calling algorithms. Of note, the performance of variant calling algorithms is directly related to the process of sequencing. Thus, current technical limitations of sequencing technology such as errors introduced by PCR amplification during library construction or mismapped reads can affect the accurate identification of somatic variants leading to detection of false variants (41). Tumor heterogeneity is an additional limitation for calling somatic variants with confidence, since it biases the detection of clonal over subclonal mutations due to differences in variant allele frequency, thus resulting in underrepresentation of somatic variants (41).

Although WES is currently the standard strategy used to identify candidate neoantigens, RNA sequencing (RNAseq) could alternatively be performed. RNAseq is currently used in combination with WES, to filter out those candidate neoantigens that do not exceed a selected threshold of gene expression. However, its usage should not be restricted to gene expression assessment as it provides additional information that might be essential for the identification of certain somatic variants that otherwise would remain undetected. For instance, low frequency somatic variants that might not be identified by WES could conversely be detected using RNAseq data if their read count is within the detection range (36). Moreover, as RNAseq surveys the entire transcriptome, it is the only method that allows the identification of peptides arising from RNA editing processes such as alternative splicing, gene fusions and post-transcriptional modifications (42, 43). Of note, unlike mutations identified using WES data, which can be assigned to the tumor but not normal DNA, alterations identified exclusively using tumor RNAseq data are not necessarily restricted to the tumor. Epitopes derived from edited RNA cannot immediately be considered candidate neoantigens until their expression in normal tissue has been ruled out. Nevertheless, the use of tumor RNAseq could provide a broader landscape of candidate neoantigens.

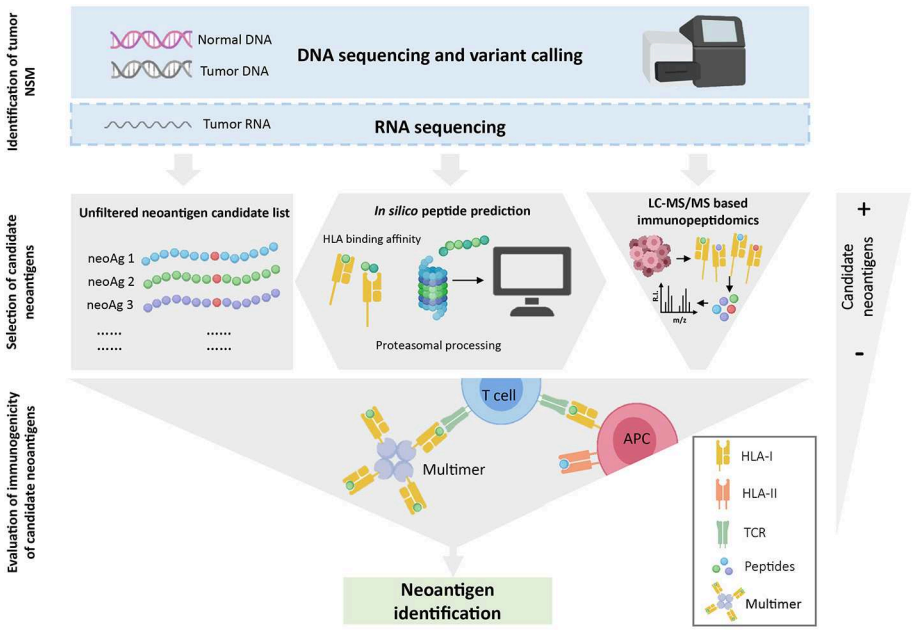

FIGURE 1 | Overview of neoantigen identification using tumor WES. WES is performed on tumor and normal DNA to identify tumor-specific NSM. When available, RNAseq is used to select mutations that are expressed. Once NSM are identified, three strategies can be used to select the list of candidate neoantigens that will be assessed for immunogenicity. The gray-filled shapes depict how each selection strategy will dictate the final number of candidate peptides to be evaluated. Note that *in silico* prediction initially increases the number of potential candidates but, after a ranking-based selection of peptides, this number decreases substantially. Finally, the immunogenicity of the selected candidate peptides is evaluated with different immunological screening assays.

Selection of Neoantigen Candidate Set of Interest

Following the identification of NSM, the neoantigen candidate set of interest can be (1) filtered using *in silico* peptide prediction algorithms, (2) selected based on the identification of specific neoepitopes eluted from tumor HLA through immunopeptidomics, or (3) left unfiltered to perform unbiased testing of all the neoantigens identified (**Table 1**).

Selection of Candidate Neoantigens Using *in silico* Peptide Prediction

The advances in computational biology and immunology have led to the development of algorithms that allow to prioritize candidate peptides that are more likely to be presented on HLA-I based on biochemical and biophysical properties of most of the steps involved in peptide processing, transport and binding to HLA-I.

While peptide processing and transport prediction tools can give important information about the nature of peptides that are presented on HLA, their predictive value alone is still limited. Tools available for proteasomal peptide processing have been trained with a combination of data sets derived from *in vitro* digestion assays with the conventional proteasome, and naturally processed HLA-I ligands, which also include those processed by the immunoproteasome (44, 45). Likewise, peptide transport prediction algorithms have been trained with data sets of experimentally validated HLA-I peptides known to bind TAP (46, 47). However, TAP-independent processing pathways also contribute to the peptide repertoire, and these cannot be predicted with currently available transport prediction tools (48). Given the yet limited predictive value of these *in silico* prediction tools, they are typically integrated with more robust predictors in pipelines for neoantigen prioritization (49, 50).

Algorithms capable of predicting peptide binding to HLA molecules are the most widely used for *in silico* prioritization of neoantigens and were instrumental for the first identification of neoantigens using tumor WES (7, 51). These tools are usually trained with large datasets of experimentally defined HLA ligands and peptides eluted from HLA molecules using mass spectrometry (MS)-based immunopeptidomics. Peptide HLA binding prediction takes into account not only the importance of anchor residues but also the influence of amino acids flanking them. Additionally, the diversity of HLA molecules, which gives rise to thousands of alleles with distinct binding preferences, are considered. Since generating experimental data for that amount of alleles is not feasible, prediction tools used to date incorporate biochemical and structural data of known alleles to infer peptide binding to rare alleles for which no or little data is available (52).

In order to predict which mutated peptides are more likely to bind to HLA, binding affinity prediction tools are commonly fed with a list of peptides in which the detected mutation is flanked by a variable number of amino acids of the wild-type sequence. Algorithms then generate small peptides (8–14 amino acids) from the input sequence for which the binding affinity to the queried HLAs is predicted. Since several peptides derived from the same 25mer sequence are likely to bind one or more

TABLE 1 | Strategies used for selection of candidate neoantigens.

Strategy	Advantages	Disadvantages
In silico peptide prediction and prioritization	Narrows down the number of candidate neoantigens Identifies minimal epitopes	Depends on accuracy of prediction algorithms Not optimal for HLA-II-presented peptides Less accurate predictions for low frequent HLA clonotypes
LC-MS/MS based immunopeptidomics	Direct identification of naturally presented HLA binding peptides Narrows down the number of candidate neoantigens Allows the identification of post-translational modified peptides and non-canonical neoantigens Identifies minimal epitopes	Limited sensitivity of mass spectrometry Biased toward detecting the more abundant peptides Relies on efficient peptide ionization and fragmentation Depends on HLA expression of tumor cells High amount of tumor tissue needed
List of all candidate neoantigens based on whole-exome sequencing data	Identification of all candidate neoantigens	Minimal epitope is not defined Limited feasibility in tumors with high mutation burden

HLA, human leukocyte antigen.

HLAs, the potential number of candidate peptides can sometimes increase (**Figure 1**). Given that *in vitro* neoantigen screening assays currently limit the number of tested peptides to the hundreds, peptides are commonly prioritized based on binding affinity ranking. Predictors such as NetMHCpan 4.0 generally report results as either IC50 values in nM units or as a percentile rank score. IC50 values reflect direct binding affinity predictions, and thresholds <500 nM can be used to define candidate peptides that are more likely to bind to HLA. Percentile rank scores reflect relative binding affinity to a specific HLA allele compared to a large set of random peptides, and ranks ≤2 are used as thresholds for selecting potential neoantigen binders (53). Although both outputs can be used, the percentile rank is preferred to select candidate peptides across multiple HLA molecules, as it is less influenced by the large differences in peptide binding affinity values among HLA molecules.

Although prediction tools for HLA-II-restricted peptides also exist, these are less reliable than HLA-I predictors for two main reasons. First, endosomal HLA-II peptide processing is complex and poorly characterized (54), limiting the development of HLA-II peptide processing algorithms. Second, prediction of binding affinity to HLA-II molecules is more complex due to its structural nature because, unlike HLA-I molecules in which the peptide-binding groove is closed, HLA-II molecules have open ends. Even though the core binding motif of both molecules comprises peptides of approximately nine amino acids, HLA-II-restricted ones have a wider length range (11–20 amino acids) compared to HLA-I-restricted ones (8–11 amino acids), and the flanking amino acids can affect binding affinity (55). Further research addressing these challenges will be crucial for improvement of HLA-II prediction tools in the future.

Despite advances in prediction algorithms, currently available tools fail to reliably predict which of the presented peptides will be immunogenic (i.e., whether a presented peptide will be recognized by T cells). This is one of the main limiting steps in neoantigen screening, and it is perfectly reflected by the fact that only few of the hundreds of peptides identified by tumor WES data and *in silico* prediction are immunogenic despite binding to HLA molecules. Although HLA binding prediction is a strong correlate of immunogenicity, accumulating data suggest that bias of *in silico* prediction toward strong binders (<500 nM) can overlook immunogenic peptides that show low-binding affinity. The first evidence of this was reported by Duan et al., who developed an algorithm, termed differential agretopicity index (DAI), which ranks mutant peptides based on their improved binding to HLA compared to the wild-type counterpart (34). Using DAI to identify neoantigens in mouse models of cancer, the study demonstrated that validated immunogenic peptides could have binding affinities up to 140-fold higher than the 500 nM threshold. These findings have been confirmed by other studies in humans (17, 18, 28, 56), highlighting that peptide selection based on the 500 nM threshold should be revisited. Additionally, other limitations of binding prediction tools have been recently identified in clinical trials of cancer vaccines. Patients with melanoma or glioblastoma receiving personalized neoantigen vaccines appear to favor $CD4^+$ over $CD8^+$ T-cell responses against the immunizing peptides, even though these were predicted and prioritized using HLA-I binding algorithms (21, 22). These data further stress the need of developing improved algorithms which can reliably predict HLA-I and II immunogenic peptides. The low number of immunogenic neoantigens validated to date [<300; reviewed in Karpanen and Olweus (32)] makes it difficult to generate a consensus for features likely to predict peptide immunogenicity. Although this is currently a matter of extensive research, only few parameters, besides the aforementioned DAI, have been suggested to improve the prediction of immunogenicity of peptides. For instance, differences in non-anchor residues (P4-P6), peptide size (i.e., large) and amino acid composition (i.e., aromatic residues) have been associated with immunogenicity (57). Additionally, peptide-HLA (pHLA) stability, measured by biochemical assays, has been proposed as a parameter to discriminate immunogenic from non-immunogenic peptides (58). Data derived from this kind of experiments led to the development of prediction tools which show that immunogenic peptides promote more stable pHLA-I than non-immunogenic peptides (59). However, the predictive value of this tool is still controversial (60). Thus, its use as a single predictor is less frequent.

It is worth mentioning that the immunogenicity of a given neoantigen does not necessarily translate into tumor rejection

and/or therapeutic benefit. Mice studies have shown that the vast majority of identified neoepitopes, despite triggering T-cell responses, fail to induce complete tumor rejections (34, 35). In humans, the previously mentioned clinical trials of personalized neoantigen vaccines have rarely shown clinical responses despite triggering strong T-cell responses against the targeted neoantigens (20–23). The development of novel algorithms or screening tools capable of identifying neoantigens capable of inducing tumor rejection could be determinant for the efficacy of personalized vaccines.

To date, there is a plethora of computational pipelines that allow the identification of NSM and the prediction of neoantigens. However, these are built on the basis of traits set by each developer, which often leads to discordant results. Strategies such as the Tumor Neoantigen Selection Alliance (TESLA), which seek to harmonize these pipelines, will be of great importance in the coming years to improve neoantigen identification (61).

Selection of Candidate Neoepitopes Using Mass Spectrometry-Based Immunopeptidomics

Another possible strategy that can be used to prioritize candidate neoantigens for screening is the use of MS-based immunopeptidomics which relies on the study of the tumor pHLA immunopeptidome (62). This method starts with the lysis and homogenization of the tumor material followed by the purification of the pHLA complexes through immunoprecipitation. After eluting the peptides bound to HLA molecules, liquid chromatography coupled tandem MS (LC-MS/MS) is performed to identify the amino acid sequence of the eluted peptides, which is commonly obtained by matching MS/MS spectra against a customized protein sequence database (63). This database is generated by combining a reference protein sequence database with genomic information derived from patient's NGS data, which is essential to identify eluted mutated peptides that are private for each patient. This method to identify neoantigens was first described in a mouse tumor model. WES and RNAseq in combination with MS analysis of peptides eluted from the cell surface MHC of two mouse tumor cell lines allowed the identification of seven candidate neoantigens, three of which turned out to be truly immunogenic (64). Since then, candidate neoantigens have also been successfully identified in human tumor cell lines (65) and, more importantly, in fresh tumor material (56, 66). Indeed, Bassani-Sternberg et al. demonstrated for the first time that this strategy could also be exploited to identify immunogenic neoantigens directly from primary human cancer tissues. In this case, the combination of WES and immunopeptidomics of tumors from five patients allowed the identification of 11 mutated peptides, and two of eight peptides tested were able to elicit antitumor T-cell responses.

MS-based immunopeptidomics is advantageous, as it substantially narrows down the list of candidate neoantigens to be screened (**Figure 1**) and, consequently, the number of false positives that are obtained using other strategies such as *in silico* prediction (67). This might be of great importance for immunogenicity screening assays, especially in tumors with high mutation burden. Additionally, this is currently the only unbiased method that directly interrogates the naturally presented HLA-bound peptides including those harboring post-translational modifications (68). Neoantigens could also derive from non-canonical or cryptic peptides, including those derived from alternative open reading frames, novel exon-exon junctions, intronic sequences, long non-coding RNAs, 5′ untranslated regions (5′UTRS; **Table 2**). These could also be identified by performing database-dependent analyses as long as the amino acid sequences of such peptides have been previously introduced into the customized protein sequence database (80, 81). This could be achieved using a customized database derived from RNAseq data as exemplified by the study of Smart et al., in which they identified epitopes derived from retained introns using RNAseq and validated their expression and presentation by MS analyses (42). Importantly, retained introns expressed in normal tissues were filtered out with the aim to exclusively identify those that are tumor-specific and can potentially be immunogenic. As an alternative to the generation of a customized sequence database, the amino acid sequence can also be directly extracted from tandem mass spectra through database-independent analysis (i.e., *de novo* sequencing). However, the use of this strategy is still limited because it is error prone and fails to determine the entire amino acid sequences due to incomplete tandem mass spectra (82).

Although MS-based immunopeptidomics offers multiple advantages, the discovery of presented immunogenic peptides using this approach is hindered by technical limitations, evidenced by the short list of human cancer neoepitopes identified through this approach to date (56, 66). The major concern is the low sensitivity of MS. The fact that MS is skewed toward detecting the more abundant peptides hampers the identification of mutated peptides among all endogenously presented peptides, especially if they are expressed at low levels or exclusively expressed in subclonal tumor populations. Because of this, and considering that tumor cells express heterogeneous levels of HLA molecules, large amounts of starting tumor material is required to identify candidate neoepitopes. Indeed, in the study by Bassani-Sternberg et al. in which they eluted HLA-I and II bound peptides from primary tumor material, tumor biopsy size seems to be associated with the number of mutated peptides detected (66). Identifying candidate neoantigens within the repertoire of HLA II-peptides in fresh tumor material can also be cumbersome probably due to their low frequency within the pool of presented peptides on APCs, which typically express HLA-II molecules. In fact, even if HLA-II peptides have been successfully eluted in different studies, neoantigens have not been identified so far among the class II tumor peptidomes (67, 83).

Another important consideration is that MS/MS relies on efficient ionization and fragmentation of the peptides. Thus, the successful identification of the sequence of a peptide will depend on its amino acid composition and the biochemical characteristics of such amino acids, which will determine their capacity to be ionized and efficiently fragmented (84). Consequently, a fraction of peptides that are naturally presented might never be detected using this approach.

Overall, this strategy yields a long list of minimal epitopes from both normal and mutated HLA-bound peptides, from

TABLE 2 | Tumor-rejection antigens derived from non-canonical protein sequences.

Epitope identified[a]	Type of tumor	Origin of non-canonical peptide	Expression in normal tissue	Gene name	HLA restriction element	Reference
MSLQRQFLR	Melanoma	aORF	Unknown	*TRP1*	HLA-A*31	(69)
VYFFLPDHL	Melanoma	Intronic	Yes	*GP100*	HLA-A*24	(70)
RSDSGQQARY	Melanoma	Intronic	Yes/low[b]	*AIM2*	HLA-A*01	(71)
VLPDVFIRC/VLPDVFIRCV	Melanoma	Intronic	No	*GNTV*	HLA-A*02:01	(72)
EEKLIVVLF	Melanoma	Intronic	No	*MUM1*	HLA-B*44:02	(4)
LPAVVGLSPGEQEY	Renal cell carcinoma	aORF	Yes	*MCSF*	HLA-B*35:01	(73)
SPRWWPTCL	Renal cell carcinoma	aORF	Yes/low[b]	*iCE*	HLA-B*07:02	(74)
EVISCKLIKR	Melanoma	Intronic	No	*TRP2*	HLA-A*68:011/HLA-A*33:01	(75)
LAAQERRVPR	Melanoma and breast cancer	aORF	Unknown	*NYESO1*	HLA-A*31	(76)
MLMAQEALAFL	Melanoma	aORF	Yes	*LAGE1*	HLA-A*02:01	(77)
CQWGRLWQL/MCQWGRLWQL	Melanoma	aORF	Unknown	*BING4*	HLA-A*02	(78)
LPRWPPPQL	Renal cell carcinoma	Intronic	Yes	*RU2*	HLA-B*07	(79)

[a]*identified by cDNA library screens;* [b]*compared to cancer tissue; aORF, alternative open reading frame; HLA, human leukocyte antigen.*

which candidate neoantigens can be selected and tested to assess their immunogenicity.

Unfiltered Neoantigen Candidate List

Once all tumor NSM are identified, one possibility is to interrogate the immunogenicity of all candidate neoepitopes identified by tumor WES, without biasing the selection of peptides based on *in silico* prediction, which may not always be accurate. This can be done using a variety of immunological screening methods, as explained in section Immunological Screening Methods Used to Evaluate Neoantigen Recognition. However, the feasibility of this approach is restricted to tumors with a limited number of mutations given the cost and effort associated with screening T cells for recognition of a large set of mutated epitopes. Alternatively, and particularly when dealing with tumors with high mutation burden, it is crucial to further filter candidate neoantigens to exclusively evaluate the immunogenicity of a selected set of candidate neoantigens.

EVALUATION OF IMMUNOGENICITY OF CANDIDATE NEOANTIGENS

Evidence arising from available studies is that the vast majority of selected candidate neoantigens identified in a tumor are not recognized by T cells (28–30, 32, 85). Thus, evaluation of the immunogenicity of candidate neoantigens using a variety of screening methods will be critical to more precisely identify and select neoantigens suitable for clinical intervention (**Table 3**).

Immunological Screening Methods Used to Evaluate Neoantigen Recognition

The first strategy employed to identify human T-cell reactivities to neoantigens was described in Coulie et al. (4). Coulie et al. identified a tumor-specific intronic mutation in MUM-1 recognized by a human cytolytic T lymphocyte (CTL) clone using an approach which involved screening melanoma-specific CTLs for recognition of target cells transfected with tumor cDNA library pools along with the appropriate HLA restriction element. Additional mutated gene products derived from CDK4 and β-catenin, capable of inducing T-cell responses, were also identified using similar strategies and were found to either enable peptide binding to HLA-I by creating an HLA-I binding motif or to modify a TCR contact residue of a peptide that was already capable of binding to HLA-I (9, 86). This strategy was widely used during the following decades to dissect the molecular nature of antigens recognized by tumor-reactive T cells, leading to the identification of additional neoantigens (10, 11, 87). However, this approach is laborious and time-consuming, it can be influenced by the size, expression levels or GC-richness of transcripts encoding for T-cell epitopes, and optimally requires the establishment of tumor-specific clones and matched tumor cell lines, which is often not possible. Furthermore, this approach unbiasedly screens T cells for recognition of both mutated and non-mutated antigens, leading to frequent identification of self-antigens, rather than neoantigens (**Table 3**).

All these limitations have incentivized the development of alternative high-throughput immunological strategies that facilitate the evaluation of T-cell reactivity against a large number of candidate neoepitopes identified by tumor WES. Yet, it is worth mentioning that a considerable number of tumor-rejection antigens identified by screening tumor cDNA libraries, including the first human neoantigen identified (4), derive from non-canonical protein sequences encoded by introns, alternative open reading frames or aberrantly spliced variants (**Table 2**). These findings are of potential concern, given that the current strategies exclusively identify NSM in exons (rarely using WGS), ignoring potential neoantigens that could arise from non-canonically

TABLE 3 | Immunological screening assays used to test for neoantigen recognition.

Strategy	Advantages	Disadvantages
cDNA libraries	Interrogates all transcribed sequences	Labor intensive and time-consuming. Biased toward highly transcribed genes. Influenced by the size, expression levels or GC-richness of transcripts encoding for T-cell epitopes. Interrogates mutated and non-mutated sequences.
Minimal epitopes	Cost-effective. HLA-matched target cells (based on *in silico* prediction) can be used instead of autologous APCs	Exclusively interrogates a selected list of mutated epitopes based on *in silico* prediction or validated by immunopeptidomics Requires autologous or HLA-matched cells as target cells Not optimal for $CD4^+$ cells
Peptide-HLA multimers	Overcomes the need of autologous APCs Allows the isolation of antigen-specific T cells	Exclusively interrogates a selected list of mutated epitopes based on *in silico* prediction or validated by immunopeptidomics Multimers are available for a limited number of HLA molecules Not optimal for $CD4^+$ cells
Tandem minigenes or peptide pools	Can be used to interrogate all or a large portion of mutated epitopes Allows potential processing and presentation of candidate neoantigens on HLA-I and HLA-II Does not require prior knowledge of the minimal epitope or HLA restriction	Cost increases in patients with high mutation burden Availability of APCs/effectors can limit this approach, especially when >250 epitopes are tested Requires autologous APCs as target cells Peptide processing by immunoproteasome in APCs might differ from processing by the proteasome in tumor cells

APC, antigen-presenting cell; HLA, human leukocyte antigen.

translated sequences. Current efforts to overcome this limitation of exome-based strategies to identify neoantigens arising from non-canonical protein sequences combine WES with RNAseq and immunopeptidomics, as previously explained in detail in section Selection of Candidate Neoepitopes Using Mass Spectrometry-Based Immunopeptidomics.

Screening of Predicted or Eluted Minimal Neoepitopes

In 2012, two reports in mouse tumor models demonstrated for the first time that tumor WES can be exploited to identify neoantigens (35, 88). In 2013, Robbins et al. performed a retrospective study to identify the molecular nature of the antigens targeted by TILs from five melanoma patients, some of which demonstrated tumor regression following TIL transfer (7). They used tumor exome sequencing to identify all NSM and synthesized neoepitopes that were predicted to bind to the patients' HLA-A class I molecules and screened the TIL infusion products for recognition of the mutated peptides individually pulsed onto COS7 monkey kidney cells or HEK293 human embryonal kidney cells transfected with the appropriate HLA-A alleles. This work led to the identification of eight mutated peptides recognized in four of five patients analyzed. Remarkably, two of the neoantigens that were identified in two independent patients using this approach, CSNK1A1 and PLEKHM2, were not identified using the tumor cDNA screening method. This work describing frequent detection of neoantigen-specific lymphocytes in responding patients together with a recent study demonstrating that patients that exhibited complete tumor regressions following tumor-infiltrating lymphocyte (TIL) therapy (6) have a higher tumor mutation burden suggest that neoantigen-specific lymphocytes play an important role in the efficacy of TIL therapy.

Although this screening strategy was initially used to interrogate reactivity to neoepitopes presented exclusively by HLA-A alleles, it can be used to identify neoantigens in any HLA of interest as long as autologous or HLA-matched antigen-negative target cells are available or by introducing the autologous HLA molecules into transfectable cells, that can be used as target cells. It can also be used to interrogate candidate neoepitopes eluted from the cell surface HLA molecules of tumor cell lines or tumor biopsies (65, 89). In a slightly higher-throughput version, it can be used to interrogate large numbers of *in silico* predicted neoepitopes by grouping these into peptide pools. It is the simplest approach available to analyze neoantigen immunogenicity, since it relies on classically available immunological techniques such as IFN-γ release by ELISA or ELISPOT assays, as well as others, and its sensitivity depends on the specific read-out chosen to measure T-cell responses. This approach has allowed to successfully identify immunogenic neo-epitopes in different malignancies including melanoma, NSCLC and ovarian cancer (56, 65, 90, 91).

A second immunological method that can be used to identify neoantigen-specific lymphocytes is the use of pHLA multimers. Since pHLA-I tetramers were described in 1996, these have become essential reagents for the visualization and isolation of antigen-specific T cells (92). However, the technically challenging generation of individual pHLA monomers coupled with the limited number of fluorochromes available for pHLA multimer detection precluded a more comprehensive analysis of T-cell immunity. Two technical innovations have contributed to facilitate large scale neoepitope discovery using HLA multimer-based detection technologies from limited biological material. First, the development of conditional HLA ligands which are cleaved upon exposure to UV-light and can be exchanged with any epitope of interest (93, 94). Using

UV-exchangeable HLA ligands, only one pHLA multimer loaded with an exchangeable peptide has to be produced for each HLA allele of interest and can be used as a stock to generate large libraries of pHLA complexes through simple manipulations. Similar strategies have been reported recently, all of them aiming at facilitating the high-throughput production of large panels of pHLA complexes (95, 96). Second, fluorochrome-based combinatorial encoding has increased the number of T-cell specificities that can be interrogated by flow cytometry in one sample (up to 28 single specificities with two-dimensional combinatorial encoding with eight fluorochromes) (97). In one study, Van Rooij et al. performed tumor WES, and expanded TILs from a melanoma patient who exhibited a partial response to ipilimumab. They used *in silico* HLA-A and HLA-B binding prediction algorithms to identify neoepitope candidates and generated a library of pHLA tetramers. TILs were screened for binding to this library of tetramers using the fluorochrome-based combinatorial encoding staining method and this led to the identification of TILs targeting two distinct neoantigens (51). Interestingly, they also monitored an increase in the frequency of one of the neoantigen-specific lymphocytes in the blood of the patient following treatment with anti-CTLA-4, suggesting the involvement of these T cells in the therapeutic efficacy of this immunotherapy.

This technology has enabled the generation of large panels of desired pHLA complexes and consequently pHLA multimer libraries are currently used for large-scale immunogenic neoepitope discovery (98), and have successfully been used to identify immunogenic mutated neoepitopes in NSCLC and melanoma (14, 99). More recently, DNA barcoding of individual pHLA molecules has enabled to screen 1031 T-cell specificities in one single reaction (100). While DNA barcodes offer the possibility of screening T cells for a full cancer mutanome using one biological sample, this technology only provides a measure of T-cell frequency, but lacks the visual assessment of the individual T-cell reactivities as well as the possibility of performing short-term culture given that T cells are lysed for DNA barcoding amplification.

High-throughput screening of T cells using multiplexed pHLA multimer staining is of particular interest as it overcomes the need of autologous or HLA-matched APCs. However, pHLA complexes are only available for a limited number of HLA allotypes. Thus, if the aim is to screen T cells for recognition of all possible predicted or HLA-eluted neoepitopes, this strategy can only be used in patients for which all or most of the HLA allotypes are available for pHLA multimer generation. The detection of $CD4^+$ specificities using HLA-II multimers represents an additional challenge in the field. Although it is feasible (101), the low accuracy of *in silico* prediction of HLA-II-restricted epitopes can result in a less precise identification of candidate minimal epitopes (see section Selection of Candidate Neoantigens using *in silico* Peptide Prediction). Furthermore, technical issues related with the production of pHLA-II multimers, and the weaker TCR binding affinities to HLA-II also hinder the use of pHLA-II multimer staining for neoantigen-specific $CD4^+$ T-cell identification (102). Consequently, the majority of screenings performed using this approach are usually focused on identifying neoepitopes presented on HLA-I molecules to $CD8^+$ T cells, which might underestimate the contribution of neoantigen-specific $CD4^+$ T cell populations.

It is worth mentioning that the immunological functional screening assay as well as the HLA multimer staining technologies described above rely on in-house or commercial production of synthetic peptides. These are frequently synthesized or ordered at <70% purity, given the relatively large number of neoepitopes obtained following *in silico* peptide prediction algorithms and the costs associated with custom peptide production. However, custom peptide libraries have been reported to contain impurities, that can affect T-cell recognition and yield false-positive results (103, 104). Hence, validation of neoantigen-specific reactivity/ies using a second batch of >70% pure peptides is highly advisable. Ultimately, the best *in vitro* evidence that a neoantigen exists is provided by showing preferential T-cell recognition of a given neoantigen expressed, processed and presented by autologous APCs or HLA transfectable target cells, compared to the corresponding wild-type (wt) counterpart.

Unbiased Screening of All Candidate Neoantigens Identified by Tumor WES

While the strategies mentioned above are frequently used to identify neoantigens and neoantigen-specific lymphocytes, they are limited by the accuracy of current *in silico* prediction algorithms, which have not been thoroughly trained to identify minimal epitopes for rare HLA-I alleles or HLA-II molecules, and do not consider post-translational modifications (see section Selection of Candidate Neoantigens using *in silico* Peptide Prediction). To overcome these limitations, Lu et al. devised a new screening assay to evaluate $CD8^+$ and $CD4^+$ T-cell responses to any of the NSM identified expressed processed and presented on the patient-specific HLA-I and HLA-II molecules, without the need for *in silico* prediction. Briefly, for each NSM identified one minigene construct was designed, encoding the mutated amino acid flanked by 12 amino acids of the wt sequence. Typically, between 6 and 24 minigenes were stringed together to generate tandem minigenes (TMG) in a single open reading frame. *In vitro* transcribed RNA generated from the TMGs was transfected into autologous APCs, such as B cells or immature dendritic cells (8, 25, 29). In addition, or as an alternative to the generation of mutated TMGs, 25-residue peptides can be synthesized and grouped into peptide pools (PPs), each containing up to 24 mutated peptides. Neoepitopes presented through intracellular (transfected TMGs) and extracellular (pulsed peptides) pathways on autologous APCs expressing all HLA-I and HLA-II molecules are then evaluated for their ability to induce T-cell responses and when reactivities are detected against a specific TMG or PP, these are subsequently deconvoluted to identify the specific neoantigen recognized.

This unbiased screening approach was used to identify two mutated antigens, KIF2C and POLA2, targeted by TIL derived from two patients that underwent complete tumor regression following TIL transfer (8). An additional study interrogated the immunogenicity of 720 non-synonymous somatic variants identified by WES, encoded by 62 TMGs,

and identified 10 neoantigens targeted by TILs presented on three different HLA molecules (33). Linnemann et al. used immortalized autologous B cells pulsed with 31-residue mutated peptides to identify neoantigen-specific $CD4^+$ T cells in two of three melanoma patients evaluated (85). Moreover, this high-throughput screening approach revealed that neoantigen-specific lymphocytes are frequently detected in TILs derived from GI cancers which have lower mutation burden (29), providing an opportunity to develop effective immunotherapies for patients with additional cancer types. Importantly, TMG and/or PP screening were used to prospectively select neoantigen-specific TILs for patient treatment and this was able to induce antitumor responses in a patient with cholangiocarcinoma treated with $CD4^+$ ERBB2IP mutation-specific lymphocytes (25), a patient with metastatic colorectal cancer treated with $CD8^+$ KRAS mutation-specific lymphocytes (26), and a patient with breast cancer treated with TILs recognizing SLC3A2, KIAA0368, CADPS2, and CTSB mutated gene products combined with anti-PD-1 (27). A diverse repertoire of lymphocytes targeting three neoantigens was also detected using this approach in one patient with cervical carcinoma who underwent complete tumor regression following TIL transfer (105). Overall, this strategy has been used to identify over 100 neoantigens in over 13 studies (8, 25–31, 33, 105–108), including both $CD4^+$ and $CD8^+$ T-cell responses. Moreover, this provides the strongest evidence that T-cell therapies targeting neoantigens can lead to antitumor responses. However, these unbiased screening strategies have also provided evidence that only a limited number of tumor somatic mutations detected by tumor WES and RNAseq are immunogenic.

The biggest advantage of the unbiased screening using TMGs and PPs is that it mimics the natural antigen processing and presentation of neoepitopes on both class I and class II patients own HLA molecules, overcoming the need of *in silico* peptide prediction algorithms. Importantly, it has enabled the identification of $CD4^+$ neoantigen-specific lymphocytes. However, it also has some limitations that should be taken into account. Although, theoretically, all mutations identified by WES can be screened, the cost associated with peptide and TMG synthesis, and *in vitro* RNA transcription greatly increases when screening tumors with high mutation load. Moreover, since the initial screening is carried out with TMGs and PPs containing multiple candidate neoantigens, a deconvolution is required to identify the neoantigen recognized. Thus, the availability of large numbers of autologous APCs and effector cells to assess the immunogenicity of neoantigens can sometimes be a limitation. The lack of autologous APCs could be overcome using HLA-matched cells or by transfecting the individual HLA alleles, although this further complicates the screening strategy. In addition, the electroporation of TMG RNA does not guarantee expression and processing of all the mutated minigenes included, which could be influenced by the position in the TMG or the 3D structure of the chimeric protein resulting from the concatenation of up to 24 minigenes. Moreover, the size of the mutated minigene or peptide to ensure proper processing and presentation is still a matter of debate. Finally, the efficiency of this approach is influenced by the APC chosen. Although immature dendritic cells and *ex vivo* stimulated B cells are the cells of preference, their proteasome can be different to that expressed in tumor cells, and the ability of each cell type to process and present TMG or cross-present peptides could differ.

In conclusion, this strategy allows to agnostically interrogate the immunogenicity of all or a large fraction of candidate neoantigens detected in a given tumor without prior knowledge of the minimal epitope or the HLA restriction element of each mutated peptide.

Novel High-Throughput Screening Strategies to Identify Neoantigens

A few novel technologies that have recently been described aim at identifying the cognate peptide recognized by a T cell through the detection of APCs that have been specifically recognized by T cells, rather than monitoring specific activation of T cells. For instance, Joglekar et al., developed a cell-based platform for T-cell antigen discovery that relies on the screening of a large number of antigens through the expression of chimeric receptors termed *Signaling and antigen-presenting bifunctional receptors* (SABRs) in NFAT-GFP-Jurkat cells through stable transduction with lentiviral vectors (109). These chimeric receptors are composed of an extracellular domain comprising a peptide tethered to an HLA fused to an intracellular CD3ζ signaling domain and a CD28 co-stimulatory domain. When recognized by a specific TCR, this interaction triggers the expression of GFP and CD69 on NFAT-GFP-Jurkat cells which can be selected and sequenced to identify the specific peptide recognized. More recently, Kisielow et al. have used a similar NFAT reporter system which is restricted to the identification of tumor-specific peptides recognized by $CD4^+$ T cells (110). In this case, the signal-triggering molecule is a MHC-TCR chimeric receptor (MCRs) which incorporates the peptide linked to the MHC domain. MCR libraries are generated by cloning fragmented tumor cell cDNA into MCR sequences and are transduced into reporter cells, which are used as target cells in co-culture assays with T cells encoding for TCR of interest. TCR interaction with a specific MCR induces reporter gene expression through NFAT activation, allowing the selection, and identification of the recognized peptide through sequencing. Although these are proof-of-concept studies and their applicability as well as advantages and limitations remain to be determined, the novel strategies described may potentially be used alone or in combination with other screening strategies for an unbiased identification of neoantigens targeted by T cells in patients with cancer.

Sources of Effector T-Cell Populations to Identify Neoantigen-Specific Lymphocytes

Once a list of candidate neoantigens is obtained, their immunogenicity is typically evaluated *in vitro*. In addition to the immunological screening methods previously described, the selection of an effector T-cell population with which screening assays will be performed is a critical determinant for neoantigen identification (**Figure 2**). Theoretically, any tissue or fluid from which T cells can be isolated and/or expanded is a potential source for neoantigen immunogenicity screenings.

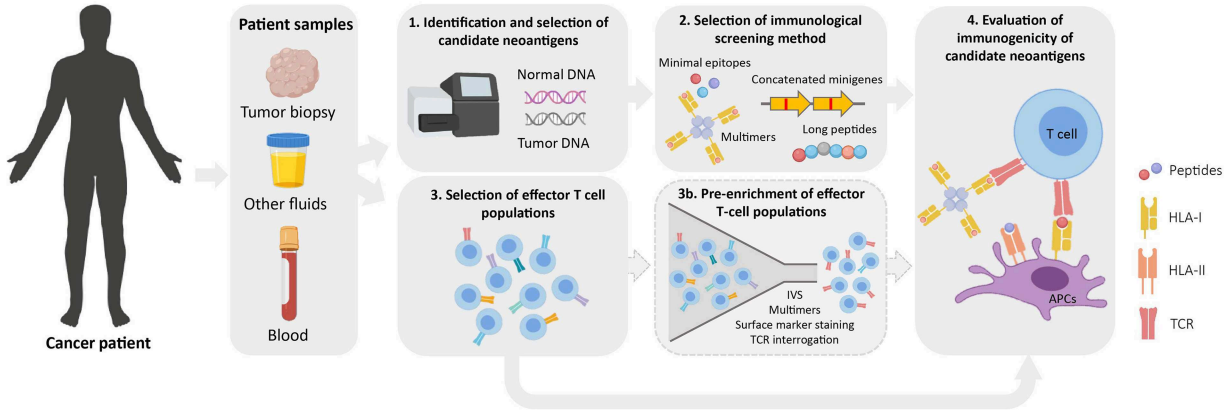

FIGURE 2 | Workflow for the identification of immunogenic neoantigens. Normal and tumor DNA obtained from cancer patients are used to identify tumor-specific NSM by WES (1). Upon selection of candidate neoantigens (see **Figure 1**), different immunological screening methods can be used to evaluate peptide immunogenicity (2). Prior to evaluating their immunogenicity, effector T-cell populations of interest are selected from cancer patient samples (3). These can either be directly used in immunological screening assays or as a starting source for an optional pre-enrichment step to increase the frequency of neoantigen-specific T cells (3b). Finally, the immunogenicity of candidate neoantigens is evaluated using the screening method and effector T-cell population/s of choice (4). IVS, *in vitro* sensitization; APC, antigen-presenting cell; TCR, T-cell receptor.

TILs and Other Tumor-Associated Populations

T cells are thought to accumulate at the tumor site, presumably as a result of local antigen-specific clonal expansion. Consistent with this, the tumor-infiltrating TCR repertoire is typically more oligoclonal as shown by intratumoral TCR deep sequencing (111, 112). It is therefore not surprising that TILs are the preferred T-cell source to detect T cells recognizing neoantigens. The optimization of TIL culture conditions in the late 1980s (113), motivated in part by the therapeutic potential of adoptive cell transfer (ACT), has facilitated the expansion of the relatively small numbers of lymphocytes that can be naturally found infiltrating human tumors. TIL cultures, which are usually expanded from tumor biopsies in the presence of high IL-2 concentrations, have been used to identify immunogenic HLA-I- and HLA-II-restricted neoantigens (7, 25, 29, 51, 111, 114). Despite being the most attractive source in terms of T-cell composition, expansion of TILs is not always successful, it can be highly heterogeneous even when expanding TILs from contiguous tumor fragments, and the generation of these cultures depends on tumor biopsies which are not always available. Furthermore, *in vitro* expansion of TILs can significantly increase or decrease the frequency of antigen-specific T cells (99, 115), thereby underestimating the initial T-cell repertoire. Recent studies have also shown that TILs are composed not only of tumor-reactive but also of cancer-unrelated T cells (e.g., virus-specific T cells) (116, 117). How these bystander cancer-unrelated T cells behave in comparison to tumor-reactive cells during TIL expansion has not been fully determined, although initial studies suggest that *ex vivo* expansion of TILs can increase the frequency of virus-specific T cells at the expense of tumor-reactive T cells (115). Therefore, other T-cell sources have been studied with the aim of complementing TILs for neoantigen validation.

Fluids directly associated with particular solid tumors, such as ascites from ovarian cancer or pleural effusions from mesotheliomas, have been used as sources for the expansion of tumor-associated lymphocytes (TALs). TALs do not fully share TCR repertoires with TILs (118), and they might thus underrepresent the tumor-reactive T-cell population of the primary tumor. Nonetheless, the potential of TALs has been demonstrated in a high-grade serous ovarian cancer patient in which a neoantigen-specific T-cell clone was detected in ascites at the time of recurrence, but not in primary ascites or tumor samples (90). Other body fluids, such as cerebroespinal fluid (CSF), although low or absent in healthy individuals, can be increased in patients with different pathologies (119). Indeed, T cells isolated and expanded from CSF of patients with diffuse intrinsic pontine glioma have been used to detect tumor-reactive T cells after dendritic-cell vaccination (120). Urine has been recently used to isolate and characterize lymphocytes from bladder cancer patients (121). Notably, urine-derived lymphocytes (UDLs) recapitulated the phenotypic and TCR landscapes of T cells from the tumor microenvironment. Given the non-invasive nature of urine collection and the similarities between UDLs and TILs, the former represents an attractive source of T cells to identify immunogenic neoantigens in bladder cancer patients.

PBMCs

One of the major challenges for the identification of neoantigens is finding non-invasive T-cell sources to perform immunological screenings. PBMCs derived from blood extractions represent the most attractive source for this purpose. The first evidence of circulating neoantigen-specific T cells was reported more than 20 years ago. Back then, effector T cells used for reactivity screenings were obtained using a mixed lymphocyte tumor culture (MLTC) (122). After successive rounds of stimulation of PBMCs from cancer patients with irradiated autologous tumor cell lines, they were tested for tumor reactivity, and positive "clones" were obtained. Using MLTC-derived clones,

Wölfel et al. identified an HLA-A2.1-restricted neoepitope derived from CDK4 (86). This strategy has been successfully exploited in other studies, but the requirement of multiple stimulations prompted the development of strategies to detect neoantigen-specific lymphocytes in unmanipulated PBMCs. The first reports of circulating neoantigen-specific T cells detected in bulk PBMCs from cancer patients are from less than a decade ago with the advent of improved multimer staining technologies (51, 99). In one of those studies, Cohen et al. used multimer libraries to screen for candidate neoantigens, resulting in the successful isolation and expansion of neoantigen-specific T cells from the blood of melanoma patients. Subsequent studies have also shown that naïve T cells from healthy donors can be used as a source to identify neoantigens from melanoma patients for which T-cell reactive clones were absent in autologous TILs (123). These studies suggest that blood-derived T cells are attractive populations for identifying immunogenic neoantigens.

Pre-enriched T-Cell Populations

Multimer-based studies have shown that neoantigen-specific T cells are present at relatively low frequencies in fresh tumor single-cell suspensions (98), and this is even more problematic when working with peripheral blood lymphocytes (PBLs) (51, 99). To overcome this challenge, different enrichment strategies have been developed to increase the odds of detecting cells which otherwise would be missed due to limited technical sensitivities. Given the low frequency of neoantigen-specific T cells, enrichment strategies rely on the selection of particular T-cell populations that are then *in vitro* expanded to achieve high cell numbers for immunogenicity screenings (**Figure 2**; **Table 4**).

One of these strategies exploits the fact that, upon recognition of their target antigen on tumor cells, T cells express co-inhibitory and co-stimulatory molecules. Furthermore, chronic exposure to target antigens may differentiate TILs into a dysfunctional (also termed exhausted) state characterized by the co-expression of exhaustion/activation markers (124, 125). This T-cell phenotype has prompted research evaluating whether the expression of these markers could be used to identify and enrich for neoantigen-specific T-cells residing in fresh tumors or peripheral blood of patients with cancer. To date, most of the co-inhibitory/co-stimulatory markers identified to associate with enrichment of tumor- or neoantigen-reactive T cells have been described in TILs from fresh tumor preparations. Initial reports demonstrated that the isolation and expansion of $CD8^+$ melanoma TILs based on either PD-1, or a combination of PD-1 TIM-3 and LAG-3 expression consistently enriched for T cells recognizing tumors and neoantigens (111, 126). Subsequent studies have confirmed that tumor-specific $CD8^+PD\text{-}1^{+/hi}$-infiltrating populations show a distinct transcriptional and metabolic profile (127). Phenotypic characterization of $CD8^+$ TILs from colorectal and lung cancer patients has revealed that CD39, rather than PD-1, could accurately distinguish between tumor-specific ($CD39^+$) and cancer-unrelated T cells ($CD39^-$) (117). In line with this, a recent study has shown that co-expression of CD39 and CD103 favors the identification of tumor-reactive T cells (128). A different approach exploits the fact that T cells express CD137 upon recognition of tumor cells (129). Consequently, isolating T cells based on CD137 expression after co-culture with autologous tumor cells leads to enrichment of neoantigen-specific T cells (130, 131). Identification of markers associated with neoantigen-specific T-cell enrichment in circulating T cells has been more challenging compared to TILs. For instance, expression levels of immune checkpoints in blood-derived T cells is lower than in TILs (111). Additionally, circulating T cells expressing immune checkpoints could result from other pathogen-specific responses. To date, only two reports have used T-cell markers for enrichment of tumor-specific T cells from peripheral blood. In contrast to $CD8^+PD\text{-}1^-$ peripheral blood T cells, sorted $CD8^+PD\text{-}1^+$ cells from melanoma patients contained lymphocytes targeting neoantigens (28). Moreover, neoantigen specificities and TCR repertoires in $CD8^+PD\text{-}1^+$ cells from blood and melanoma tumors were very similar. More recently, isolation of circulating memory T cells based on CD62L and CD45RO expression enabled the identification of neoantigen-specific T cells (108). Enriching T cells based on marker expression is advantageous as no foreknowledge of T cell-specific reactivities or HLA restriction is required, thereby theoretically broadening its application to any patient. However, marker expression is variable among patients. Furthermore, the low frequency of marker-expressing cells demands an additional *in vitro* expansion step after sorting in order to achieve reasonable cell numbers for *in vitro* immunological screening assays, which could change the repertoire compared to the initial population. Although there is no direct evidence of this for marker-sorted cells in humans, mouse antigen-specific T cells among sorted $CD8^+PD\text{-}1^+$ TILs have been shown to decrease in frequency after *in vitro* expansion (132). Despite these challenges, this approach is attractive not only for neoantigen screening but also as a source of T cells for therapeutic applications such as ACT. Open questions regarding this strategy that still need to be addressed include: (i) which marker best recovers most of the neoantigen-specific T-cell repertoire, and (ii) whether the co-expression of multiple markers can improve enrichment based on single-markers.

Other enrichment strategies rely on the detection of the interaction between the TCR and its cognate pHLA complex. Staining of T cells with fluorescently-labeled pHLA multimers allows the simultaneous detection and sorting of pure antigen-specific populations, which can then be interrogated for validation of neoantigens in functional assays. Using this approach, multimer-enriched T cells from either PBMCs or fresh tumor digests have been used for validation of neoantigens derived from solid and hematological malignancies (99, 133, 134). Besides the disadvantages related to pHLA multimers mentioned in section Screening of Predicted or Eluted Minimal Neoepitopes, one that limits multimer-based enrichment of T cells is the fact that positive signals after multimer staining not necessarily determine functional T-cell responses (134–136).

Another frequently used enrichment strategy is *in vitro* sensitization (IVS), which exploits antigen-specific stimulation and expansion to increase the frequency of specific T-cell

TABLE 4 | Strategies to enrich for neoantigen-specific lymphocytes.

Strategy	Advantages	Disadvantages
Surface marker-based selection	Prior knowledge of the specific reactivity or HLA restriction is not required Universal (Can be used for every patient) Increases the frequency of neoantigen-specific lymphocytes	Expression of surface markers varies among patients May not capture all reactivities Does not exclusively select neoantigen-reactive lymphocytes
Multimer staining	Allows isolation of T cells with one specific reactivity with high purity	Requires generation of HLA multimer for each reactivity Limited number of HLA multimers Prior knowledge of the specific reactivity and HLA restriction required Not optimal for isolating tumor-reactive $CD4^+$ T cells Sensitivity limited by the frequency of the neoantigen-specific population
In vitro sensitization	Increases the frequency of T cells with a specific reactivity	Requires multiple rounds of *in vitro* sensitization Requires autologous or HLA matched APCs Laborious depending on the number of peptides screened

APC, antigen-presenting cell; TCR, T-cell receptor; HLA, human leukocyte antigen.

reactivities. The most frequently used approach of IVS involves the co-culture of either PBMCs or TILs, with or without irradiated feeders and a pool of candidate peptides in the presence of cytokine cocktails [usually combinations of interleukin (IL)-2, IL-7, IL-15, and IL-21]. Co-cultures are usually incubated for 10–14 days, after which the resulting T-cell populations can be screened for neoantigen recognition or for subsequent rounds of stimulations. A modified version of this approach involves the stimulation of TILs or PBMCs with autologous APCs electroporated or pulsed with TMGs or peptides (long or minimal epitopes), respectively, under similar culture conditions as the ones mentioned above. Alternatively, if the patient's autologous tumor cell line is available, it can be used instead of APCs for stimulation. These three strategies have proven to be useful for enrichment of neoantigen-specific T cells and for the subsequent validation of candidate neoantigens (86, 108, 137, 138). However, the simultaneous presentation of multiple epitopes during IVS may favor the enrichment of T-cell populations specific for immunodominant peptides, leading to underrepresentation of the true neoantigen-specific T-cell repertoire present in the starting population. To overcome the potentially biased enrichment of T cells and in order to detect the broader repertoire of neoantigen-specific T cells, a more reliable but also more cumbersome approach involves the stimulation of T cells with APCs pulsed with every single predicted minimal epitope for separate (91). It is important to note, however, that this strategy has been limited to tumors with low mutational load, or those whose neoantigen candidate list has been prioritized using *in silico* prediction algorithms.

The methods described in this section have been commonly used as single enrichment strategies. However, the combination of such enrichment strategies (e.g., marker-based selection and IVS) can result in highly enriched populations of neoantigen-specific T cells (108). Furthermore, recent efforts aim at combining enrichment methods, such as IVS with or without CD137-based T-cell selection, with other sensitive technologies such as TCRβ deep sequencing by NGS to screen for neoantigens (91, 139–141).

T-Cell Clones and TCR-Transduced Lymphocytes

The antitumor responses observed upon adoptive transfer of TILs targeting neoantigens has provided rationale to develop personalized T-cell therapies. However, the differentiated status of the administered cells has been associated with limited antitumor activity in mouse models, suggesting that TCR-gene engineered T cells could be more efficacious. This, combined with recent progress in the non-viral delivery of TCRs into PBLs (142), has made personalized neoantigen-specific TCR-gene engineering a true possibility.

The rapid identification of neoantigen-specific TCRs, a pre-requisite for the development of such therapies, can be performed through multiple strategies. First, T-cell clones can be established either from TILs, peripheral blood subsets or enriched populations (as described above) and can be screened for recognition of neoantigens. TCRα and TCRβ sequencing can be carried out from the neoantigen-specific clones to isolate the variable regions of the TCR. These can then be cloned into a vector of choice to transduce or transfect PBLs to express and test the specificity of the TCRs identified. This approach led to the rapid identification of six neoantigen-specific TCRs from two patients, including a high affinity HLA-II-restricted $KRAS_{p.G12D}$-specific TCR in a recent report (30). However, the limited proliferative capacity of some clonotypes may result in a biased representation of the starting TCR repertoire.

A second approach to isolate neoantigen-specific TCRs uses the oligoclonality of specific tumor-resident TCR clonotypes as a surrogate to select for candidate neoantigen-specific TCRs that may have undergone clonal antigen-specific expansion. As exemplified in the work by Pasetto et al. the most frequent TCRβ clonotypes identified by TCRβ deep sequencing were selected as candidate tumor or neoantigen-specific lymphocytes and were paired with the most dominant TCRα sequences, leading to the cloning, expression and immunological testing of a few TCRα-β pairs (112). However, the inefficient allelic exclusion of TCRα chains during somatic recombination in T cells frequently leads to T cells harboring two TCRα sequences and this can hinder construction of the correct pairs. Alternatively, the TCRα

sequence that pairs with the oligoclonal TCRβ clonotype selected can be identified using pairSEQ, a high-throughput strategy combining TCRα and TCRβ sequencing with statistical analysis to infer TCRα-β pairs from bulk PBLs or TILs (143). In addition, single-cell TCRα and TCRβ sequencing of cells directly isolated from the tumor can be carried out either using conventional sanger sequencing (112) or NGS (116), to identify TCRα-β pairs from TILs. Once the sequence of the selected TCRα-β pairs are identified, T cells can be screened for recognition of candidate epitopes in functional assays that require autologous target cells (116). Using this approach, Pasetto et al. generated PBLs expressing 68 TCRα-β pairs derived from melanoma-resident $CD8^+PD\text{-}1^+$ T cells from 10 patients and successfully identified 9 neoantigen-specific TCRs. Furthermore, recently, single-cell transcriptomics has enabled to couple specific TCRα-β sequences to specific differentiation and functional traits. Although this technology has not yet been exploited to isolate neoantigen-specific TCRs from TILs, it could further improve our understanding of the functional and phenotypic traits of TILs and the accuracy of existing biomarkers to select for candidate neoantigen-specific lymphocytes. The major limitation of this approach is the high amount of TCR clonotypes that can be retrieved from all the sequencing data. Hence, high-throughput platforms to gene-engineer and test the specificity of such high number of TCRs is currently a matter of extensive research (144).

CONCLUDING REMARKS

Virtually all cancers harbor genetic alterations, some of which can give rise to mutated, non-self peptides presented by HLA molecules and elicit T-cell responses, referred to as neoantigens. Recent data suggests that neoantigen-specific lymphocytes can be detected in the vast majority of cancer patients, regardless of their tumor mutation burden. Moreover, they appear to have a central role in the clinical activity of cancer immunotherapies. Thus, neoantigens have emerged as promising targets for personalized immunotherapies. However, mounting evidence suggests that only a small fraction of the NSM identified by tumor WES are actually immunogenic. While inherent difficulties can limit neoantigen identification, such as tumor heterogeneity or as a result of *holes* in the TCR repertoire, the success or failure of neoantigen identification is, in great part, determined by the identification of candidate neoantigens and the immunological screening assays required to identify *bona fide* neoantigens, all with their own advantages and disadvantages.

Whilst *in silico* peptide prediction strategies have led to the identification of neoantigens, they can inaccurately predict peptides, and they are not efficiently trained to identify HLA-II neoantigens. Immunopeptidomics can be used to discover novel neoantigens or validate those obtained using *in silico* peptide predictors, but MS based identification of peptides is limited by its current sensitivity and by the fact that some peptides may never be detected using this approach. To date, the safest, albeit, the most laborious, and costly strategy to identify neoantigens requires the unbiased screening of all neoantigens identified using TMGs or PPs, as demonstrated by the growing number of neoantigens identified using this approach in the last five years. This strategy has provided a broader idea of the frequency of neoantigen reactivities in cancer patients and is capable of detecting $CD4^+$ T-cell responses targeting neoantigens, which may be important to develop effective treatments. Moreover, the specific immunological screening method and read-outs selected, as well as the choice of effector population screened can also greatly impact on neoantigen identification.

Thus far, clinical trials testing vaccines targeting neoantigens have demonstrated they are safe and well tolerated, and personalized T-cell based therapies targeting neoantigens have shown antitumor responses in selected cases. However, whether individualized immunotherapies targeting neoantigens can mediate effective antitumor responses in a broader patient population, remains an open question. Despite all the technological innovation and development of novel screening assays, the rapid and precise identification of the *bona fide* neoantigens in any given patient remains a major hurdle that will need to be overcome to translate the potential of neoantigen targeting into effective therapies for patients with cancer.

AUTHOR CONTRIBUTIONS

AG conceived the review. All authors listed made a substantial, direct, and intellectual contribution to the work. Figures were created by AG-G with BioRender.com.

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Induced Pluripotent Stem Cell-Based Cancer Vaccines

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Over a century ago, it was reported that immunization with embryonic/fetal tissue could lead to the rejection of transplanted tumors in animals. Subsequent studies demonstrated that vaccination of embryonic materials in animals induced cellular and humoral immunity against transplantable tumors and carcinogen-induced tumors. Therefore, it has been hypothesized that the shared antigens between tumors and embryonic/fetal tissues (oncofetal antigens) are the key to anti-tumor immune responses in these studies. However, early oncofetal antigen-based cancer vaccines usually utilize xenogeneic or allogeneic embryonic stem cells or tissues, making it difficult to tease apart the anti-tumor immunity elicited by the oncofetal antigens vs. graft-vs.-host responses. Recently, one oncofetal antigen-based cancer vaccine using autologous induced pluripotent stem cells (iPSCs) demonstrated marked prophylactic and therapeutic potential, suggesting critical roles of oncofetal antigens in inducing anti-tumor immunity. In this review, we present an overview of recent studies in the field of oncofetal antigen-based cancer vaccines, including single peptide-based cancer vaccines, embryonic stem cell (ESC)- and iPSC-based whole-cell vaccines, and provide insights on future directions.

Keywords: cancer vaccine, iPSC, ESC, oncofetal antigen, cancer stem cell

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INTRODUCTION

Cancer cells have the capability to proliferate indefinitely and metastasize to different parts of the body. Embryonic stem cells (ESCs) have the ability to undergo rapid clonal proliferation and self-renew, and can inhabit and thrive in various environments of the human body. The similarities between fetal development and cancer have long been recognized (1) following the discovery of oncofetal proteins and antigens such as α-fetoprotein (AFP) (2), carcinoembryonic antigen (CEA) (3), and human chorionic gonadotropic (HCG) (4) (**Supplemental Table 1**). These proteins are tumor associated proteins or antigens (TAA) that are synthesized during embryonic development and appear again in adults during cancer development. Furthermore, these proteins are well-known biomarkers for cancer detection and monitoring (3, 5–9). Induced pluripotent stem cells (iPSCs) can be generated by introducing four transcription factors into adult somatic cells, which transform their transcriptional and epigenetic state to a pluripotent one that closely resembles ESCs (10). Similar to ESCs, iPSCs share genetic and transcriptomic signatures with cancer cells, including protein markers that can be recognized by the immune system (11, 12).

Schöne recognized over a century ago that immunization with embryonic/fetal tissue could lead to the rejection of transplanted tumors in animals (13). Later studies indicated that vaccination of embryonic materials in animals elicited humoral and cellular immunity against transplantable

tumors and carcinogen-induced tumors, supporting the idea that anti-tumor immunity may arise from the antigens shared between fetal tissue and cancer cells. Recent studies provided evidence that oncofetal antigen-based cancer vaccines could elicit potent T cell responses (5–9). However, there are problems associated with utilizing embryonic/fetal materials for the development of anti-cancer vaccines. Ethical issues, tumorigenicity, and alloimmunity have been the main limitations of using ESCs for clinical applications. Therefore, a substitute for ESCs is needed for overcoming these obstacles. A recent study using an irradiated autologous iPSC-based cancer vaccine has started to address these issues (14). Moreover, the use of ESCs/iPSCs alone as an anti-cancer vaccine only showed moderate anti-tumor effects in some of the early studies (13, 15, 16), suggesting that vaccine adjuvants may be needed in combination with ESCs/iPSCs to enhance innate immunity and increase antigen presentation. Here, we summarize and compare recent studies in addressing these challenges.

CANCER CELLS ARE REMARKABLY SIMILAR TO ESCS AND iPSCS

Cancer cells and ESCs share many cellular and molecular features. These include a rapid proliferation rate (17), upregulated activity of telomerase (18), increased expression levels of oncogenes such as c-*MYC* (19) and krupple-like factor 4 (*KLF4*) (20), and similar overall gene expression profiles (21, 22), microRNA signatures (23), and epigenetic status (24). Similar to cancer cells, after long-term culture the ESC lines will continue to proliferate actively and express high levels of telomerase activity, allowing them to maintain telomere length and cellular immortality (18, 25, 26). These features of ESCs resemble the hallmarks of cancer cells that have "sustaining proliferative signaling" and "replicative immortality" (27).

The discovery of iPSCs in 2006 (10, 28) has revolutionized the field of stem cell research. Human iPSCs reprogrammed from a patient's somatic tissues share almost the same gene expression profiles with that patient's ESCs (29–32), providing a possible solution to the ethical objections that have obstructed the use of human ESCs in many countries. Similar to ESCs, iPSCs share genetic and transcriptomic signatures with cancer cells (14). Human iPSCs were first generated by the transduction of fibroblasts with four transcription factors: *OCT4*, *SOX2*, c-*MYC*, and *KLF4* (28). *C-MYC* is a well-known oncogene (33, 34), and the other three factors are also known to be upregulated in multiple cancers types (35–40). Indeed, one study showed significant overexpression of at least one of these factors in 18 of the 40 cancer types that were evaluated (41). Also, these genes are associated with tumor progression and poor prognosis in certain tumor types (41), suggesting that targeting these genes in cancers may be therapeutically beneficial.

A recent study analyzed and compared the epigenomic and transcriptomic signatures of human tumors from The Cancer Genome Atlas (TCGA) and ESCs, as well as iPSCs and other progenitor cells from Progenitor Cell Biology Consortium (PCBC) (42). In this study, the authors applied machine learning algorithms to reveal a positive correlation between tumor dedifferentiation status and stemness indices for most of the tumor cases they analyzed (42). Importantly, they also demonstrated that the cancer stemness indices are higher in recurrent and metastatic tumors than primary tumors, supporting the concept that cancer stem cells play essential roles in cancer recurrence and metastasis (43, 44). In addition, using single-cell transcriptome analysis the authors identified a heterogeneous expression of stemness-associated markers in patient tumors, suggesting the need for multi-target strategies when targeting cancer stem cells.

IMMUNOGENICITY OF ESCS AND iPSCS

Embryonic stem cells are usually obtained from an unrelated donor due to their limited availability. Therefore, these cells often express mismatched major histocompatibility complex (MHC) and/or minor histocompatibility (miH) antigens and will trigger alloimmune responses when transplanted in the host. ESCs express low levels of HLA class I molecules (45) and almost undetectable levels of HLA class II and costimulatory molecules (46). Although expressed at a low level, HLA class I molecules in ESCs are sufficient to trigger xenorejection of human ESCs mediated by cytotoxic T cells (47, 48). ESCs induce potent humoral and cellular immune responses, leading to the infiltration of inflammatory cells that is followed by ESC rejection (49). So far, most immunogenicity studies of ESCs have focused on a scenario that involves MHC mismatches, implicating alloimmunity as one of the main players in the immune responses after ESCs transplantation. However, whether embryonic antigens in ESCs could induce an immune response is less clear.

Induced pluripotent stem cells are somatic cells that were reprogramed back to a pluripotent state. Autologous iPSCs can be generated from the person receiving therapy. Since the initial discovery of iPSCs, researchers immediately assumed that these cells would be a potential cell source of autologous cell-based therapies to bypass the issues of alloimmunity caused by allogeneic sources such as human ESCs or donated tissue (50, 51). However, later studies investigating iPSC immunogenicity in autologous settings raised questions about this assumption. Araki et al. (52) showed that autologous iPSC-derived teratomas were rejected by immune-competent mice and found a comparable level of rejection of autologous ESC-derived teratomas. These data suggest that in autologous transplantation models with minimized alloimmunity, other antigens such as embryonic antigens in ESCs and iPSCs could still induce an immune response. In 2014, we noticed that autologous iPSCs are immunogenic (11), contradicting earlier studies claiming they are immune privileged. We showed in murine models that undifferentiated autologous iPSCs elicited an immune response with increased lymphocytic infiltration and elevated granzyme-B, IFN-γ, and perforin intragraft. In contrast, autologous iPSC-derived endothelial cells were accepted by immune mechanisms similar to self-tolerance. These studies suggest that undifferentiated autologous iPSCs may express

antigens of embryonic origin that can trigger an immune response, whereas fully differentiated cells derived from iPSCs have lower levels of immunogenicity. Based on these data and the similarity between iPSCs and cancer cells, we reached the conclusion that undifferentiated iPSCs are immunogenic and hypothesized that they can be used as a cancer vaccine.

ONCOFETAL PEPTIDE VACCINES AND WHOLE-CELL VACCINES

Oncofetal Peptide-Based Vaccines

A wide range of vaccines based on the aforementioned oncofetal antigens have been tested in pre-clinical studies, and some single antigen vaccines have been tested in clinical trials. Among all oncofetal antigens, many well-studied ones belong to a class of proteins called cancer testis antigens (CTAs) (**Supplemental Table 1**). CTAs are expressed within the immune-privileged environment of the testes as well as by tumor cells. Targeting CTAs can induce highly tumor-specific immune responses and thus provide an ideal strategy for anti-cancer vaccines. For example, a series of clinical trials have evaluated the CTA melanoma-specific antigen A3 (MAGE-A3) as a cancer vaccine target. MAGE-A3 is highly expressed in many different tumor types (53, 54). An early phase clinical trial demonstrated that adjuvant-mixed, recombinant MAGE-A3 proteins or peptide vaccines could elicit potent anti-tumor T cell and antibody responses which are associated with objective responses (54). However, a phase III trial in non-small-cell lung carcinoma (NSCLC) evaluating MAGE-A3 as an adjuvant treatment demonstrated no significant improvement in disease-free survival compared with placebo in MAGE-A3-positive patients. So far, no further clinical trials testing the MAGE-A3 targeting immunotherapies in NSCLC have been approved based on these results (55).

Another example of a single-peptide-antigen vaccine in clinical trial targeting glypican-3 taught us a similar lesson (56). In this phase II clinical trial, the investigators observed that two patients had tumor relapse despite significant numbers of vaccine-induced peptide-specific CTLs in their blood. Interestingly, they found that although glypican-3 was expressed in the primary tumor, the recurrent tumors lost the antigen expression. The investigators concluded that "the peptide vaccine may eradicate tumor cells that express such antigen, [and] cancer cells that do not express or lose the same antigen may then proliferate. In such cases, vaccines that target multiple shared antigens would be effective."

Upon learning the lessons from failed early clinical trials using single-peptide cancer vaccines, later clinical trials evaluating peptide antigen-based cancer vaccines have focused mostly on multiple-peptide and antigens and/or are administered in combination with immunostimulatory adjuvants and other targeted therapies (57).

These results indicate that targeting one antigen alone may not be able to generate a sufficiently effective and durable anti-tumor immune response to mediate tumor rejection because of tumor heterogeneity and the rapid appearance of escape mutants. Therefore, it has been suggested that strategies that could target multiple tumor-associated antigens at once would induce a broader spectrum of anti-tumor immunity and possibly provide more effective and durable protection against cancer.

ESC-Based Whole-Cell Cancer Vaccines

Since the establishment and characterization of human ESC lines, researchers have attempted to evaluate ESC-based whole-cell cancer vaccines due to their ability to deliver multiple oncofetal antigens in one treatment. In addition, unlike defined antigen-based vaccines, the whole-cell vaccine is universally applicable to all patients regardless of their HLA type. Li et al. found that human ESCs were able to induce a moderate anti-tumor effect (16). Both humoral and cellular immunity were activated by H9 ESC line, as evidenced by the production of colon carcinoma cell line-specific antibodies and IFNγ-producing cells, respectively. It was speculated that oncofetal antigens shared by the ESCs and tumors might have contributed to the vaccine-induced anti-tumor response. However, these immune responses were induced by a xenogeneic human ESC line injected into mice, and it is very likely that the incompatibility of the MHC antigens between the human ESCs and mouse cells contributed to a large portion of the immune responses. Furthermore, the anti-tumor effects produced by the xenogeneic ESC-vaccine were not as potent as those induced by immunization with the syngeneic murine colon cancer cells. A similar approach using xenogeneic human ESCs as a cancer prevention vaccine was evaluated by Zhang et al. (58) in an ovarian cancer model in rats, and a moderate tumor prevention effect was observed in this study.

These results raise the question of whether allogeneic or autologous ESCs are better than xenogeneic ESCs as an anti-cancer vaccine. A later study by Dong et al. (59) evaluated an allogeneic ESC cancer vaccine in mice. They investigated the ESC vaccine both as a prophylactic vaccine and as a therapeutic treatment in a transplantable lung cancer model by showing it could inhibit tumor growth in mice by enhancing lymphocyte proliferation and cytokine secretion, suggesting the potential of utilizing allogeneic ESC vaccines as a therapeutic strategy. However, they observed a stronger tumor inhibitory effect in the prophylactic group compared with the therapeutic group, which may be due to the immunosuppressive environment in established tumors.

To test the prophylactic ESC cancer vaccine in a physiologically relevant setting, Yaddanapudi et al. (60) employed a spontaneous mouse tumor model. Allogenic ESCs along with GM-CSF were used to provide immunostimulatory adjuvant activity. GM-CSF can stimulate and activate antigen-presenting cells (APCs), which can process and present tumor antigens to CD4+ helper T cells and CD8+ cytotoxic T lymphocytes (CTL) (61, 62). The authors observed more potent and durable protection against tumor growth than that found in earlier studies using ESCs alone, corroborating the immunostimulatory effects of the GM-CSF in the cancer vaccine. Moreover, this combinatory vaccination could inhibit carcinogen and chronic pulmonary inflammation induced lung cancer, which is a physiologically relevant spontaneous lung cancer model in mice.

iPSC-Based Whole-Cell Cancer Vaccines

Embryonic stem cells and iPSCs share nearly identical gene expression and epigenetic profiles (29–32). Based on the similarities between cancer cells and ESCs, Li et al. (16) evaluated one human iPSC line TZ1 as an anti-cancer vaccine in a transplantable mouse colon cancer model. They found that although these iPSCs induced significant numbers of IFNγ- and IL-4-producing splenocytes against the mouse colon cancer cells, no evidence of tumor rejection was seen, possibly due to the accumulation of myeloid-derived suppressor cells in TZ1-immunized groups. These data suggest that modifications of the iPSC-based cancer vaccine are needed to increase the immune response against tumors. For example, autologous iPSCs may contain a more representative and accurate panel of tumor antigens than xenogeneic iPSCs, and therefore, autologous iPSCs may be better than xenogeneic iPSCs as anti-cancer vaccines, pending further confirmatory studies. In addition, an immunostimulatory vaccine adjuvant may enhance the anti-tumor immunity of the iPSC-based vaccines.

Embryonic/fetal materials or ESCs often come from unrelated donors and may express mismatched MHC that could trigger an immune response. To study the immunogenicity of oncofetal proteins, alloimmunity stimulated by MHC mismatches will need to be eliminated. In addition, tumorigenicity associated with ESCs has been one of the major obstacles in using ESCs as cancer vaccines for clinical applications. Recently, a study by our lab (14) addressed these issues using an irradiated autologous iPSC-based cancer vaccine. In this study, we first demonstrated that human and murine iPSCs express a list of tumor-associated and tumor-specific antigens by comparing expression profiles of 11 different human iPSC clones with human ESCs, cancer tissues, and healthy tissues using RNA sequencing. We showed that human iPSCs cluster with human ESCs and the cancer tissues, revealing significant gene expression overlap in cancer genes among different cancer types and iPSCs. To evaluate whether the oncofetal antigens in iPSCs rather than MHC mismatches could induce immune responses, we minimized alloimmunity by utilizing autologous iPSCs as the source of the anti-cancer vaccine. To enhance the anti-tumor immunity induced by the vaccine, we included an immunostimulatory adjuvant, CpG oligodeoxynucleotide, a toll-like receptor 9 (TLR 9) agonist that can induce the maturation of APCs (**Figure 1**). We then irradiated iPSCs before vaccination to prevent teratoma formation, as studies have shown that gamma irradiation could inhibit the tumorigenicity of iPSCs (63, 64). We irradiated iPSCs at 60 Gy, which is a lethal dose to human iPSCs *in vitro* and known to significantly decrease teratoma formation ability of human iPSCs in mice (63, 64). We generated autologous iPSCs by introducing Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) into mouse fibroblasts from the same mouse strain. Vaccinations with irradiated iPSCs mixed with the immunostimulatory CpG were administered weekly for a month, inducing antibodies that bound to iPSCs and tumor cells. Vaccination with iPSC-based cancer vaccine also induced CD4+ and CD8+ T cells that could recognize tumor cells *in vitro*, suggesting the induced immune responses are tumor specific. Vaccination increased APCs and activated T cells in mice, resulting in a favorable ratio of CD8+ T cells over CD4+CD25+FoxP3+ regulatory T cells (T-regs). As a result, vaccinated mice rejected transplanted breast cancer, melanoma, and mesothelioma tumor cells, indicating that the stimulated immune activity was tumor-specific and functional. Importantly, adoptive transfer of T cells isolated from vaccine-treated tumors could transfer this tumor protection to naïve mice, proving that the tumor protection effect was mediated by T cells (**Figure 1**).

Because preventive treatment of cancer is clinically uncommon for non-viral associated cancers, we also investigated the therapeutic effects of the iPSC-vaccine in established tumors. Here, the vaccination with iPSC vaccine did not stop the growth of established melanomas, which may be due to the established immunosuppressive tumor microenvironment. We then examined a clinically relevant scenario involving the surgical removal of the majority of tumors but left some residual tumor remains at the margins; we found that the iPSC + CpG vaccine could inhibit tumor relapse. These data are consistent with the finding that cancer stemness features are more highly expressed in recurrent tumors (42).

Because adult stem cells are also present, although rare, in some adult organs such as skin, liver, bone marrow, and digestive system (65), we evaluated auto-immunity by monitoring the animal body weight, organ histology, and antinuclear antibody levels. All of these measurements were normal, suggesting the absence of gross toxicity and autoimmunity in vaccinated mice. The iPSC vaccine could break the self-tolerance of the immune system to oncofetal antigens yet did not induce significant auto-immunity, which was possibly due to the higher abundance of these oncofetal antigens in tumors than in resident stem cells within organs. Taken together, our data support further assessing the value of iPSC-based whole-cell therapy as an anti-cancer immunotherapy.

CONCLUDING REMARKS

Oncofetal antigen-based cancer vaccines have demonstrated therapeutic potential in preclinical and some clinical studies. As presented by several examples in this review, various oncofetal antigen-based vaccine strategies, particularly approaches that combine an autologous iPSC vaccine with an immune adjuvant, have demonstrated great promise to elicit potent anti-tumor responses for cancer treatment. Despite these advances, challenges remain. For instance, many early clinical studies using oncofetal antigen-based vaccines focused on single oncofetal antigens with or without immune adjuvants, limiting the level, and duration of the induced anti-tumor immune response due to tumor heterogenicity and fast adaptation of cancer cells. Unlike the defined antigen-based vaccines, whole-cell vaccines are universally applicable to all patients without concerns on HLA type mismatches. Therefore, whole cell-based cancer vaccines, with the epitope heterogeneity of wholes cells of ESCs and iPSCs, may prove more potent, durable, and easier to apply than single-antigen targeted vaccines.

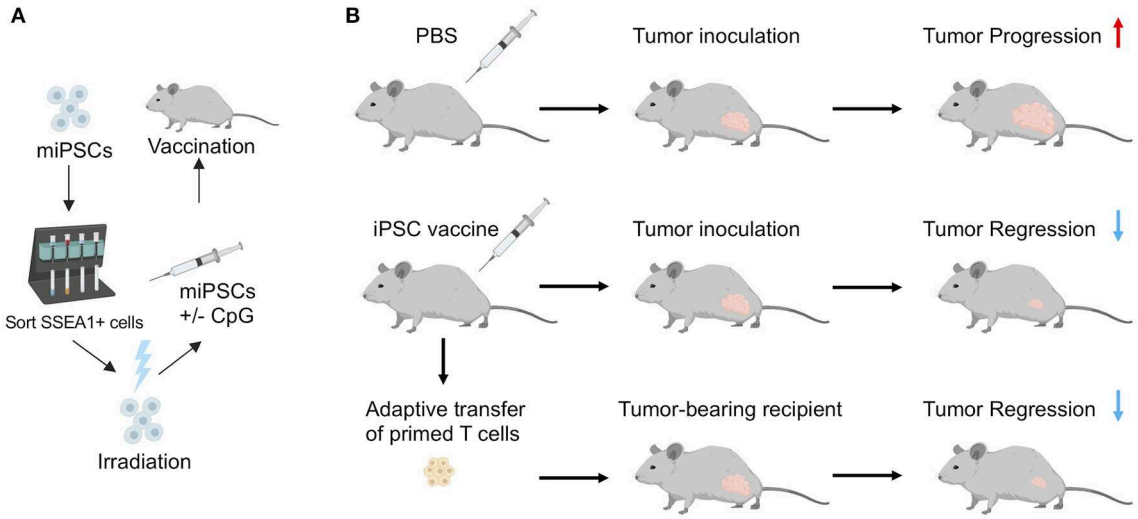

FIGURE 1 | (A) Schematic illustration of vaccine preparation consisting of sorting murine iPSCs for a pluripotent marker, irradiation, resuspension in adjuvant solution (CpG), and subcutaneous injection in mice. **(B)** In a prophylactic setting, autologous iPSC vaccines prevent tumor growth in syngeneic murine models. Adoptive transfer of T cells isolated from vaccine-treated mice inhibited tumor growth in unvaccinated tumor-bearing recipients, indicating that the iPSC vaccine promotes an antigen-specific anti-tumor T cell response. Adapted from Kooreman et al. (14) with permission from Elsevier.

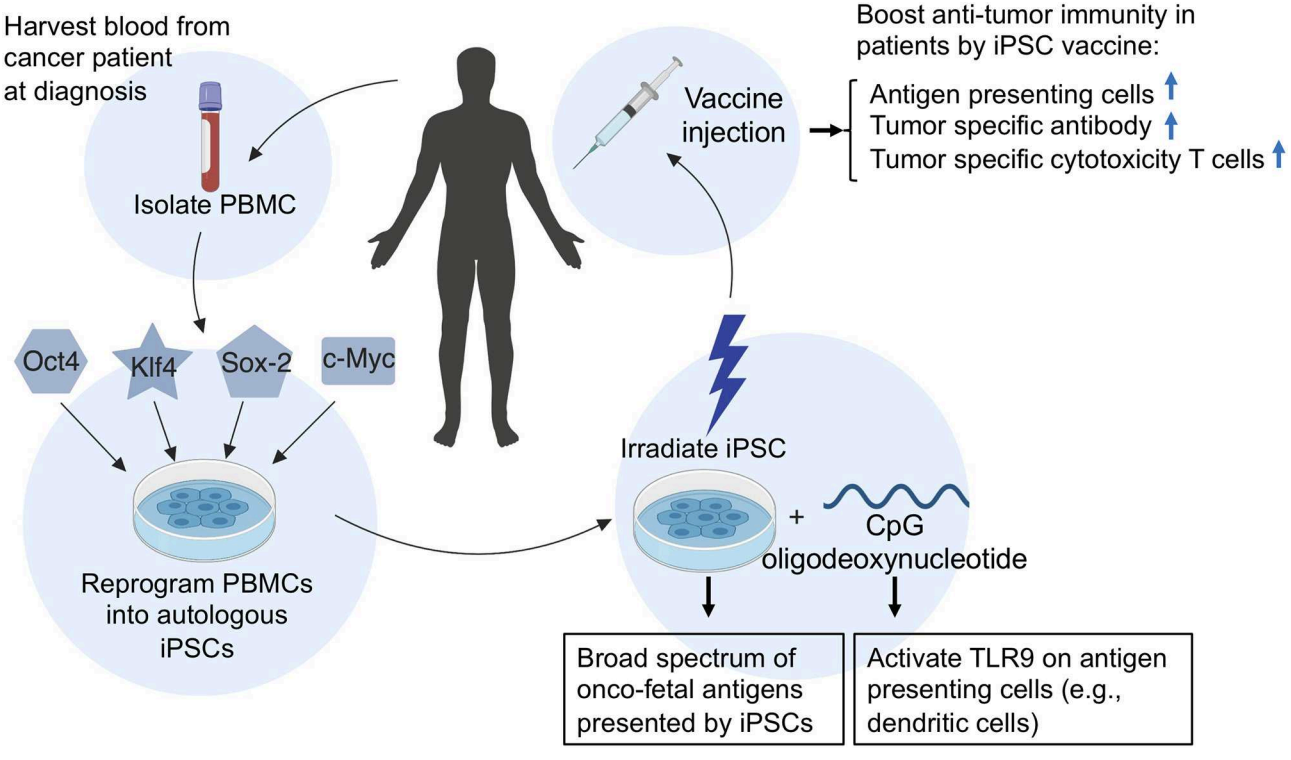

FIGURE 2 | A schematic illustration of the generation and application of an autologous iPSC-based cancer vaccine in patients. To generate an autologous iPSC-based cancer vaccine, peripheral blood mononuclear cells (PBMCs) are isolated from the patient's blood and reprogrammed into induced pluripotent stem cells (iPSCs) by the introduction of four Yamanaka factors (Oct4, Klf4, Sox2, and c-Myc). The resulting patient-derived iPSCs are then irradiated and prepared in combination with CpG oligodeoxynucleotides and injected into patients as an anti-cancer vaccine. Upon vaccination of iPSC-autologous vaccine in patients, the irradiated iPSCs will provide a broad spectrum of oncofetal antigens, while CpGs will activate toll-like receptor 9 (TLR9) on antigen-presenting cells such as dendritic cells, B cells, and macrophages, which can process and present oncofetal antigens to helper T cells and cytotoxic T lymphocytes, thus conferring anti-tumor immunity.

The only FDA approved non-antiviral cancer vaccine, Sipuleucel-T (Provenge), was developed as a TAA pulsed autologous dendritic cell-based cancer vaccination for prostate cancer (66). In 2010, it was approved as an autologous whole-cell cancer vaccine that utilizes a TAA and GM-CSF fusion protein pulsed autologous peripheral blood mononuclear cells (PBMCs). It prolonged patient survival rate by 50% at 3 years in a phase III study, thus has been approved for treating patients with castration -resistant metastatic prostate cancer (67), supporting the efficacy of TAA-based cancer vaccine and the feasibility of using autologous whole-cell cancer vaccine in clinical settings.

In addition, because autologous iPSC-based cancer vaccines are relatively easy to generate (**Figure 2**), iPSC vaccines can be made available at short notice after a diagnosis, ready to be dispensed soon after surgery, chemotherapy, or radiation therapy when cancer cells are most vulnerable. Vaccination of iPSC-vaccines at this time could prime the immune system to target a broad spectrum of cancer-specific antigens to prevent recurrence of cancer, because recurrent and metastatic tumors have a higher level of stemness phenotype (42).

Concerns such as teratoma formation and auto-immunity must be addressed in evaluating the use of iPSC-based cancer vaccines in humans. Although the iPSC-based cancer vaccine did not induce significant auto-immunity in mice and injection of irradiated miPSCs did not result in teratoma formation in mice (14), differences in mouse and human iPSCs and immune systems should be carefully considered before moving this treatment to the clinical settings.

Approaches to further enhance the efficacy of iPSC-based cancer vaccines include concurrent treatment with PD-1/CTLA-4 checkpoint inhibitors, chemotherapy, or radiation therapy. Additional approaches include immunostimulatory agents that can more potently activate APCs, including agonistic CD40 monoclonal antibodies and other TLR agonists such as PolyI:C. These approaches offer powerful combination therapies with possible synergistic effects that may be more effective in patients who have a high risk of disease recurrence after receiving initial standard-of-care therapy.

AUTHOR CONTRIBUTIONS

XO wrote the manuscript. XO, MT, and JW revised the manuscript and provided the critical input.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01510/full#supplementary-material

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REVIEW
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CIITA-Driven MHC Class II Expressing Tumor Cells as Antigen Presenting Cell Performers: Toward the Construction of an Optimal Anti-tumor Vaccine

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Construction of an optimal vaccine against tumors relies on the availability of appropriate tumor-specific antigens capable to stimulate CD4+ T helper cells (TH) and CD8+ cytolytic T cells (CTL). CTL are considered the major effectors of the anti-tumor adaptive immune response as they recognize antigens presented on MHC class I (MHC-I) molecules usually expressed in all cells and thus also in tumors. However, attempts to translate in clinics vaccination protocols based only on tumor-specific MHC-I-bound peptides have resulted in very limited, if any, success. We believe failure was mostly due to inadequate triggering of the TH arm of adaptive immunity, as TH cells are necessary to trigger and maintain the proliferation of all the immune effector cells required to eliminate tumor cells. In this review, we focus on a novel strategy of anti-tumor vaccination established in our laboratory and based on the persistent expression of MHC class II (MHC-II) molecules in tumor cells. MHC-II are the restricting elements of TH recognition. They are usually not expressed in solid tumors. By genetically modifying tumor cells of distinct histological origin with the MHC-II transactivator CIITA, the physiological controller of MHC-II gene expression discovered in our laboratory, stable expression of all MHC class II genes was obtained. This resulted in tumor rejection or strong retardation of tumor growth *in vivo* in mice, mediated primarily by tumor-specific TH cells as assessed by both depletion and adoptive cell transfer experiments. Importantly these findings led us to apply this methodology to human settings for the purification of MHC-II-bound tumor specific peptides directly from tumor cells, specifically from hepatocarcinomas, and the construction of a multi-peptide (MHC-II and MHC-I specific) immunotherapeutic vaccine. Additionally, our approach unveiled a noticeable exception to the dogma that dendritic cells are the sole professional antigen presenting cells (APC) capable to prime naïve TH cells, because CIITA-dependent MHC-II expressing tumor cells could also perform this function. Thus, our approach has served not only to select the most appropriate tumor specific peptides to activate the key lymphocytes triggering the anti-tumor effector functions but also to increase our knowledge of intimate mechanisms governing basic immunological processes.

Keywords: MHC-II, CIITA, CD4+ TH cells, APC, tumor vaccines

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INTRODUCTION

In recent years, tumor immunology has witnessed a dramatic development mostly due to the possibility of applying the acquired knowledge in the field to the development of concrete and realistic approaches to fight cancer. The interest of many investigators has been concentrated mainly on ways to activate and maintain those effector cells of adaptive immunity that are believed be the major actors in eliminating the tumor cells, the CD8+ cytolytic T cells (CTL). This was justified by the fact that CTL recognize directly the tumor cells *via* their specific receptors (TcR) directed against "tumor antigens" [here defined as peptides derived from both overexpressed or mutated (neoantigens) proteins in the tumor], presented by MHC class I (MHC-I) molecules of tumor cells (1). At the variance with MHC class II (MHC-II) molecules that are constitutively expressed only in few cell types (2), MHC-I molecules are expressed, with few exceptions, in all cell types including tumor cells (3). Moreover, the intracellular pathway through which MHC-I molecules are loaded with peptides favors the binding of peptides from endogenously synthesized proteins (4, 5), as potential tumor antigens are. Unfortunately, CTL suffers of important extrinsic and intrinsic limitations in the fight against tumors. Often the tumor cells down-regulate their MHC-I expression to elude recognition by the CTL (3, 6–8); moreover tumor cells secrete in the tumor microenvironment suppressive mediators that limit the functional activity of CTL (9). Finally, and importantly, maturation, proliferation and functional activity of CTL require the continuous support of CD4+ T cells (T helper cells or TH) and this makes TH cells the master officers and the regulators of all adaptive immune responses (10, 11). Thus, the efficacy of the adaptive immune response against the tumor is strongly conditioned by the initial priming and activation of TH cells. To become fully active, TH cells must recognize antigens, including tumor antigens, via their TcR that interact with the antigen only if it is presented within the context of MHC-II molecules expressed on the surface of professional antigen presenting cells (APC), mainly dendritic cells (DC) and macrophages. At variance with MHC-I, loading of peptides on MHC-II molecules preferentially takes place in endosomal compartments (4), rich of degraded products from endocytosed external materials. Hence, it is believed that MHC-II molecules cannot present peptides derived from the processing of endogenously synthesized molecules. As mentioned above, due to their relatively restricted tissue distribution MHC-II molecules are not expressed on the majority of tumor cell types. For all these reasons, tumor cells would be prevented to stimulate TH cells and consequently to initiate the cascade of event leading to anti-tumor effector functions. The inability of tumor cells to trigger TH cells has contributed to substantiate the immunological dogma, verified for a wide variety of antigens, including pathogens, that tumor antigens could trigger the response of TH only if endocytosed, processed and presented by professional APC (12, 13). However, while for pathogens the mechanism of phago-endocytosis, digestion, processing, and presentation on the MHC-II molecules by professional APC is part of the normal physiology to eliminate the non-self external aggressors, the same is not true for tumor cells as in general these cells are not phagocytosed and degraded by APC. Thus, processing and presentation of putative immunogenic tumor antigens is strongly limited to tumor cell debris and possibly secreted tumor cell products that APC can capture in the tumor microenvironment. It is clear that in this condition the potential repertoire of tumor antigens that professional APC can process and expose via their MHC class II molecules is relatively limited both in quality and in quantity.

RE-ORIENTING THE FOCUS

On the basis of the above considerations, it was not so surprising that attempts to translate to clinics vaccination protocols based only on tumor-specific MHC-I-bound peptides resulted in very limited, if any, success (7). In our opinion the failure of this vaccination attempts was mostly due to inadequate triggering of the TH arm of adaptive immunity. In this review, we focus on a novel strategy of anti-tumor vaccination established in our laboratory and based on the persistent expression of MHC-II molecules in tumor cells. Our approach started by asking a relatively naïve question: *should tumor cells have the possibility to express in a "physiological way" MHC-II molecules, would they be capable to process and present putative tumor antigens, and would they even have the capacity to trigger naïve CD4+ TH cells specific for tumor?*

CANONICAL MHC CLASS II EXPRESSION IN TUMOR CELLS CAN RESULT IN TRIGGERING OF PROTECTIVE ANTI-TUMOR IMMUNE RESPONSE *IN VIVO*

Although, MHC-II molecules can present preferentially peptides originated from protein processing in endosomal compartments and thus derived from exogenously endocytosed material, endogenous proteins could also access the MHC-II pathway of antigen presentation, as demonstrated by previous important studies (14–16) and peptides of these proteins could be recognized and serve as immunogens for TH cell triggering (17, 18). On this ground, we hypothesized that tumor cells, modified to express MHC-II molecules in an appropriate way, could present their own tumor antigens in a MHC-II-restricted fashion to tumor-specific TH cells.

As mentioned above, normally, tumor cells do not express MHC-II genes constitutively because this expression is developmentally regulated and restricted to few cell types. Nevertheless, a vast array of cell types can transiently express MHC class II genes after induction with immune cytokines, particularly IFNγ (19). Both constitutive and inducible MHC class II gene expression are under the control of the MHC class II transcriptional activator encoded by the AIR-1 locus discovered in our laboratory (20–23) and also designated CIITA (24). CIITA regulates also the expression of other fundamental genes necessary for MHC-II transport to endosomal compartments

and loading of peptides, including the invariant chain (In chain) and DM (25–28). When experiments were performed to stably express CIITA in both human and mouse tumor cells, we could demonstrate the constitutive expression of MHC-II genes and corresponding molecules and, importantly, the acquisition of antigen processing and presentation to primed TH cells (29). These findings were the ground to verify *in vivo* the hypothesis that MHC-II positive tumor cells could be specifically recognized by the host immune system and establish a protective immune response. Indeed, we could demonstrate that CIITA-transfected tumor cells of distinct histological origin can be efficiently rejected or strongly retarded in their growth when injected into immunocompetent syngeneic mice (30, 31). Importantly, capacity to reject the tumors and/or strongly retard their growth was directly related to the amount of CIITA-driven MHC class II molecules expressed on the cancer cell surface (30–32). Furthermore, it was shown that CIITA-tumor vaccinated mice develop an anamnestic response not only against the CIITA-transfected tumor but, most importantly, against the parental tumor leading to a very efficient rejection of the parental tumor as well. The expression of MHC class II molecules driven by CIITA was an obligatory requirement to induce the anamnestic protective response against the parental tumor, and this received confirmation also by experiments using as a vaccine non-replicating CIITA-transfected tumor cells (33).

Careful analysis of the mechanisms of protection highlighted several crucial aspects. First, enduring immunity was generated in CIITA-tumor vaccinated as shown by the fact that these mice remained immune from further challenge with parental tumor cells for many months. Moreover, anti-tumor effector mechanisms were specifically mediated by CD4+ TH cells and CTL, since elimination of these cell subpopulation *in vivo* by injecting anti-CD4 or anti-CD8 specific antibodies, abrogated the capacity of the animals to generate protective immunity after administration of CIITA-tumor cells. On the other hand, elimination of B cells or NK cells did not affect the capacity of the animals to reject CIITA-tumor cells. Finally, the crucial importance of CD4+ TH cells as key players in the generation of protective anti-tumor immunity was substantiated by adoptive cell transfer experiments of CD4+ cells from vaccinated mice into naïve recipients and consequent acquisition of protection from tumor growth when challenged with parental tumor cells.

Cumulatively, these findings demonstrated the that the expression of MHC class II molecules driven by CIITA in tumor cells was key in triggering an adaptive and protective immunity.

These results were at variance with respect to those obtained by the group of Ostrand-Rosenberg and colleagues, who studied the function of MHC class II expression in tumors by focusing however mostly on a single tumor model, the H-2^K SaI sarcoma, and on MHC class II alpha-beta transfected genes, in absence of invariant chain, reaching the conclusion that class II-transfected cells could be better rejected as compared to CIITA-transfected cells (34, 35). We have extensively discussed in a previous publication (36) the immunological constraints and limitations of this approach and the consequent biological conclusions, due mostly to the fact that MHC class II molecules are highly unstable in absence of invariant chain and therefore they can hardly go to the cell surface end present antigenic peptides for appropriate recognition by CD4+ T cells (6, 37).

THE TUMOR MICROENVIRONMENT SWITCH IN CIITA-TUMOR VACCINATED MICE

The comparative study of the tumor microenvironment and tumor draining lymph nodes of animals injected with parental tumor or CIITA-tumor cells gave additional and crucial hints for understanding the mechanism through which CIITA-tumor cells triggered a protective immune response (32). Little infiltration composed mostly by macrophages and neutrophils, and virtually no CD4+ T cells, CD8+ T cells, and DC was observed in tumors derived from parental cells. In contrast a rapid infiltration of CD4+ T cells, followed by DC and CD8+ T cells was observed at the tumor site when mice were injected with CIITA-tumors. Interestingly the CIITA-tumor microenvironment was characterized by extensive areas of tumor cell necrosis. Furthermore, in CIITA-tumor vaccinated mice challenged with parental tumors, the number of infiltrating lymphocytes and the extension of necrotic tissue were clearly larger than those found in naïve mice injected with CIITA-tumor cells (31).

The above histological aspect in parental tumor-injected vs. CIITA-tumor injected mice was indeed representative of what is generally described as a "non-inflamed or cold" vs. an "inflamed or hot" tumor microenvironment, respectively (38, 39). Thus, forcing the "physiological" expression of MHC-II molecules by transfecting CIITA into tumor cells resulted in a dramatic modification of the tumor microenvironment which was associated to specific tumor rejection and/or strong retardation of tumor growth (**Figure 1**). Within this frame it is tempting to speculate that in spontaneous tumors characterized by an inflamed microenvironment, tumor infiltrating CD4+ as well as CD8+ T cells by actively secreting IFNγ may transiently induce CIITA expression and consequently MHC class II gene expression in naïve tumor cells resulting in further recognition and killing of the tumor. Additionally, tumor-draining lymph nodes of mice vaccinated with CIITA-tumor cells showed a more polarized TH1-type phenotype with respect to a rather polarized TH2-type phenotype observed in similar lymph nodes of mice injected with parental tumor cells. It should be underlined the strong anti-tumor T cell immunity was not accompanied by manifestations of autoimmunity, suggesting tumor-specific and not self-antigens were the target of the observed anti-tumor response. The subversion of the tumor microenvironment affected also the number CD4+/CD25+ regulatory T cells (Tregs) in draining lymp nodes. It is generally accepted that Tregs play an important role in regulating the activity of CD4+ TH cells. In tumor-bearing, hosts is often observed an increase in number and corresponding function of Tregs (40). In our tumor model, we found an increase in draining lymph nodes of parental tumor-bearing mice not paralleled, however, by a functional increase in suppressive function *in vitro* and *in vivo* (41). On

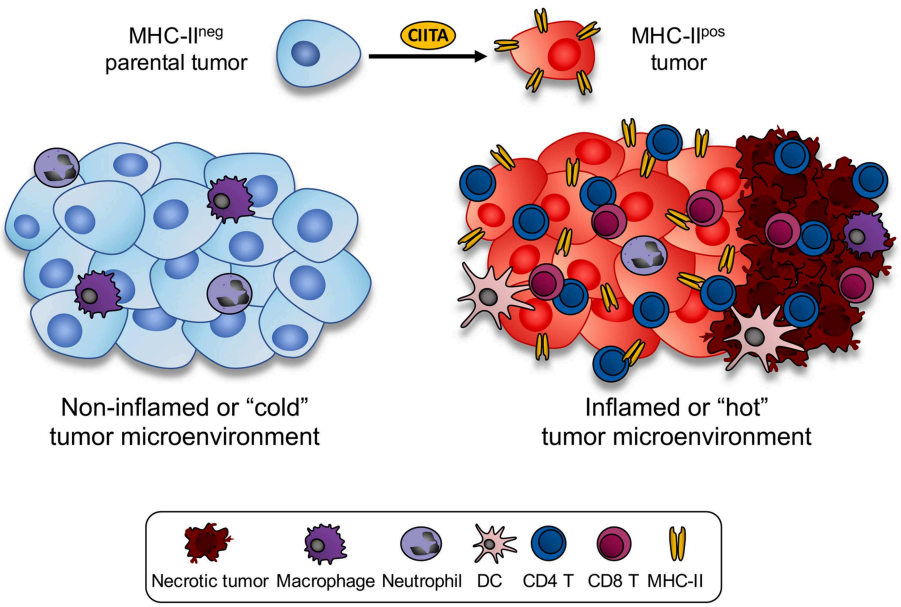

FIGURE 1 | Expression of CIITA-driven MHC-II expression in tumor cells drastically modify the histology of the tumor microenvironment. MHC class II negative mouse tumors of distinct histologic origin and H-2 genotype (left side) are very little infiltrated by blood-derived cells (cold or non-inflamed tumor). In the mouse tumor models analyzed in our studies, scarce infiltration of neutrophils and monocyte-macrophages was detected *in vivo* in the microenvironment of parental tumors. Upon stable transfection with CIITA and consequent expression of MHC class II molecule, tumors became rapidly infiltrated by CD4+ T cells, followed by CD8+ T cells and only later by dendritic cells and macrophages (inflamed or hot tumor). As result of the intense lymphocyte infiltration, large areas of tumor necrosis were generated. Thus, the tumor microenvironment was drastically modified by the CIITA-driven MHC class II expression in the tumor cells.

the other hand in CIITA-tumor vaccinated mice, the number of Tregs was clearly reduced and comparable to the number of naïve animals (33). This led us to conclude that vaccination with CIITA-tumor cells affected also a crucial component of the regulatory circuit, the Tregs, by preventing their increase in number in the tumor microenvironment and in so doing facilitating the triggering and persistence of anti-tumor CD4+ TH cells (36).

CIITA-DRIVEN MHC-II EXPRESSING TUMOR CELLS ARE THE MAJOR APC *IN VIVO*

Cumulatively, the above described studies clearly demonstrated that CIITA-driven MHC-II expressing tumor cells are strongly recognized *in vivo* and trigger tumor specific CD4+ TH cell responses that are protective against subsequent rechallenge with parental tumors. Nevertheless, they did not formally prove that CIITA-tumor cell could function as classical APC in triggering the priming of naïve tumor antigen-specific TH cells. The possibility remained that priming of naïve TH cells could be still mediated by professional APC capturing of MHC-II-peptide complexes derived from dying CIITA-tumor cells or from cellular debris.

The final demonstration that CIITA-mediated MHC-II expressing tumor cells could indeed function as classical APC came recently by using a transgenic mouse model, in which professional APC can be transiently deleted. These transgenic C57BL/6 H-2^b mice, designated CD11c.DTR, carry the diphteria toxin receptor under the control of the CD11c promoter, which is strongly expressed in DC. Thus, in these animals dendritic cells can be conditionally deleted by administration of diphteria toxin (42). Two highly tumorigenic MHC-II-negative C57BL/6 H-2^b tumor cell lines, MC38 colon carcinoma and LLC Lewis lung carcinoma, were stably transfected with CIITA and selected for expression of MHC class II molecules. When injected *in vivo* in CD11c.DTR mice both these CIITA-tumors were rejected or strongly retarded in their growth. Importantly the same behavior was observed after treatment with diphtheria toxin to eliminate DC (43).

The mice rejecting the tumor were immune to MHC-II-negative parental tumors and their CD4+ TH cells protected naïve H-2^b C57Bl/6 mice in adoptive cell transfer experiments. To exclude that additional professional APC like macrophages, in absence of DC could serve as main subpopulation to prime tumor-specific naïve CD4 T cells, CD11c.DTR transgenic mice were treated with liposomal Clodronate, a compound that is selectively engulphed by macrophages. Upon phagocytosis, liposomal Clodronate kills the cells by apoptosis (44). Interestingly, in the spleen liposomal Clodronate is engulphed by and kill quite selectively the marginal zone and the metallophilic macrophages considered the predominant APCs (45). Even after treatment with liposomal Clodronate, mice injected with CIITA-tumor cells could reject or strongly retard tumor growth with a behavior very similar to the one observed in liposomal Clodronate-untreated mice (43). Thus, CIITA-driven MHC-II positive tumor cells can perform not only antigen processing and presenting function *in vitro* at least for primed T cells of either human (29) or mouse (32)

but, more importantly, they can prime *in vivo* naïve CD4+ TH cells and thus serve as *bona fide* APC to generate a strong adaptive immune response capable to protect against the tumor (43, 46).

Of relevance, recent work indicated that the MHC class II-positive H-2^d A20 B cell lymphoma cells expressing GFP (A20-GFP), but not the MHC class II-negative H-2^d 4T1-GFP mammary carcinoma cells, can indeed prime directly and be killed *in vitro* by syngeneic CD8 T cells specific for GFP, although in this particular system *in vivo* cross-priming by dendritic cells may also be required (47). These experiments underline the importance of MHC class II expression on tumors to elicit optimal antigen priming, although *in vivo* they may not apply to all tumor histotypes.

Collectively, our findings have not only practical but also conceptual consequences because they challenge the widely accepted view of the exquisite supremacy of DC and, to lesser extent, macrophages to serve as sole APC for priming antigen-specific naïve CD4+ TH cells (9). Whether CIITA-driven MHC class II expressing tumor cells may also spontaneously acquire or be endowed in part with phagocytotic function and thus eat the other dead tumor cells and cross-present their tumor antigen to the naïve lymphocytes just like human immature DCs, remains to be investigated.

Another interesting consideration derived from the above results relates to the genetic characteristics of C57BL/6 H-2^b and their transgenic derivative CD11c.DTR mice. These mice express only one subclass of MHC class II molecule, the I-A molecules because of a defect of the Eα gene (48). Thus, not only tumor cells of distinct genetic background and distinct histotype origin can become immunogenic when expressing CIITA-driven MHC class II molecules (31, 43) but they can also do so by presenting relevant and sufficient tumor derived-peptides within a single MHC-II restricting element, the IA molecule. Very similar results were obtained by other investigators in a pancreatic ductal adenocarcinoma model of C57BL/6 H-2^b (49).

The capacity of CIITA-dependent MHC class II expressing tumor cells to serve as APC *in vivo* raises the question of whether these cells possess or acquire the expression of co-stimulatory molecules, such as B7.1 (CD80) and B7.2 (CD86) that may serve as "signal 2" in triggering antigen-specific naïve TH cells upon interaction with CD28 (50), as previous studies of another group has shown that prevention of tumor growth *in vivo* of CIITA-modified tumor cells in a distinct model of mammary carcinoma in H-2^q model required also expression of CD80 (51). We found that MC38 and LLC tumor cells do not express CD80 and CD86 costimulatory molecules and this phenotype is not modified by CIITA expression. Thus, either CIITA-tumors do not need necessarily accessory molecules to perform their APC function *in vivo,* or other accessory molecules are involved to provide the second signal, or tumor-specific, and possibly organ-specific constraints limit the immune stimulating function of CIITA-driven MHC class II expressing tumor cells.

This important issue should certainly deserve detailed investigation in the future.

As outlined earlier and in relation to the peculiar modification of the tumor microenvironment generated by CIITA-driven MHC class II positive tumor cells, our studies raise another relevant question related to the anatomical location in which the anti-tumor immune response against CIITA-modified cancer cells takes place. It is generally assumed that TH cell priming mediated by professional APC, namely DC, takes place in the lymph nodes, where DC that have captured and processed the antigens in the periphery migrate and present antigenic peptides within the context of MHC-II molecules (12). As the tumor microenvironment was drastically modified in presence of CIITA-tumor cells, with a profound change both in number and compartmentalization of the leukocyte infiltration (32) we may speculated that it could be the ideal site for the formation of ectopic lymphoid-like structures or tertiary lymphoid organs (TLO), neoformations that are often detected in chronic inflamed tissues and in tumor tissues (36, 52). TLO share many characteristics with lymph nodes associated with the generation of an adaptive immune response (53). If this will be confirmed in future studies, tumors cells expressing MHC class II molecules not only act as APC for priming naïve tumor-specific CD4+ T cells but also perform APC activity ectopically with respect to the canonical site represented by the lymph nodes.

FROM THE BENCH TO THE BEDSIDE: THE CONSTRUCTION OF AN OPTIMAL ANTI-TUMOR THERAPEUTIC VACCINE… AND BEYOND

A major corollary of the studies related to the high *in vivo* immunogenicity of CIITA-driven MHC-II expressing tumors is that MHC class II molecules should be loaded with sufficient quantity of tumor specific peptides, derived from either overexpressed or mutated genes, to generate an efficient functional triggering of tumor-specific CD4+ T cells, an event that we have defined as Adequate Antigen Availability (AAA) (54). Thus, these cells can be instrumental to identify the key tumor antigens which may serve to develop new generation anti-tumor vaccines (**Figure 2**).

This strategy has indeed been applied recently by a European Consortium of nine institutions, including our laboratory (the Hepavac Consortium, www.hepavac.eu), as part of the construction of an innovative vaccine against human hepatocarcinomas (HCC). HCC was selected because ranks sixth in terms of incidence but fourth in term of deaths/year worldwide (GLOBOCAN 2018, http://gco.iarc.fr/). Given the current lack of available effective treatments, the overall prognosis for patients with HCC is poor with a dismail 5-year survival of <25%, making the disease a highly important and relevant target for the development of innovative therapies (55).

By using a well-established experimental protocol and purification platform (56), the relevant MHC II-bound tumor specific peptides were selected from CIITA-driven MHC-II expressing human HCC cells. These peptides, along with a number of highly specific HCC MHC-I-bound tumor peptides, contributed to the formation of a peptide cocktail to be used

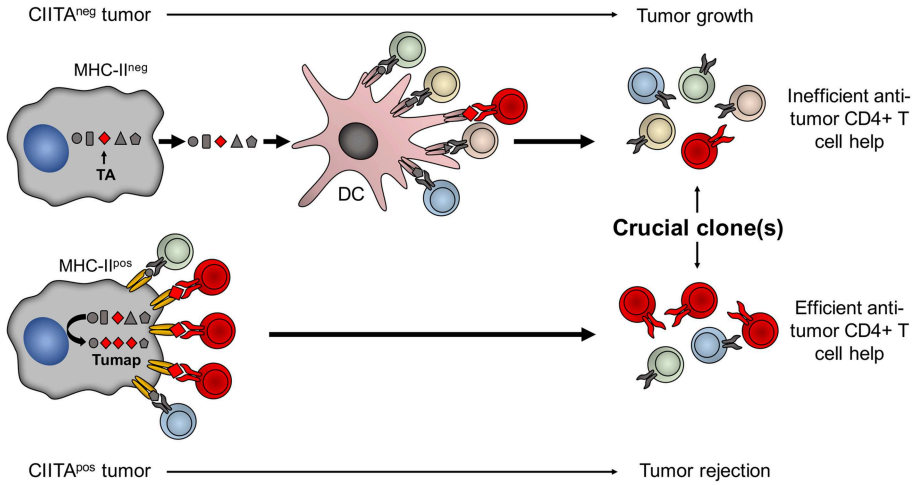

FIGURE 2 | Tumor cells expressing CIITA-driven MHC-II molecules are potent surrogate APC to prime relevant tumor-specific TH cells. The MHC class II-bound tumor peptidome (Tumap, red symbols) derived from CIITA-driven MHC-II expressing cells is highly enriched of tumor-specific epitopes (Lower part) as compared to the one of classical APC, such as dendritic cells (DC), that may capture tumor antigens (TA) only after phagocytosis of dying MHC-II-negative tumor cell debris (Upper part). As a result, in CIITA-tumors, MHC-II-tumor peptide complexes efficiently stimulate and amplify higher number of tumor-specific TH cell clones (in red) to generate a strong immune response capable to reject the tumor (Lower part). On the contrary, classical APC do not efficiently select sufficient tumor-specific peptides from MHC-II-negative tumor cells to be presented within the context of their MHC-II. As a consequence DC cannot efficiently prime tumor-specific TH cell clones and tumor takes off and grows (Upper part).

as the first multi-epitope, multi-target, and multi-allele cancer vaccine against HCC aimed at stimulating both CD4+ and CD8+ T cells. This vaccine is, at present, in a phase I/II clinical trial whose results on safety, tolerability, and immunogenicity (primary endpoints) and possibly overall survival (secondary endpoint) are expected by the end of 2019.

In studying patient's HCC tumor tissues as well as normal liver tissues, we observed two important features that bear relevance not only for applying profitable vaccination approaches as the one described here but also to better understand old and recent observations on the immunologically tolerant environment of the liver (57, 58). The first important observation was related to the expression of MHC-I and MHC-II in liver cells. While both these molecules were virtually absent in normal liver cells, MHC-I cell surface molecules were expressed at very high level in HCC cells (59). This of course was relevant to purify the MHC-I tumor peptidome and select the appropriate peptides for the vaccine compositions. The second important observation was that MHC-II expression, instead, remained silent in HCC cells both *in vivo* and in patients' derived tumor cell lines. Importantly MHC-II expression could not be rescued even by treatment with IFNγ, the most potent inflammatory cytokine that induces MHC-II expression indirectly via the primary transcriptional activation of CIITA (19). In depth analysis of the molecular mechanism responsible of this finding demonstrated that the CIITA promoter IV, the specific promoter activated by the IFNγ (60), was silenced by hypermethylation of its sequence and thus rendered developmentally unresponsive in liver cells (59). This finding may have important effects on the interpretation of the tolerogenic environment of the liver, because the impossibility to express MHC-II molecules by liver cells, continuously in contact with massive concentrations of antigenic materials derived from the digestive tract, would prevent accidental co-participation of these cells to APC function and activation of immune system against potential food antigens as well as other antigens including self antigens.

Thus, as it was the case for the discovery of the surrogate APC function of CIITA-driven MHC-II expressing tumor cells, we also believe that unveiling the tissue constraints at the basis of CIITA-driven MHC class II expression or non-expression may serve to better understand important aspects of basic immunology.

AUTHOR CONTRIBUTIONS

GF and RA conceived and wrote the paper. All authors revised and approved the final manuscript.

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Development of a Human Cytomegalovirus (HCMV)-Based Therapeutic Cancer Vaccine Uncovers a Previously Unsuspected Viral Block of MHC Class I Antigen Presentation

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Human cytomegalovirus (HCMV) induces a uniquely high frequency of virus-specific effector/memory CD8+ T-cells, a phenomenon termed "memory inflation". Thus, HCMV-based vaccines are particularly interesting in order to stimulate a sustained and strong cellular immune response against cancer. Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor with high lethality and inevitable relapse. The current standard treatment does not significantly improve the desperate situation underlining the urgent need to develop novel approaches. Although HCMV is highly fastidious with regard to species and cell type, GBM cell lines are susceptible to HCMV. In order to generate HCMV-based therapeutic vaccine candidates, we deleted all HCMV-encoded proteins (immunoevasins) that interfere with MHC class I presentation. The aim being to use the viral vector as an adjuvant for presentation of endogenous tumor antigens, the presentation of high levels of vector-encoded neoantigens and finally the repurposing of bystander HCMV-specific CD8+ T cells to fight the tumor. As neoantigen, we exemplarily used the E6 and E7 proteins of human papillomavirus type 16 (HPV-16) as a non-transforming fusion protein (E6/E7) that covers all relevant antigenic peptides. Surprisingly, GBM cells infected with E6/E7-expressing HCMV-vectors failed to stimulate E6-specific T cells despite high level expression of E6/E7 protein. Further experiments revealed that MHC class I presentation of E6/E7 is impaired by the HCMV-vector although it lacks all known immunoevasins. We also generated HCMV-based vectors that express E6-derived peptide fused to HCMV proteins. GBM cells infected with these vectors efficiently

stimulated E6-specific T cells. Thus, fusion of antigenic sequences to HCMV proteins is required for efficient presentation via MHC class I molecules during infection. Taken together, these results provide the preclinical basis for development of HCMV-based vaccines and also reveal a novel HCMV-encoded block of MHC class I presentation.

Keywords: human cytomegalovirus, therapeutic cancer vaccine, glioblastoma, cancer immunotherapy, viral immune evasion

INTRODUCTION

Glioblastoma multiforme (GBM) is one of the most frequent and devastating brain tumors (1, 2). In fact, GBM is incurable and has a bad prognosis even after aggressive standard treatment that combines radiation, surgery and chemotherapy (3). Accordingly, there is a need to develop novel therapeutic strategies to combat this deadly disease.

Different forms of immunotherapy have been implemented or explored in a variety of human malignancies including GBM (4). Adoptive transfer of genetically modified T cells may be an option in treatment of GBM (5–8). In recent clinical trials, checkpoint inhibitors have failed to prolong the overall survival of patients with recurrent GBM (9–11). As a neoadjuvant therapy, however, PD-1 monoclonal antibody blockade improves local and systemic antitumor T cell responses (12). Therapeutic cancer vaccines stimulating tumor-reactive CD8+ T cells represent another form of immunotherapy that has also been tested in GBM patients (4, 13).

Successful tumor immunotherapy requires preexisting CD8+ T cells in the tumor microenvironment (TME) (14, 15) and genetic mutations that generate tumor neoantigens (16, 17). GBM, however, provides a "cold" TME with low numbers of infiltrating immune cells (15, 18) and scarce somatic mutations (19, 20). *In situ* vaccination with viral vectors can turn "cold" TME into "warm" through the adjuvant effect resulting from triggering multiple pattern recognition receptors (PRRs) (21–25). This inflammatory response may increase TME infiltration with immune cells. A large fraction of tumor-infiltrating immune cells are in fact memory CD8+ T lymphocytes specific for common viruses such as human cytomegalovirus (HCMV) (26–29). These cells are neither tolerized nor exhausted by continuous stimulation and can be repurposed for tumor immunosurveillance (27).

Human cytomegalovirus (HCMV) inflates memory by intermittent reactivation from latency or reinfections (30–32). In HCMV-infected humans, on average 10% of the circulating T cells with an effector-memory phenotype are in fact HCMV-specific (33, 34). Thus, HCMV-based vectors represent a very promising novel platform for therapeutic vaccination (35, 36). HCMV persists in immunocompetent individuals without causing disease (37). Intriguingly, HCMV infects GBM cells *in vitro* (38). Moreover, HCMV is detected in GBM tumor tissue but not in the surrounding normal brain tissue (39). Thus, immunotherapy may leverage HCMV-encoded tumor antigens to induce elimination of tumor cells by cytotoxic CD8+ T cells (40–42). Several strategies to achieve this goal have been explored including adoptive transfer of *in vitro*-expanded HCMV-specific T cells and vaccination with autologous dendritic cells (DCs) stimulating HCMV-specific T cells *in vivo* (39).

In this study, we designed novel HCMV-based therapeutic viral vaccines to exploit the patient's own immune system for elimination of tumor cells. We increased the immunostimulatory capacity of the HCMV-based vector by deleting important viral immune evasion genes. Moreover, we expressed a well-characterized epitope from human papillomavirus (HPV) that functions as a neo-epitope after infection of GBM cells. Finally, we tested whether genetically altered T cells specific for HCMV-encoded epitope or neo-epitope are stimulated by GBM cells infected with the HCMV-based vaccines.

MATERIALS AND METHODS

Ethics Statement

Buffy coat preparations were purchased from German Red Cross (Dresden, Germany). Blood samples were taken with the approval of the ethics committee of the Charité–Universitätsmedizin Berlin. Written informed consent was obtained from all donors.

Cells

The GBM cell lines U343 and LN18 were kindly provided by the Department of Neurosurgery, Charité-Universitätsmedizin Berlin, Berlin, Germany. The GBM cell line U251 was a kind gift of L. Wiebusch from the Children's Hospital, Laboratory for Molecular Biology, Charité-Universitätsmedizin Berlin, Berlin, Germany. Human embryonic lung fibroblasts (Fi301) and GBM cell lines were cultured in Eagle's minimum essential medium (EMEM) from Lonza supplemented with 1 mM sodium pyruvate, 2 mM L-alanyl-L-glutamine, non-essential amino acids, 50 μg/ml gentamicin, and 10% heat inactivated FBS (hiFBS) (HyClone). PBMCs and reporter Jurkat cell lines were cultured in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine, 25 mM HEPES Buffer, 50 μg/ml gentamicin, and 10% hiFBS.

Flow Cytometry of Surface Molecules

Cells were harvested, washed and stained as previously described (43). Cell surface expression of HLA-A2 molecules was detected by using PE-conjugated anti-HLA-A2 antibody BB7.2 (BioLegend). For quantifying fluorescence of labeled cells, a FACSCalibur® (BD Biosciences) was used. Results were evaluated with the software programs CellQuestPro® (BD Biosciences) and FlowJo V10 (Tree Star, Inc).

Viruses

HCMV strain TB40/E and the corresponding bacterial artificial chromosome TB40/E-BAC (clone 4) as well as RV-TB40-BAC_{KL7}-SE-EGFP, an enhanced green fluorescent protein (EGFP)-expressing virus derived from TB40/E (44), were kindly provided by Christian Sinzger, University of Ulm, Ulm, Germany. The advantages of TB40/E are high titer growth in cell culture similar to lab strains and cell tropism resembling recent clinical isolates (45). TB40/E and the mutants derived from TB40/E-BAC were propagated in Fi301 cells. For generation of virus stocks, cells and medium were collected at various times after infection, after which cells were disrupted by three freeze-thaw cycles and cell debris was pelleted by centrifugation.

Generation of Recombinant Viruses

As a neoantigen for expression in TB40/E-BAC derived vectors, we used human papillomavirus type 16 (HPV-16) consensus E6/E7 fusion protein (ConE6E7, GenBank accession number: FJ229356) (46). In addition, the HLA-A2-binding peptide $E6_{29-38}$ (TIHDIILECV) derived from the E6 protein of HPV-16 (47) was fused with an AA-linker (AATIHDIILECV) to the C-terminus of HCMV IE1 (E6peptideIE1) or HCMV UL83 (E6peptideUL83). The corresponding sequences were synthesized and verified by Integrated DNA Technologies (IDT). The synthesized E6/E7 encoding sequence was digested with EcoRI and Kpn-I and cloned into the expression vectors pEF6/V5-His A and pcDNA^{TM}3.1 (+). These constructs were named pEF6E6/E7EcoRI and pcDNAE6/E7Kpn-I, respectively. Recombinant HCMV was generated using BAC technology as previously described (48). All recombinant BAC clones were confirmed by PCR and DNA-sequencing of the target area. Viruses were reconstituted from BACs by electroporation of 1×10^6 Fi301 cells using program A24 of the Nucleofector II (Amaxa) and a basic Nucleofector kit (Lonza), according the manufacturer's instructions.

Virus Titration and Growth Kinetics

Virus titers of virus stocks and multi-step growth kinetics were quantified by 50% tissue culture infectious dose (TCID50) assay on Fi301 cells. The TCID50 values were calculated using the method of Reed and Muench (49).

Stable Transfection of U251

U251 cells were stably transfected with pcDNAE6/E7Kpn-I by electroporation as previously described (50). Transfected cells were selected by G418 for neomycin resistance and different clones were isolated and separately cultured for E6 and E7 expression assays.

Detection of HPV-16 E7 Protein

For detection of E6/E7 fusion protein, 1×10^6 cells were trypsinized and aliquots covering a range of different cell numbers were prepared (7×10^2 to 16×10^4 cells). In these aliquots, the E6/E7 fusion protein was detected by using recomWell HPV 16/18/45 ELISA Kit (Mikrogen GmbH, Neuried, Germany) according to manufacturer's instructions. The optical density was measured at 450 nm in a microplate photometer (Multiskan FC, Thermo Fisher Scientific, USA). The absorbance detected for experimental probes was expressed relative to the absorbance measured for the same number of CaSki cells, an E6- and E7-expressing cervical carcinoma cells that served as positive control.

Generation of TCR Expression Vectors

For HLA-A2-restricted HPV $E6_{29-36}$-specific TCR (51) transgene cassettes were codon-optimized for human expression and synthesized by GeneArt/Life Technologies. TCR-α/β chains with human TCR constant regions replaced by their murine counterparts were linked via 2A "self-cleaving" peptide sequence from *Porcine teschovirus-1* (P2A) and cloned in the configuration TCRβ-P2A-TCRα into pMP71-PRE using *Not*I and *Eco*RI restriction sites as described recently (52). The HCMV-specific TCR (NLV3) detecting a HLA-A2-restricted epitope derived from pp65 (NLVPMVATV; aa 495-503) was used in its original configuration as described by Schub et al. (53).

TCR Gene Transfer

TCR gene transfer was carried out as described (54) with minor modifications. In brief, HEK-293 cells stably expressing GALV-env and MLV-gag/pol were grown to ∼80% confluence and transfected with 3 μg of pMP71-TCR vectors in the presence of 10 μg Lipofectamine2000 (Life Technologies). At 48 and 72 h after transfection, 3 ml of retrovirus containing supernatant were harvested. 1×10^6 human PBMCs, that had been frozen after isolation from healthy donors by ficoll gradient centrifugation, were thawed and stimulated with 5 μg/ml anti-CD3 (OKT3) and 1 μg/ml anti-CD28 (CD28.2) (Biolegend) coated plates in the presence of 300 U/ml recombinant human interleukin 2 (hIL-2) (Peprotech). Transductions at 48 and 72 h after stimulation were performed by addition of retrovirus containing supernatant and 4 μg/ml protamine sulfate followed by spinoculation for 90 min at 800 g and 32°C (1st transduction). For second transduction, retrovirus was preloaded onto retronectin (Takara)-coated plates followed by spinoculation for 30 min at 800 g and 32°C. Transduced PBMCs were maintained in the presence of 300 U/ml hIL-2 for a total of 2 weeks. At least 2 days prior to use in experiments, transduced PBMCs were cultured in the presence of 30 U/ml hIL-2.

Functional Assays With TCR-Transduced T Cells

IFN-γ production was measured by ELISA after 16 h coculture of 1×10^5 TCR-transduced T cells with 1×10^5 target cells (HCMV-vector infected or HCMV-vector infected and pulsed with the corresponding peptide). As a negative control, 1×10^5 TCR-transduced T cells were cocultured with 1×10^5 target cells that had been left uninfected. Stimulation with phorbol myristate acetate and ionomycin (P+I) was used as a positive control.

Reporter Cell Lines

For detection of NFAT activation, a previously described cellular platform for analysis of TCRs was used (55, 56). In the human T cell lymphoma cell line Jurkat 76 (J76), the response elements of transcription factor nuclear factor of activated T-cells (NFAT)

drive the expression of EGFP (55). The J76 cell line is a subline of cell line Jurkat E6.1 (JE6.1), which lacks expression of the TCR alpha and beta chains (57). The J76 cell line was transduced with a retroviral vector encoding HLA-A2-restricted HPV $E6_{29-36}$-specific TCR (51). Moreover, J76 cells were co-transduced to express a HLA-A2-restricted HCMV pp65-specific TCR (NLVPMVATV; aa 495-503) and CD8 (56).

For measuring of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) activation a single T cell reporter cell line was used, in which the responsive element for NF-κB controls EGFP expression (58). This single reporter cell line was transduced with retroviral vector encoding HLA-A2-restricted HPV $E6_{29-36}$-specific TCR (51) or with retroviral vector encoding the HCMV-specific TCR (NLV3), which recognizes a HLA-A2-restricted epitope derived from pp65 (NLVPMVATV; aa 495-503) (53).

Antigen Presentation Assays Using Reporter Cell Lines

For stimulation of reporter cell lines 5×10^4 GBM cells (LN18, U343, or U251 cells) were infected with HCMV-based vaccines (MOI of 5). After 2 days and 4 days, respectively, infected cells were co-cultured with HPV E6-specific reporter cells and HCMV pp65-specific reporter cells, respectively, for 24 h at a ratio 2:1. Subsequently, EGFP expression of reporter cells was determined by FACS analysis.

U251 cells stably transfected with pcDNAE6/E7Kpn-I (U251-E6/E7 cells) were used to assess the impact of HCMV infection on MHC class I presentation of the E6/E7 fusion protein. For this purpose, U251 cells were left uninfected or infected with RVTB40ΔUS11 for 3–24 h at different MOIs. RVTB40ΔUS11 lacks all known HCMV-encoded immunoevasins (US2, US3, US6, and US11) that target MHC class I presentation and does not downregulate MHC class I molecules. On uninfected and infected U251-E6/E7 cells, the existing peptide-MHC class I complexes on U251 cells were removed by acid wash as previously described (59). Briefly, 1×10^6 cells were harvested, washed with PBS and subsequently washed with ice-cold citric acid buffer (pH 3) for 2–3 min. Afterwards, stripped U251-E6/E7 cells were pelleted, washed twice with EMEM, resuspended in RPMI 1640 medium and subsequently co-cultured for 18 h with the HPV $E6_{29-36}$-specific reporter cell line, in which the responsive element for NF-κB controls EGFP expression (58). Finally, EGFP expression of reporter cells was determined by FACS analysis. In parallel, the maximal peptide stimulation was always determined by pulsing a cell aliquot with the E6 peptide (1 μg/ml) during coculture with the E6-specific reporter cell line.

Peptide Synthesis

The peptides used for pulsing antigen-presenting cells (1 μg/ml) were synthesized by peptides & elephants GmbH (Hennigsdorf, Germany).

Statistical Analysis

Statistical significance was determined by one-way ANOVA analysis or unpaired *t*-test. *P* values below 0.05 (95% confidence) were considered to be significant. Prism 6 software (GraphPad) was used for statistical analysis.

RESULTS

Susceptibility of GBM Cells to HCMV Infection

In order to construct therapeutic vaccines targeting GBM we first investigated whether GBM cells are susceptible to HCMV infection. For this purpose, we used RV-TB40-BAC_{KL7}-SE-EGFP. This EGFP-expressing virus is derived from low-passage HCMV strain TB40/E and contains an intact US-gene region encoding all immunoevasins (US2, US3, US6, and US11) that downregulate MHC class I presentation (44). We infected the GBM cell lines LN18, U343, and U251 with RV-TB40-BAC_{KL7}-SE-EGFP at a multiplicity of infection (MOI) of 0.3. At different time points of infection, we determined the percentage of EGFP-expressing GBM cells (**Figure 1**, left graphs). In addition, we analyzed the presence of virus in the supernatant of infected GBM cell cultures (**Figure 1**, right graphs). Although all GBM cell lines tested were susceptible to HCMV, infection the virus remained mostly cell-associated during the observation period of 12 days. Thus, LN18, U343, and U251 cells are susceptible to HCMV infection as previously reported for other GBM cell lines (38, 60). Taken together, these experiments indicate that HCMV-based vectors can be used to mark GBM cells for attack by CD8+ T cells.

Construction of HCMV-Based Therapeutic Vaccines

Next, we generated HCMV-based vectors that lack immunoevasins (US2, US3, US6, and US11) and efficiently stimulate CD8+ T cells. We used a bacterial artificial chromosome (BAC) clone of the HCMV strain TB40/E (TB40-BAC4), which lacks the *US1-US6* region due to insertion of the BAC (45). We obtained RVTB40ΔUS11 from TB40-BAC4 by deleting *US11*. RVTB40ΔUS11 does not downregulate MHC class I molecules as recently described (**Figure 2A**) (61).

We now pursued two strategies to equip RVTB40ΔUS11 with neo-epitopes. Firstly, we used a consensus sequence encoding the E6 and E7 protein of human papillomavirus type 16 (HPV-16) as a fusion protein (E6/E7). E6/E7 covers all relevant antigenic peptides but is non-transforming (46). Vaccination of mice with a plasmid encoding E6/E7 induces a strong CD8+ T cell response and prevents growth of E6/E7 tumors (46). In accordance, we observed that E6/E7-expressing clones derived from stably transfected U251 cells (U251 cells) stimulate reporter T cells that recognize a HLA-A2-restricted peptide ($E6_{29-38}$: TIHDIILECV) (47) (**Figure 2B**). Thus, we inserted the E6/E7 sequence into the RVTB40ΔUS11 at different locations ensuring that endogenous or exogenous promotors control E6/E7 expression (**Figure 3**). The E6/E7 expression level in cells infected with E6/E7-expressing HCMV-based vaccines was in the same order of magnitude as observed for U251 cell transfected with an E6/E7-expressing plasmid (**Figure 2C**).

Secondly, we fused a single neo-epitope flanked by an Alanine spacer to the C-terminus of a viral protein as

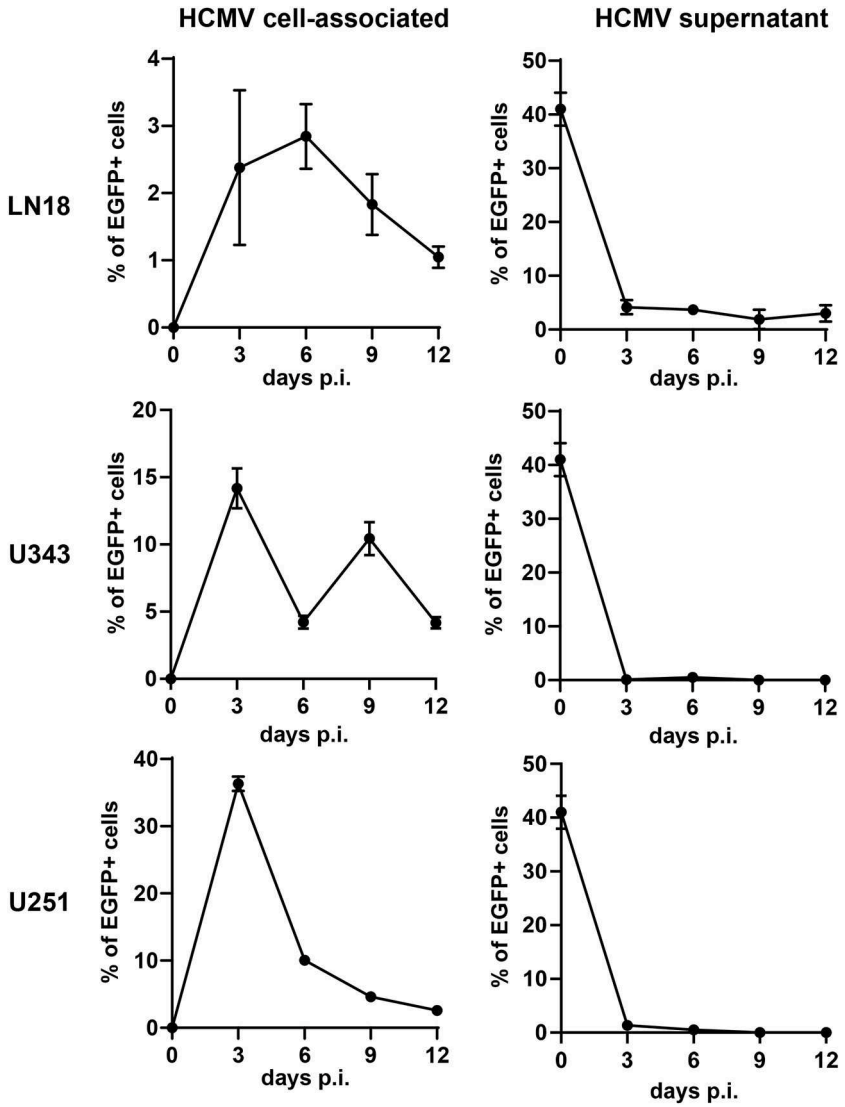


FIGURE 1 | Susceptibility of GBM cells to HCMV infection. The GBM cell lines LN18, U343, and U251 were infected with RV-TB40-BAC$_{KL7}$-SE-EGFP (MOI of 0.3). At different time points cells were tested for cell-associated virus by FACS analysis of EGFP expression (Left graphs). For detection of cell-free HCMV (Right graphs) supernatants from infected GBM cell lines were collected at different time points. Subsequently, Fi301 cells were infected with the supernatants and tested for EGFP expression by FACS 2 days after infection. Results are derived from three technical replicates; error bars represent the mean ± SEM.

recently reported for murine cytomegalovirus (MCMV) (62, 63) (**Figure 4**). We used HPV-16 E6$_{29-38}$ as CD8+ T cells specific for this peptide recognize and kill HLA-A2+ tumor cells expressing E6 despite tumor-associated immune evasion mechanisms (64). This E6 peptide was fused to the C-Terminus of IE1 (RVTB40ΔUS11_E6peptideIE1) or UL83 (RVTB40ΔUS11_E6peptideUL83). We also generated a mutant virus with both the full E6/E7 sequence inserted into UL83 and the E6 peptide linked to IE1 (RVTB40ΔUS11_E6/E7intoUL83_E6peptideIE1). All generated HCMV-based vaccines showed growth kinetics similar to WT TB40/E and control virus (RVTB40ΔUS11) (**Figure 5A**). The relevant features of the different HCMV-based vaccines are summarized in **Figure 5B**.

HCMV-Based Vaccines Expressing E6 Peptide Fused to Viral Protein but Not E6/E7 Expressing HCMV-Based Vaccines Stimulate E6-Specific T Cells

Now we investigated whether the different HCMV-based therapeutic vaccines could stimulate antigen-specific T cells after infection of GBM cells. To this end, we used a recently developed T cell reporter platform, in which the response elements for NFAT control EGFP expression (55, 56). These cells were transduced either with a retroviral vector encoding a HPV-specific TCR recognizing the HLA-A2-restricted peptide HPV E6$_{29-36}$ (51) or with a retroviral vector encoding HCMV-specific TCR detecting the HLA-A2-restricted HCMV epitope

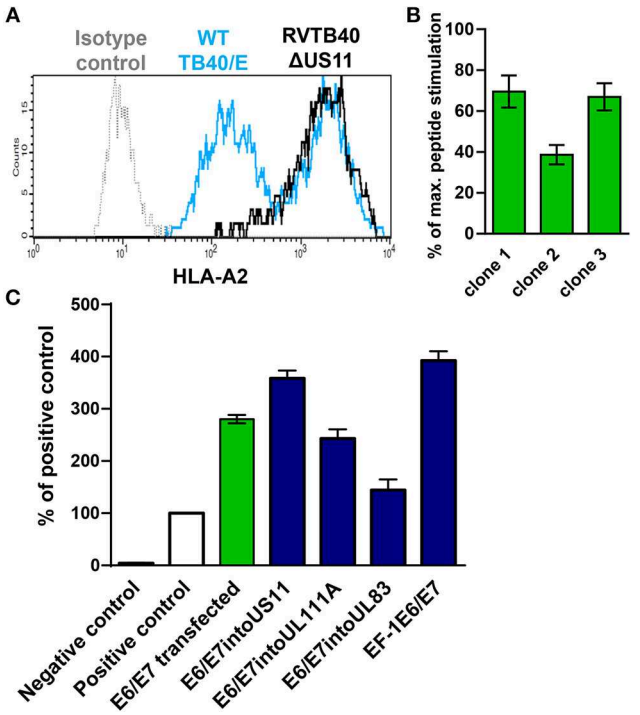


FIGURE 2 | Rationale for generation of HCMV-based vectors expressing E6/E7 fusion protein. **(A)** Prevention of virus-induced MHC class I downregulation in cells infected with HCMV-based vectors lacking *US2*, *US3*, *US6*, and *US11*. Fi301 cells were infected with WT TB40/E or RVTB40ΔUS11 at MOI of 0.5. After 2 days, cells were stained with HLA-A2-specific antibody or isotype control and analyzed by flow cytometry. The results shown are representative of three experiments. **(B)** Stimulation of specific reporter T cells by U251 cells expressing E6/E7. Clones of U251 cells stably transfected with E6/E7 expressing plasmid were incubated with E6 peptide-specific reporter cell line, in which EGFP expression is driven by the responsive elements of NF-κB. Stimulation of reporter cells is given as percentage of maximal peptide stimulation, i.e., stimulation of reporter cells incubated with peptide pulsed cells. **(C)** Detection of E6/E7 in cells infected with HCMV-based vectors driving E6/E7 expression under control of endogenous or exogenous promotors. For quantification of the E6/E7 fusion protein expressed by different HCMV-based vectors (blue bars) an ELISA detecting the HPV-16 E7 protein was used. For each experimental group 6×10^4 cells were used. RVTB40ΔUS11 served as a negative control. The absorbances detected for experimental probes were expressed relative to the absorbance measured for 6×10^4 CaSki cells, a well-characterized E6- and E7-expressing cervical carcinoma cell line (Positive control, white bars). We also included U251 cells stably transfected with E6/E7 encoding plasmid in our analysis (green bar).

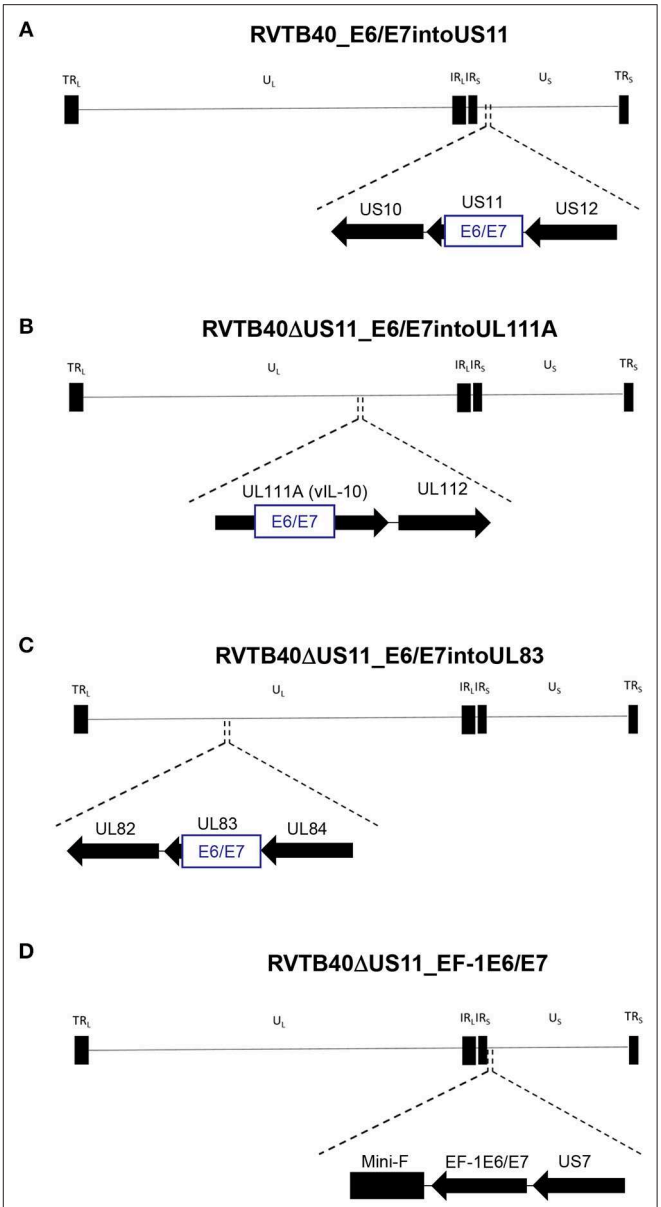


FIGURE 3 | Construction of HCMV-based vaccines expressing E6/E7 fusion protein. The HCMV genome has a length of ~235 kB and contains a unique long (U_L) and a unique short (U_S) region each flanked by terminal (TR_L and TR_S), and internal (IR_L and IR_S) inverted repeats. The E6/E7 encoding sequence was inserted into **(A)** US11 (RVTB40_E6/E7into US11), **(B)** UL111A (RVTB40ΔUS11_E6/E7intoUL111A), or **(C)** UL83 (RVTB40ΔUS11_E6/E7intoUL83) in such a way that endogenous promotors control E6/E7 expression. **(D)** The E6/E7 consensus sequence was put under the control of the elongation factor-1 alpha (EF-1 alpha) promotor, a strong constitutive promotor of human origin, and inserted between TB40-BAC4 Mini-F sequence and US7 (RVTB40ΔUS11_EF-1E6/E7).

$pp65_{495-503}$ (53) together with CD8. In addition, we used another set of reporter cell lines with the same TCR specificities, in which EGFP expression is driven by the responsive elements of NF-κB (65). These reporter cell lines were incubated with HLA-A2+ LN18, U343, and U251 cells that had been infected with the different HCMV-based therapeutic vaccines for 2 or 4 days, respectively. Surprisingly, GBM cells infected with E6/E7-expressing vectors stimulated neither NFAT (**Figure 6**, left side, blue columns) nor NF-κB (**Figure 7**, left side, blue columns) in E6-specific reporter T cell lines. In stark contrast, all GBM cells infected with an HCMV-based vector expressing the E6 peptide fused with an Alanine-linker to the C-terminus of HCMV IE1 (E6peptideIE1) nicely activated NFAT (**Figure 6**, left side, red columns) and NF-κB (**Figure 7**, left side, red columns) in E6-specific T cells. Although to a lesser extent stimulation of reporter cell lines was also observed with all GBM cells that had been

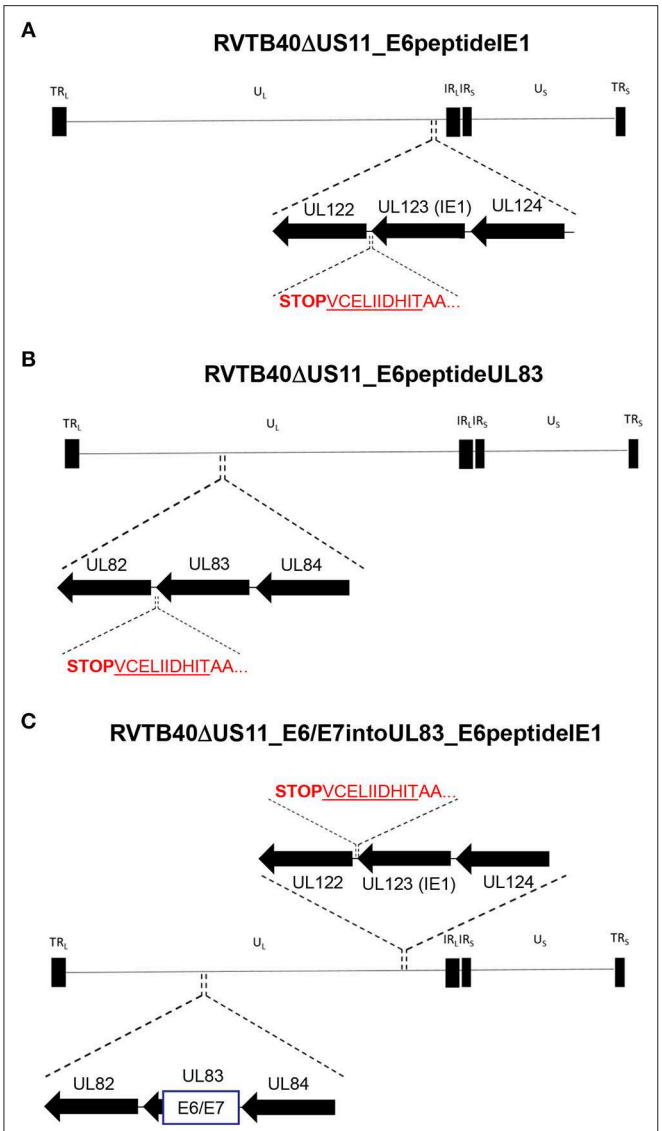
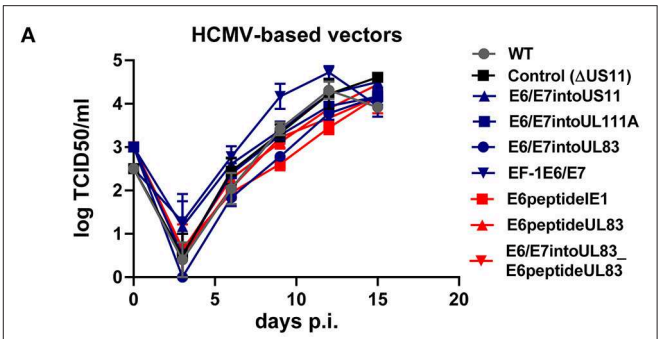


FIGURE 4 | Construction of HCMV-based vaccines expressing E6 peptide fused to the C-terminus of HCMV IE1 or HCMV UL83. The HLA-A2-binding peptide $E6_{29-38}$ (TIHDIILECV) derived from the E6 protein of HPV-16 was fused with an AA-linker (AATIHDIILECV) to the C-terminus of **(A)** HCMV UL123 (IE1) (RVTB40ΔUS11_E6peptideIE1) or **(B)** HCMV UL83 (RVTB40ΔUS11_E6peptideUL83). **(C)** In addition, a recombinant virus expressing both the E6/E7 fusion protein inserted into UL83 and the E6-peptide fused to the C-terminus of HCMV IE1 was generated (RVTB40ΔUS11_ E6/E7intoUL83_E6peptideUL83).



B

	WT	Control (ΔUS11)	E6/E7intoUS11	E6/E7intoUL111A	E6/E7intoUL83	EF-1E6/E7	E6peptideIE1	E6peptideUL83	E6/E7intoUL83_ E6peptideIE1
Immunoevasins	+	-	-	-	-	-	-	-	-
E6/E7	-	-	+	+	+	+	-	-	+
UL111A	+	+	+	-	+	+	+	+	+
UL83	+	+	+	+	-	+	+	+	-
E6 peptide	-	-	-	-	-	-	+	+	+

FIGURE 5 | Features of HCMV-based therapeutic vaccines used in this study. **(A)** Growth curve kinetics of E6/E7 expressing vaccines (blue) and E6 peptide-expressing vaccines (red). Fi301 cells were infected at MOI of 0.01. Supernatant was collected at different time points after infection and titrated on Fi301 cells to calculate the TCID50. WT TB40/E and RVTB40ΔUS11 served as a control. Results are derived from three experiments; error bars represent the mean ± SEM. **(B)** Summary of all HCMV-based vectors used in this study.

infected with a HCMV-based vector expressing the E6 peptide fused with an Alanine-linker to the C-terminus of HCMV UL83 (**Figures 6** and **7**, left side, red columns). As expected, all HCMV-based therapeutic vaccines with the exception of those deficient of pp65 (UL83) could stimulate pp65-specific reporter cell lines to a similar extent after infection of GBM cells (**Figures 6** and **7**, right side). Taken together, E6 peptide fused to the C-terminus of HCMV proteins but not the complete E6/E7 fusion protein expressed separately from HCMV proteins stimulated E6 peptide-specific T cells.

A Novel HCMV-Encoded Block of MHC Class I Presentation

The finding that GBM cells infected with HCMV vaccines failed to stimulate E6-specific T cells despite abundant E6/E7 protein expression was surprising. It suggested that MHC class I presentation of E6/E7 is impaired by the HCMV-vector although RVTB40ΔUS11 lacks all known immunoevasins (US2, US3, US6, US11). To address this issue, aliquots of transfected U251 cells, which stably express the E6/E7 protein, were left uninfected or infected at different MOIs with the HCMV-vector. Thereafter, cells were acid washed as described previously (59) to remove all existing peptide-MHC class I complexes from the cell surface. Subsequently, cells were co-cultured for 18 h with HPV $E6_{29-36}$-specific reporter cells, which express EGFP under the control of NF-κB responsive elements (58). Maximal peptide stimulation was assessed in parallel by pulsing cells with E6 peptide during coculture with the reporter cells. **Figure 8A** shows that U251-E6/E7 cells (Positive control) but not untransfected U251 cells (Negative control) stimulated E6 peptide-specific reporter cells. Strikingly, acid washed U251-E6/E7 cells that had been infected with different MOIs of the HCMV-vector showed a significantly reduced capacity to stimulate E6-specific reporter cells as compared to acid washed uninfected U251-E6/E7 cells (**Figure 8A**). After additional pulsing with exogenous

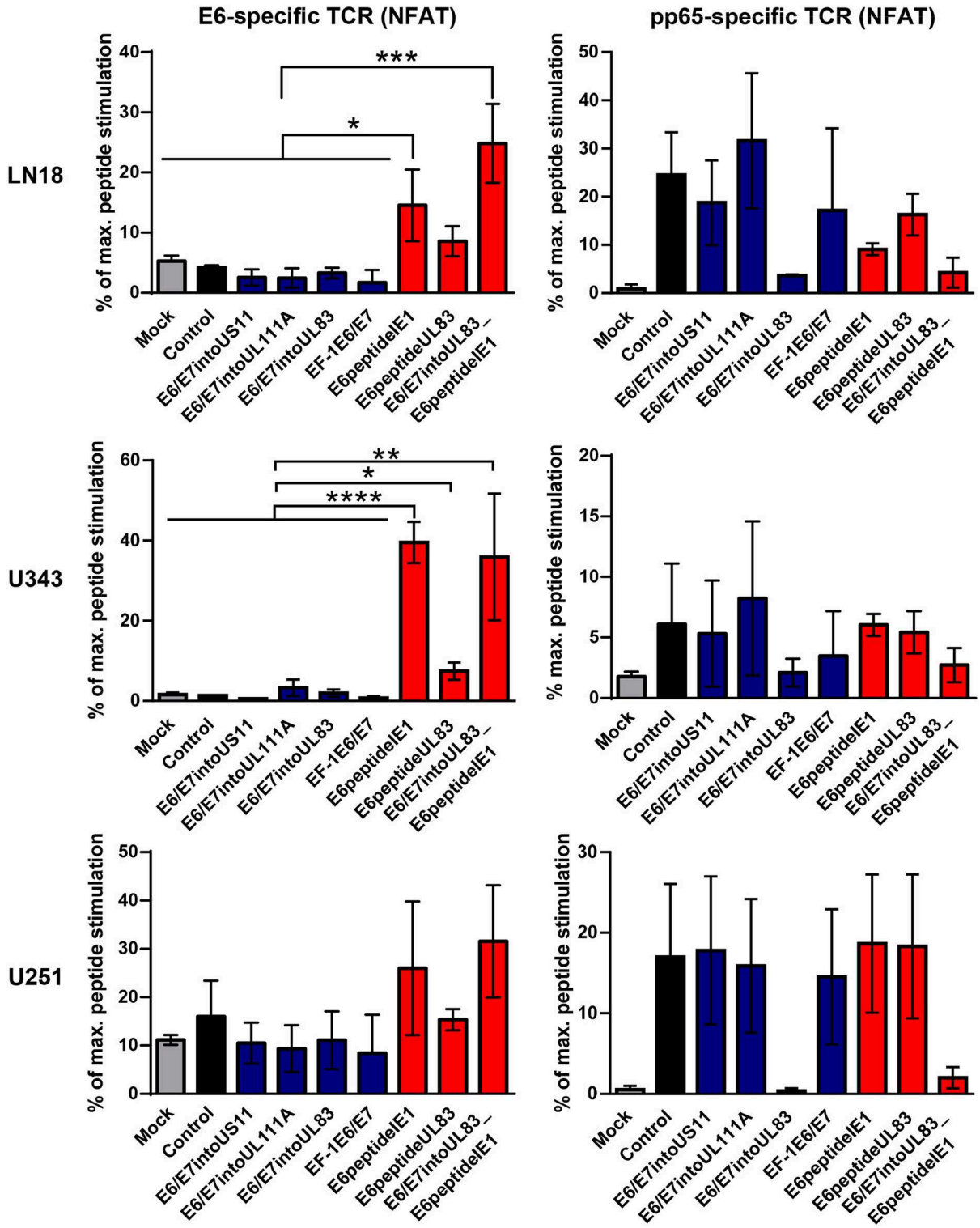


FIGURE 6 | NFAT-driven EGFP expression in reporter cell lines stimulated by infected GBM cells. 5×10^4 GBM cells (LN18, U343, or U251 cells) were infected with HCMV-based vaccines (MOI of 5). After 2 and 4 days, respectively, infected cells were co-cultured with HPV E6-specific reporter cells (left graphs) and HCMV pp65-specific reporter cells (right graphs), respectively, for 24 h at a ratio 2:1. Subsequently, EGFP expression of reporter cells was determined by FACS analysis. Uninfected cells (Mock) and cells infected with RVTB40ΔUS11 (Control) were also included in this type of analysis. Stimulation of reporter cells is given as percentage of maximal peptide stimulation, i.e., stimulation of reporter cells incubated with peptide pulsed cells. Results are derived from three technical replicates; error bars represent the mean ± SEM. $^{****}P < 0.0001$; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, one-way ANOVA test.

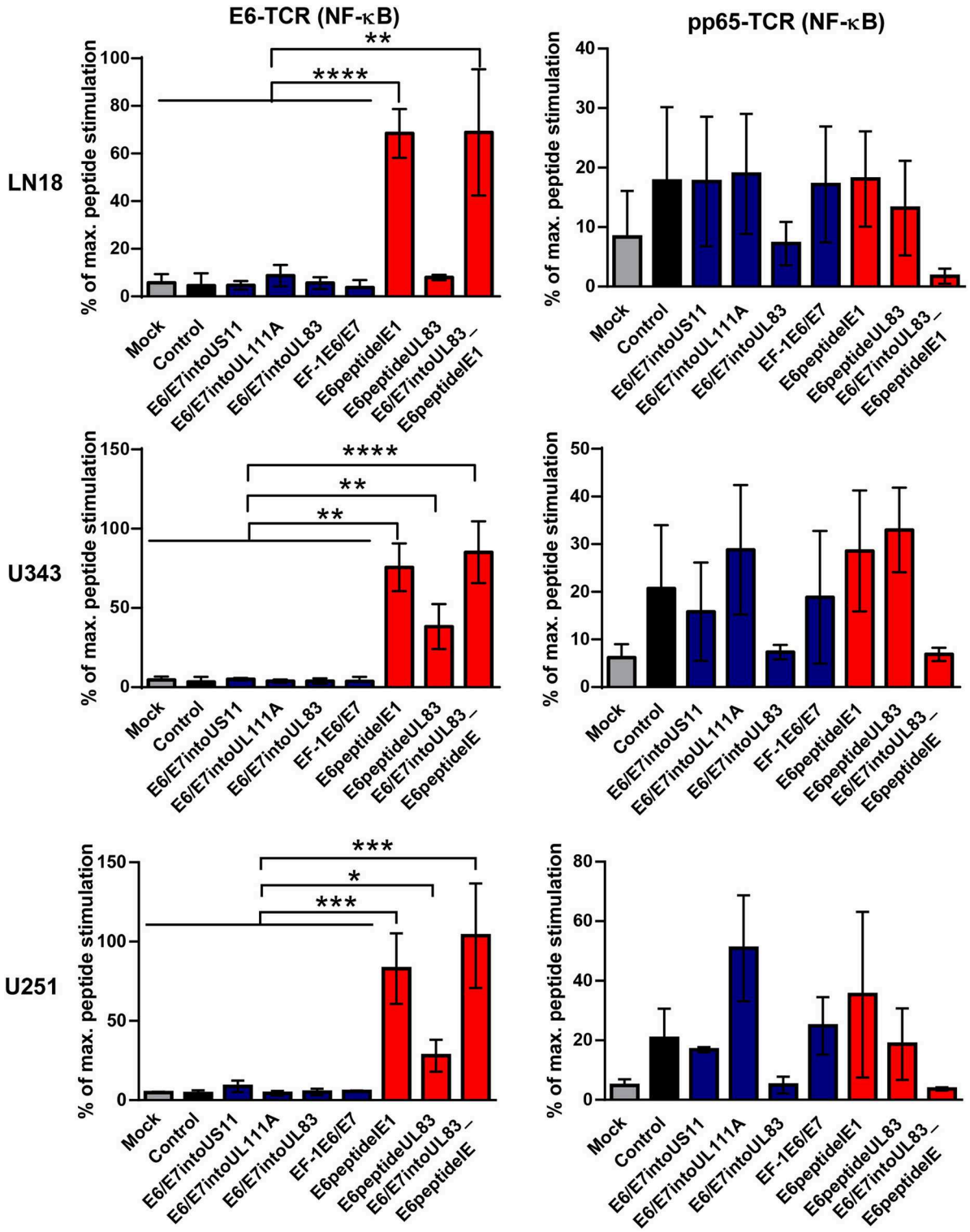


FIGURE 7 | NF-κB-driven EGFP expression in reporter cell lines stimulated by infected GBM cells. 5×10^4 GBM cells (LN18, U343, or U251 cells) were infected with HCMV-based vaccines (MOI of 5). After 2 and 4 days, respectively, infected cells were co-cultured with HPV E6-specific reporter cells (left graphs) and HCMV pp65-specific reporter cells (right graphs), respectively, for 24 h at a ratio 2:1. Subsequently, EGFP expression of reporter cells was determined by FACS analysis. Uninfected cells (Mock) and cells infected with RVTB40ΔUS11 (Control) were also included in this type of analysis. Stimulation of reporter cells is given as percentage of maximal peptide stimulation, i.e., stimulation of reporter cells incubated with peptide pulsed cells. Results are derived from three technical replicates; error bars represent the mean ± SEM. $^{****}P < 0.0001$; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, one-way ANOVA test.

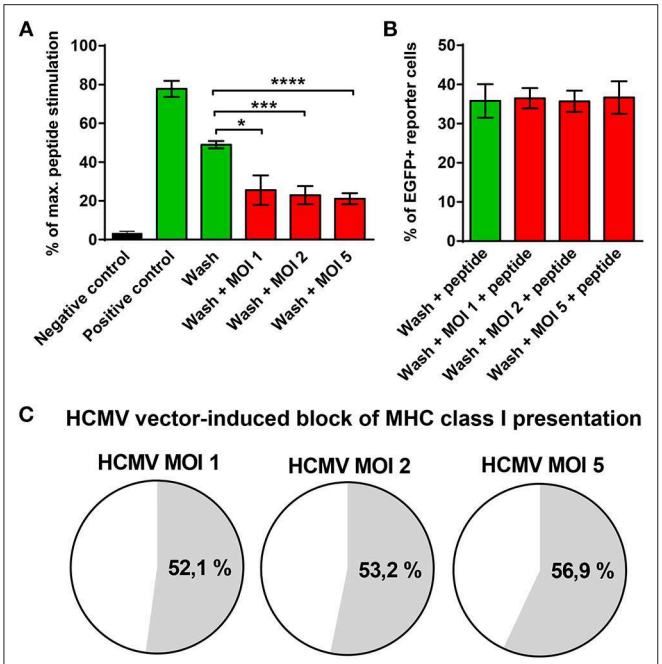


FIGURE 8 | Block of MHC class I presentation induced by immunoevasin-deficient HCMV. U251 cells stably expressing the E6/E7 fusion protein (U251-E6/E7 cells) were left uninfected or infected with RVTB40ΔUS11, a mutant HCMV lacking all known immunoevasins, at the indicated MOIs for 3–24 h. Subsequently, cells were harvested, washed with ice-cold citric acid, to remove all preexisting peptide-MCH complexes and cocultured at a ratio of 2:1 with HPV E6-specific reporter cells, in which NF-κB drives EGFP. After 18 h EGFP expression was assessed by FACS analysis. Unwashed U251 cells (Negative control) and unwashed U251-E6/E7 cells (Positive control) were also cocultured with HPV E6-specific reporter cells. In parallel, maximal peptide stimulation was determined for each experimental group by pulsing cells additionally with E6 peptide (1 μg/ml) before coculture with HPV E6-specific reporter cells and subsequent FACs analysis. **(A)** The stimulation in each experimental group is given as percentage of maximal peptide stimulation. **(B)** The % of EGFP+ reporter cells after pulsing with E6 peptide (maximal peptide stimulation) is shown for washed U251-E6/E7 cells left uninfected and washed U251-E6/E7 cells infected with mutant HCMV at the indicated MOIs. **(C)** The block of MHC class I presentation after infection with mutant HCMV at the indicated MOIs is given as a percentage. The results shown are derived from three independent experiments. Error bars represent the mean ± SEM (****$P < 0.0001$; ***$P < 0.001$; *$P < 0.05$; unpaired t-test).

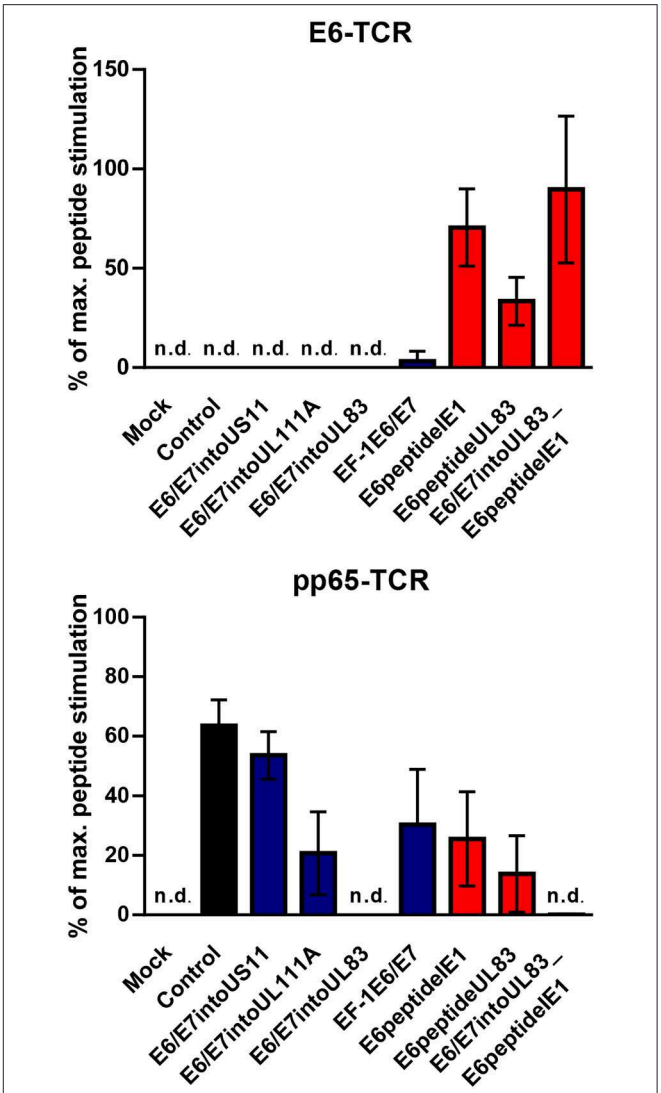


FIGURE 9 | Release of IFN-γ by TCR transduced PBMCs after stimulation with infected cells. 1×10^5 human PBMCs, transduced with HPV E6-specific TCR and HCMV pp65-specific TCR), respectively, or non-transduced (Background), were co-cultured for 16 h with 1×10^5 fibroblasts that had been infected for 48 h with HCMV-based vaccines. PBMCs as described above were also co-cultured with infected fibroblasts that had been additionally pulsed with the corresponding peptide (maximal peptide stimulation). Subsequently, IFN-γ production was measured by ELISA. Release of IFN-γ is shown as percentage of maximal peptide stimulation after subtraction of the background. Uninfected cells (Mock) and cells infected with RVTB40ΔUS11 (Control) were also included in this type of analysis. Results are derived from three experiments; error bars represent the mean ± SEM; n.d., not detectable.

E6 peptide, however, acid washed infected U251-E6/E7 cells stimulated E6-specific reporter cells to a similar extent as acid washed uninfected U251-E6/E7 cells (**Figure 8B**). In fact, the block of MHC class I antigen presentation induced by the HCMV-vector was more than 50% (**Figure 8C**). Taken together, we discovered a previously unsuspected HCMV-encoded block of MHC class I presentation.

Genetically Altered Human T Cells Secrete IFN-γ in Response to E6 Peptide but Not E6/E7 Expressing HCMV-Based Vaccines

HCMV-based vaccines enabling presentation of a neo-epitope by tumor cells could be combined with adoptive transfer of genetically modified T cells specific for the vectored neo-epitope. In order to test this option and verify our results obtained with the reporter cell lines, we transduced human PBMCs with either retroviral vector encoding E6-specific TCR or retroviral vector encoding pp65-specific TCR. Subsequently, we co-cultured these cells for 16 h with vaccine-infected fibroblasts that express HLA-A2. Untransduced PBMCs were included as a negative control and treated in the same way. After co-culture the release of IFN-γ was measured as a read out of T cell function.

Moreover, we pulsed aliquots of the vaccine-infected fibroblasts with the corresponding E6-derived and pp65-derived peptides, respectively. These cells were also co-cultured with transduced PBMCs to assess the maximal peptide-stimulated IFN-γ release. In **Figure 9**, the specific IFN-γ release induced by vaccine-infected fibroblasts is given as a percentage of IFN-γ release after stimulation with cells that had been additionally pulsed with exogenous peptide (maximal peptide stimulation). As observed for reporter cell lines, cells infected with HCMV-based vaccines expressing E6/E7 protein did not stimulate PBMCs transduced with E6 peptide-specific TCR (**Figure 9**, upper graph, blue columns). In contrast, cells infected with HCMV-based vaccines expressing the E6 peptide fused to the C-terminus of HCMV IE1 or HCMV UL83 induced IFN-γ release by E6-specific PBMCs (**Figure 9**, upper graph, red columns). Moreover, all HCMV-based vaccines with intact *UL83* (pp65) were able to activate PBMCs transduced with pp65-specific TCR (**Figure 9**, lower graph). Taken together, PBMCs transduced with E6-specific TCR could be used for adoptive transfer to detect tumor cells targeted by E6 peptide-expressing HCMV-based therapeutic vaccines.

DISCUSSION

In this study, we generated HCMV-based therapeutic vaccines that lack immunoevasins for *in situ* vaccination of GBM patients. We pursued two different strategies to channel a defined vector-encoded neo-epitope into the processing machinery of antigen-presenting cells. In one set of HCMV-based vaccines, we expressed the consensus sequence encoding an immunogenic but non-transforming E6/E7 fusion protein under the control of endogenous or exogenous promoters. In another set, we fused a single E6-epitope to the C-terminus of HCMV IE1 or HCMV UL83. Surprisingly, GBM cells transfected with an E6/E7 expression plasmid but not cells infected with the E6/E7 expressing HCMV-vectors were recognized by E6-specific T cells despite comparable E6/E7 expression. In contrast, cells infected with HCMV-based vaccines expressing an E6-epitope fused to HCMV proteins by an Alanine linker nicely stimulated E6 peptide-specific T cells. Subsequent analysis demonstrated a previously unnoticed HCMV-encoded block of MHC class I presentation that could explain the failure of E6/E7-expressing vaccines.

The central nervous system is subjected to continuous immunosurveillance through special gateways that allow exchange of immune cells and antigens with the periphery (66). As outlined in a recent review (67), antigens in the CNS are transported to cervical lymph nodes either in a soluble form or via APCs that take up antigen in the meningeal linings. After priming in the CNS-draining lymph nodes, antigen-specific T cells home back to the CNS to kill their target. Thus, *in situ* vaccination with a HCMV-based therapeutic vaccine in the brain can activate specific cytotoxic T cells in the CNS-draining lymph nodes. These in turn can migrate back to the CNS to eliminate tumor cells. We found that LN18, U343, and U251 cells are susceptible to HCMV infection as previously reported for other GBM cell lines (38, 60). Moreover, it has been recently shown that HCMV targets Glioma stem–like cells (GSCs) (60, 68). GSCs are radioresistant and chemoresistant and play a crucial role in progression and recurrence of tumor cells. Accordingly, they represent attractive targets for novel GBM therapies (69). HCMV-based therapeutic vaccines expressing E6 peptide as a neo-epitope and lacking immunoevasins could render these tumor-driving cells vulnerable to cytotoxic attack by E6-specific CD8+ T cells. After killing of GSCs release of apoptotic debris containing further tumor-specific antigens could be phagocytosed by resident microglia or brain endothelial cells, which efficiently cross prime CD8+ T cells (70, 71). In addition, many viruses including HCMV can trigger bystander activation of antiviral memory CD8+ T cells as part of an early line of antiviral defense (72–77). Thus, therapeutic HCMV-based vaccines as described in this study could amplify the anti-tumor response in GBM patients by several distinct mechanisms.

HCMV-based vaccines expressing the E6-epitope fused to the C-terminus of HCMV IE1 or HCMV UL83 could easily activate E6-specific T cells. In accordance, MCMV-vector expressing a HPV E7-derived peptide at the C-terminus of MCMV IE2 protein could efficiently protect mice from lethal tumor challenge (62, 78). In contrast, cells infected with HCMV-based vaccines expressing the E6/E7 protein separately from viral proteins did not stimulate E6-specific reporter cell lines or E6-TCR transduced PBMCs despite strong E6/E7 expression. The fusion protein E6/E7, however, was not *per se* resistant to processing. Uninfected U251 cells stably transfected with pcDNA-E6/E7 (U251-E6/E7 cells) expressed E6/E7 at the same order of magnitude and stimulated E6-TCR expressing reporter cells. Thus, the E6 epitope is naturally processed and presented by HLA-A2 in the absence of HCMV.

After infection of U251-E6/E7 cells with HCMV, however, the MHC class I presentation of the E6 peptide derived from the E6/E7 fusion protein was impaired. This was not due to known HCMV-encoded immunoevasins as we used RVTB40ΔUS11 as a vector. This mutant HCMV lacks US2, US3, US6, and US11, the known immunoevasins. It is well-described that cytosolic and nuclear proteasomes have to degrade viral proteins to generate the viral peptides that are presented by MHC class I molecules on the cell surface (79). On the other hand, viral pathogens such as herpes simplex viruses and HCMV highjack and relocalize the proteasomal machinery of the host cells to facilitate their own replication (80–82). Thus, these pathogens may diminish the proteasomal activity for processing of antigens thereby reducing the presentation of peptides by MHC class I molecules. The precise mechanism underlying this novel virus-induced block of MHC class I presentation remains to be elucidated.

We observed that GBM cells infected with HCMV-based therapeutic vaccines stimulate IFN-γ release by pp65-sepcific T cells. In fact, pp65 is the most abundant HCMV-encoded protein (83) and represents a major target for the CD8+ T cell responses in infected human individuals (84, 85). It may be a useful target for immunotherapeutic interventions in GBM patients as pp65-specific cytotoxic T cells lyse HCMV-infected GBM cell lines *in vitro* (86, 87). Thus, PBMCs derived from GBM patients could be transduced *in vitro* with retroviral vectors encoding pp65-TCR and adoptively transferred back to

eliminate GBM cells. Experiments with rhesus CMV in rhesus macaques, an animal model for HCMV infection of humans, have demonstrated that pp65-specific T cell responses are important for limiting viral dissemination during primary infection (88). This result implies that simultaneous application of pp65-specifc T cells with *in situ* vaccination of HCMV-based therapeutic vaccines prevents unwanted side effects due to virus spread. Thus, although pp65 helps HCMV to subvert host defense (89–93) and is not required for viral replication (94) it should not be eliminated from a HCMV-based therapeutic vaccine. On the other hand, it is important to use HCMV-based vectors, which do not express cmvIL-10 (UL111A) for several reasons. Firstly, cmvIL-10 dampens the antiviral immune response (95–100). Secondly, cmvIL-10 produced by HCMV-infected GSCs can induce immunosuppressive macrophages and microglia, which subsequently support tumor growth (42, 101).

Autologous DC vaccines generated *ex vivo* from peripheral blood monocytes represent another promising novel approach in immunotherapy of GBM patients (40, 41, 102–104). They can complement adoptive T cell transfer and *in situ* vaccination and play a role in adjuvant treatment of cancer including GBM (105). HCMV-based vectors may be useful for generation of DC vaccines because HCMV infects DCs (43, 106). However, cmvIL-10 confers an immunosuppressive function upon HCMV-infected DCs (95–100, 107). Thus, HCMV-based vaccines lacking cmvIL-10 may be suitable for generation of autologous DCs that stimulate pp65-specific T cells and neo-epitopes expressed by the HCMV-based vector.

Besides GBM cells HCMV also infects cells from other malignant human tumors including colorectal carcinoma and prostate cancer (108–110). Accordingly, patients with these malignancies could also benefit from vaccination with HCMV-based therapeutic vaccines expressing neo-epitopes.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

MA designed research, performed experiments, analyzed data, and contributed to figure preparation. SO performed experiments. AK designed experiments and provided intellectual input. JL and PS contributed new reagents, analytic tools. GW contributed new reagents, analytic tools, designed experiments, analyzed data, and provided intellectual input. MR designed research, analyzed data, provided intellectual input and contributed to manuscript writing. GS was involved in experiment conception, wrote the paper, analyzed data, provided intellectual input and prepared figures.

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REVIEW
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Immune Conversion of Tumor Microenvironment by Oncolytic Viruses: The Protoparvovirus H-1PV Case Study

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Cancer cells utilize multiple mechanisms to evade and suppress anticancer immune responses creating a "cold" immunosuppressive tumor microenvironment. Oncolytic virotherapy is emerging as a promising approach to revert tumor immunosuppression and enhance the efficacy of other forms of immunotherapy. Growing evidence indicates that oncolytic viruses (OVs) act in a multimodal fashion, inducing immunogenic cell death and thereby eliciting robust anticancer immune responses. In this review, we summarize information about OV-mediated immune conversion of the tumor microenvironment. As a case study we focus on the rodent protoparvovirus H-1PV and its dual role as an oncolytic and immune modulatory agent. Potential strategies to improve H-1PV anticancer efficacy are also discussed.

Keywords: oncolytic viruses, H-1PV, immunotherapy, immunogenic cell death, combination therapy, tumor microenvironment, checkpoint blockade

INTRODUCTION

After the market approval of Imlygic® (Talimogene laherparepvec, T-Vec, Amgen, Thousand Oaks, CA, USA) (1), oncolytic viruses (OVs) are gaining tangible momentum as a new class of anticancer agents. This is apparent from the fact that more than 40 OVs belonging to at least ten viral families are currently undergoing clinical trials against various malignancies, as monotherapy or in combination with other anticancer modalities (2). Most likely, other OVs will soon be approved for use as novel therapeutics for cancer patients.

OVs selectively replicate in and kill tumor cells in a multimodal fashion while sparing normal tissues. Productive virus infection ends with the lysis of the cancer cell and the release of progeny viral particles. In this way, OVs have the ability to multiply and spread throughout the tumor bed. Importantly, OV-mediated cell death is often immunogenic and accompanied by the activation of anticancer immune responses (3). The relevance of this immunological facet of oncolytic virotherapy is further emphasized by the limited OV propagation observed in cancer patients (4).

In this review we provide a brief introduction of the tumor microenvironment, its immune components and the different strategies developed by tumors to avoid attack from the immune system, before focusing on the ability of OVs to act as immune adjuvants and contribute to the induction of systemic antitumor immunity. We also discuss possible ways to enhance the anticancer activity of OVs by combining them with

other anticancer treatments and in particular with other forms of immunotherapy (e.g., checkpoint blockade). We use the protoparvovirus H-1PV, one of the OVs presently under evaluation in the clinic, as a case study.

THE TUMOR MICROENVIRONMENT

Solid malignant tumors comprise not only a heterogeneous population of neoplastic cells but also a multitude of resident and infiltrating non-transformed cells, secreted factors and extracellular matrix (ECM) proteins, which altogether constitute the tumor microenvironment (TME) (5). The non-transformed cells of the TME consist in particular of cancer-associated fibroblasts (CAFs), adipocytes, stromal, and vascular endothelial cells, pericytes, lymphatic endothelial cells, and recruited cells of the immune system. Tumor-infiltrating immune cells include T-lymphocytes [$CD8^+$ cytotoxic (memory) T-cells, $CD4^+$ helper (Th1, Th2) T-cells, and regulatory T-cells (Tregs)], B lymphocytes (B-cells), tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), and natural killer (NK) cells (5, 6).

Non-neoplastic cells may account for more than 50% of the total tumor mass, and their composition varies between different tumors. Like cancer cells, non-malignant cells produce, and release cytokines, chemokines, growth factors, matrix remodeling enzymes, vesicles, and other soluble factors into the tumor mass, often supporting tumor growth (5). Metabolic interactions between cancer and non-malignant cells influence all stages of carcinogenesis.

The ECM network, an important TME component, consists of a flexible deposit of collagen and fibronectin fibrils associated with glycoproteins, proteoglycans, and polysaccharides within and around tumor areas (7). The ECM not only serves as a physical scaffold for all cells of the TME, but also provides biochemical signals by hosting growth factors and chemokines modulating tumor cell growth, migration, and metastasis (7, 8). Although the formation of the ECM is primarily the responsibility of CAFs, cancer cells also contribute. Cancer development and progression are associated with increased ECM deposition (7).

Recent findings from whole-genome sequencing and microRNA expression profiling studies (9, 10) have further highlighted the key role of non-malignant cells and other TME components in influencing tumor growth, immune tolerance, metastasis, and therapeutic resistance (11, 12). It follows that targeting these "normal" elements may represent a new approach to complement conventional therapies and develop innovative and more efficient treatments against cancer.

ANTI-TUMOR IMMUNE RESPONSE

Among the non-transformed cells of the TME, immune cells have attracted the most attention in the past decade and have become the subject of intense preclinical and clinical research. In a healthy body, the immune system is able to detect and eliminate malignant cells (13), a phenomenon referred to as immune surveillance against tumors. The two main components of this surveillance are activated cytotoxic $CD8^+$ T cells (13, 14) (also called cytotoxic T lymphocytes, CTLs) and NK cells (15) which belong, respectively, to the adaptive and innate arms of the immune system.

(i) In order to exert their tumoricidal activity, CTLs have to recognize tumor-associated antigen (TAAs) motifs presented by major histocompatibility complex class I molecules (MHC-I) on tumor cells. To become activated, naïve CTL need to be previously primed by professional, antigen-presenting cells (APCs) which expose TAA motifs through MHC-I molecules to T-cell receptors on CTLs. CD28 molecules expressed at the surface of CTLs bind to CD80 or CD86 polypeptides exposed on APCs (DCs or macrophages), providing a co-stimulatory signal for CTL killing activation. CTL tumoricidal activity is carried out both directly through the release of cytotoxic granules containing perforin and granzymes, and indirectly through the secretion of cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-2. These cytokines induce apoptosis of tumor cells and/or activation of anticancer immune responses (16). $CD4^+$ T helper cells also contribute to the cytotoxic anticancer immune response mediated by CTLs, by stimulating CTL priming through the release of cytokines, particularly IFN-γ (17).

(ii) In contrast to CTLs, NK cells do not require specific TAA recognition to interact with tumor cells nor MHC-dependent cross-priming. A repertoire of inhibitory and activating receptors on these cells, makes their activity dependent on the down- or up-modulation of various ligands exposed on tumor cells, respectively. Similarly to CTL, NK cells are able to kill neoplastic cells directly by releasing perforin and granzymes as well as indirectly by secreting death receptor ligands (FasL and TRAIL) and cytokines [IFN-γ, TNF-α, and granulocyte macrophage-colony-stimulating factor (GM-CSF)] (15). NK cell functions can be activated or exacerbated in presence of cytokines released by DCs and monocytes (IL-12 and IL-15) as well as T-cells or NK cells themselves (IL-2) (18).

In conclusion, the immune system appears to play a major pleiotropic role in the surveillance against tumors.

TUMOR STRATEGIES OF IMMUNE EVASION

As mentioned above, $CD8^+$ T-cells, NK cells, and monocytes populate TMEs. The presence of these cells was found to correlate with a better prognosis and treatment responsiveness of various tumors including brain, hepatocellular, lung, breast, renal, colorectal cancers, and melanoma (19, 20). TMEs containing these immune cell populations are called inflamed. Unfortunately, the immunosuppressive ecosystem prevailing in many TMEs suppresses NK and CTL cytotoxic activities, thereby precluding long-standing protective immunity. In addition,

the TME often inhibits T-cell proliferation, promotes T-cell apoptosis, down-regulates expression of MHC molecules and antigen processing machinery components on most cells within tumors (in particular neoplastic cells, DCs, and $CD4^+$ T helper cells) and corrupts TAMs toward an M2 immunosuppressive phenotype, thereby allowing tumor cells to escape attack from the immune system. For a comprehensive discussion of the strategies developed by cancer cells to escape immune surveillance, we redirect readers to excellent recent reviews by Muhn and Bronte (21) and Fearon (22). Briefly, a main mechanism by which tumors prevent attack from the immune system consists in the release within TMEs of immunosuppressive molecules such as growth factors [e.g., transforming growth factor (TGF)-β], cytokines [e.g., interleukin-10 (IL-10)], chemokines, inflammatory, and matrix-remodeling enzymes as well as metabolites. These molecules contribute to establish complex and dynamic communication networks between all the cells composing a tumor in order to promote its survival, development, and metastasis. These molecules are produced not only by tumor cells, but also by non-malignant cells of the TME including CAFs (23, 24), adipocytes (25), and infiltrating immune cells such as Tregs (26, 27), Bregs (28, 29), MDSCs (30, 31), and TAMs (32). Thus, diverse cell subtypes depending on their activation state by producing and secreting these molecules simultaneously participate in establishing an immune-suppressive TME *via* multiple mechanisms [e.g., Tregs through the production of IL-10 and TGF-β inhibit CTL and NK cytolytic activity, promote Treg survival, and expansion and modulate the activity of other immunosuppressive cells within the TME such as Bregs, MDSCs, TAMs and CAF, which in their turn concur to augment immunosuppression (26, 27)]. The activity of these cells may change from tumor to tumor and during the different phases of tumorigenesis and even between different regions within the same tumor.

A second immune-inhibitory mechanism relies on a natural process developed by the immune system to regulate the amplitude and the quality of the T-cell response. This mechanism is triggered to prevent the immune response from getting over-activated and causing autoimmune reactions that could damage healthy tissues. The factors involved in this inhibitory process are collectively referred to as immune checkpoint molecules (ICs) and are expressed at the surface of several cell populations of TME. The mechanism is triggered upon interaction of ICs acting as receptors and located on tumor-infiltrating effector T-cells, B-cells and NK cells, with specific ICs behaving as ligands and often expressed at the surface of APCs, Tregs, TAMs, and MDSCs. Interestingly, ICs ligands are overexpressed in many tumor cells. Well-known examples of IC-receptors include the CTL-associated antigen 4 (CTLA-4/CD152), the programmed death receptor 1 (PD-1/CD279), and the molecules lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin, and mucin domain containing protein 3 (TIM-3), and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (33–35). The corresponding ligands are CD80 and CD86 for CTLA-4, and programmed death receptor ligand 1 and 2 (PD-L1/CD274, PD-L2/CD273) for PD-1. These IC receptor-ligand interactions play a critical role in blocking anticancer immune responses mediated by cytotoxic T-cells and NK cells in TMEs (35). The underlying molecular mechanisms involved in these inhibitory signaling pathways are complex and beyond the scope of this review (36–38). Within the TME, tumor cells and myeloid cells are considered to be the main cell types responsible for T-cell suppression through the expression of PD-1 ligands (39).

CANCER IMMUNOTHERAPY

To overcome tumor-driven immune evasion and suppression, a new appealing therapeutic strategy, namely cancer immunotherapy, emerged, and was recognized as the breakthrough of the year 2013 (40). Presently, the field is rapidly expanding, yielding continuously growing evidence of clinical efficiency in patients with various types of solid and hematological tumors. Cancer immunotherapy is generally based on two approaches. Passive immunotherapy aims at enhancing an already existing antitumor immune response; active immunotherapy attempts to trigger the latter *de novo*. Administration of immunomodulating antibodies (e.g., immune checkpoint inhibitors, ICIs) and the adoptive transfer of tumor-infiltrating lymphocytes or chimeric antigen receptor (CAR) T-cells represent the passive immunotherapy approach, while the active one is exemplified by anticancer vaccination [discussed in this issue by Fennemann et al. (41)]. Current cancer immunotherapeutic strategies, their molecular bases, challenges, and future directions and prospects are extensively reviewed in various recent publications to which the reader is redirected (41–44). Special attention is paid in the present review to immune checkpoint blockade using ICIs, given its relevance to the oncolytic virotherapy approaches discussed below.

IMMUNE CHECKPOINT BLOCKADE

The discovery of the aforementioned immunosuppressive pathways represented a breakthrough for oncology, as illustrated by the 2018 Nobel Prize in Physiology or Medicine jointly awarded to Allison and Honjo for their contribution to novel cancer therapy approaches based on the inhibition of negative immune regulation. Indeed, these findings have paved the way for the development of innovative treatments that aim to restore or boost anticancer immune responses in TMEs through alleviation of the immunosuppressive signals inhibiting the cytotoxic activities of CTL and NK cells (35). Like other promising cancer immunotherapies using DC-based vaccines (45) and CAR T-cell therapy (46), the application of immune checkpoint inhibitors is currently the subject of intense efforts worldwide to harness the power of the immune system against cancers.

While small-molecule immune checkpoint inhibitors are under development (47), immune checkpoint blockade (ICB) has been successfully achieved using monoclonal antibodies that interfere with the interactions between checkpoint receptors and cognate ligands by targeting either of these molecules (48). Examples of ICB include nivolumab and pembrolizumab directed against PD-1; ipilimumab specific for CTLA-4; and

atezolizumab, durvalumab, and avelumab developed against PD-L1. These market-approved antibodies, alone or in combination, showed impressive results against several types of cancer including melanoma and lung carcinomas (48, 49), with some patients experiencing a durable and complete anticancer response. New antibodies targeting the more recently discovered immune checkpoint molecules Tim-3 (50) and LAG-3 (33) have shown pre-clinical efficacy and are now entering clinical trials (51, 52).

Despite these successes, it should be stated that only a fraction (10–40%) of treated patients responds positively to checkpoint blockade with PD-1 or PD-L1 specific antibodies (53). In addition treatment resistance is common (54, 55) influenced at least in part by patient HLA class I genotype (56). Furthermore, the appearance of severe immune-related adverse events due to an exacerbated activation of the global immune system (57, 58) hampers (combinatorial) treatments with checkpoint blocking antibodies.

The clinical outcome of checkpoint blockade is thought to depend on the neoantigen load of tumors as well as the size and composition of the immune cell population present in the tumor bed. Inflamed tumors (also referred to as hot tumors) that contain $CD8^+$ and $CD4^+$ T-cells, monocytes and pro-inflammatory cytokines, show the best response rate to ICB (59). Indeed, the immune landscape of inflamed tumors is indicative of a pre-existing antitumor immune response that has been silenced by the tumor-bed suppressive environment, as revealed by prominent Treg and MDSC infiltration, production of anti-inflammatory cytokines or T cell exhaustion. Another common feature of inflamed tumors is the elevated expression of PD-L1 by neoplastic or immune cells. A PD-1/PD-L1 signature in tumors generally correlates with a positive response to anti-PD-1 therapy (19), although PD-L1 expression is not a prerequisite for successful checkpoint therapy.

In contrast, immune-excluded or deserted tumors (cold tumors) are characterized by poor or almost no T-cell infiltration in the stroma, and they respond poorly to ICB (20, 60). Therefore, it is clear that the development of new strategies to convert a cold TME into a hot one, is essential for improving the clinical outcome of ICB and increasing the proportion of patients who benefit from this treatments. One of the most promising strategies in this respect is the use of OVs.

ONCOLYTIC VIRUSES

In recent years, OVs have attracted significant attention as anti-cancer agents because they preferably replicate in, and eventually lyse, tumor cells while sparing normal counterparts. Tumor cells offer a favorable environment for the lytic replication of many OVs that exploit various physiological alterations occurring in cancer cells. These tumor cell defects are often associated with: (i) rapid proliferation and dysregulated metabolism (61); (ii) impairment of antiviral immune responses (62); (iii) production of immune suppressive factors in the TME (63, 64), (iv) intracellular signaling pathway alterations that promote survival under stress conditions (65, 66). Besides directly killing tumor cells through activation of different cytocidal programs ranging from apoptosis, pyroptosis, and necroptosis to autophagy and lysosome-dependent cell death, OVs proved able to convert a cold TME into an inflamed one, thereby reawakening antitumor immune responses. Due to their multimodal activity, OVs have become a major focus of interest in cancer therapy research. As a result of their oncosuppressive activities, more than forty OVs are presently in clinical testing against various malignancies and a number of OVs are undergoing phase III clinical trials (67).

This list of OVs under investigation includes herpes simplex virus (HSV), adenoviruses (Ad), vaccinia virus (VV), measles virus (MV), coxsackie virus, poliovirus, protoparvovirus, reovirus, Newcastle disease virus, vesicular stomatitis virus (VSV), and Seneca Valley virus. Some of the OVs undergoing clinical trials are based on human pathogens (e.g., Ad, HSV, MV, poliovirus) and are engineered to reduce their toxicity and compel their lytic multiplication in response to factors and/or pathways specifically active in tumor cells.

The therapeutic potential of OVs can be best exemplified by the clinical benefit of the prototypical drug in this class, the genetically modified type 1 HSV designated talimogene laherparepvec (T-Vec). For a recent T-Vec review, the reader is referred to Conry et al. (68). Based on encouraging clinical results, T-Vec became the first oncolytic virus to receive regulatory approval by FDA in 2015 with an indication for advanced melanoma (1). This virus was engineered to prevent production of both its neurovirulence protein ICP34.5 required for lytic infection of normal cells (in particular neurons), and its ICP47 protein that reduces MHC class I expression and virus/tumor antigen presentation by infected cells. These changes also brought the viral US11 gene under control of an early/intermediate promoter, partially reinvigorating the virus lytic activity in tumor cells. Furthermore, two copies of the human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene were introduced into the virus genome to enhance it immunogenicity, knowing that GM-CSF production by cancer cells attracts DCs in the tumor niche and enhances their antigen presentation function. T-Vec propagates preferentially in neoplastic cells in which the malignant transformation process has impaired the PKR (e.g., through oncogenic Ras activation) and/or type I IFN pathways, features characterizing in particular many melanoma cells.

In a phase III randomized clinical trial (OPTiM) conducted in 436 patients with stage IIIB to IV melanoma, intralesional injection of T-Vec resulted in significantly better durable and overall response rates compared with subcutaneous administration of GM-CSF alone, with superior overall survival for patients with stage III or IV M1a disease. The virus treatment proved also to be beneficial against non-injected lesions, demonstrating its ability to stimulate an anticancer immune response. In T-Vec-treated patients with cutaneous melanoma arising in the head and neck, a complete response rate of 30% was achieved, and 73% of responses persisted longer than 1 year. Based on these results, phase IB/II and IB/III clinical trials combining T-Vec with the immune checkpoint inhibitors ipilimumab (anti-CTLA-4) or pembrolizumab (anti-PD-1), respectively, were undertaken in patients with advanced

melanoma (see below section Strategies to improve OVs and optimize their immune stimulatory activities).

Another promising candidate for clinical applications is the oncolytic poliovirus PVSRIPO in which the internal ribosomal entry site of poliovirus is replaced with that of human rhinovirus type 2, to ablate neurovirulence. PVSRIPO uses for its entry the poliovirus receptor CD155, which is highly expressed on the surface of neoplastic cells and in other cells of the TME. There is preclinical and clinical evidence that this OV has strong ability to activate DCs and promote formation of tumor-specific CTLs (69). The results of a clinical trial in which 61 patients with grade IV malignant glioma were treated with PVSRIPO, showed increased survival rate (at 24 and 36 months) in 21% of patients in comparison with historical controls (70).

Other OVs are endowed with an intrinsic oncotropism, which can be traced back to their elevated sensitivity to the antiviral innate immune responses developed by normal human cells but often deficient in their neoplastic derivatives (e.g., VSV) and/or to the depending of their lytic multiplication on oncogenic pathways [e.g., activated Ras signaling for reovirus (66)]. The group of genuinely oncotropic OVs also includes non-human animal viruses (e.g., Newcastle disease virus or protoparvovirus whose natural hosts are avian or rodent species, respectively). One advantage of animal OVs lies in the lack of pre-existing antiviral immunity in contrast to human pathogen-based OVs against which patients may have developed neutralizing antibodies prior to virotherapy. For a complete list of OVs undergoing clinical testing we redirect the reader to these recent reviews (4, 71).

It is worth noting that no champion has emerged yet among the various OVs under investigation. Each OV has indeed its own peculiar modes of replication, action, and tumor specificity. This variation justifies the continued development and optimization of these ground-breaking anticancer agents.

ONCOLYTIC VIRUSES AS TOOLS TO HEAT UP TUMORS

OVs evoke anticancer immune responses through different mechanisms. In addition to releasing progeny virions into the TME, virus-mediated tumor cell lysis disseminate a wide repertoire of both cellular tumor-associated antigens/neo-antigens (TAAs/TANs), danger-associated molecular patterns (DAMPs) and viral pathogen-associated molecular patterns (PAMPs) which lead to an inflammatory immune response. In an ideal scenario, TAAs and TANs are captured and processed by infiltrating APCs, in particular DCs. DCs loaded with antigens migrate to draining lymph nodes where they mature and acquire the capacity to prime T-cells, thus leading to a cancer-specific T-cell response potentially directed against a wide spectrum of tumor antigens.

PAMPs consist of viral RNA, DNA, or proteins that are sensed by pattern recognition receptors (PRRs) expressed by DCs. PRRs include Toll-like receptors, RIG-like receptors, NOD-like receptors, and cGAS (72–74). As a consequence of PRR engagement, DCs produce pro-inflammatory (e.g., TNF-α and IL-12) and antiviral [type I IFNs (IFN-α and IFN-β)] cytokines (75). These cytokines contribute to TAA/TAN cross-presentation and priming of CTL, among other effects (76). It is noteworthy that the cGAS-Sting pathway in tumor-infiltrating DCs can also sense tumor-derived genomic DNA, leading to IFN-β production and eventually CTL activation (77), highlighting the relevance of this antiviral pathway in cancer development and therapy (78).

Interestingly, OV-infected cancer cells may sense PAMPs and contribute in a direct way to the production and release of pro-inflammatory cytokines into the TMEs. This is exemplified by the type I IFN response. Neoplastic transformation is often associated with defects in antiviral innate immunity, with cancer cells unable to produce type I IFN and/or to respond to these cytokines. However, in the context of heterogeneous tumors, a fraction of cancer cells may still be able to detect viral PAMPs through their PRRs and sustain significant type I IFN production. The virus-induced type I IFN response is pleiotropic and comprises facets which are undesirable (antiviral effects) and desirable (anticancer effects) in the context of oncolytic virotherapy. Type I IFNs thus act as a double-edged sword: on the one hand, they are directed against the virus by blocking its multiplication and inducing its neutralization and elimination and on the other hand, they have anticancer properties. The oncosuppressive potential of type I IFNs relies in part on their ability to arrest tumor cell proliferation and exert anti-angiogenic effects (79–81). Furthermore, type I IFNs may promote the activation of anti-tumor immune reactions (76). It is well-documented that type I IFNs are important regulators of NK cell and CTL functions. In particular, type I IFNs stimulate NK cell cytotoxic activity and NK cell-mediated production and secretion of IFN-γ (82, 83). Besides inhibiting angiogenesis and inducing cell cycle arrest and apoptosis of tumor cells (84), IFN-γ is a strong immune stimulant. In particular, INF-γ induces the expression of MHC class II molecules on DCs, activates and increases the phagocytic activity of macrophages, and promotes antigen-specific Th1 and CTL responses (84). Type I IFNs also have a crucial role in mediating the interplay between innate and adaptive immunity. Type I IFNs induce maturation of DCs, in particular upregulating the surface expression of MHC class I and co-stimulatory CD40 and CD86 molecules, which are essential for CTL activation (85). Type I IFNs support CTL differentiation and expansion (86, 87). Accordingly, virus-induced type I IFNs directly stimulate the cross-priming of CTLs by DCs (88), and are essential for the protection of activated T cells from NK cell cytotoxicity (89). Notably, IFN-α enhances the induction and maintenance of a Th1 response through its direct action on Th-cells (90).

OVs differ markedly in the ability to trigger production of type I IFNs. The type I IFN response is, for instance a main determinant of whether melanoma cells are resistant or sensitive to oncolytic MV (91). Whereas, NDV is a strong inducer of type I IFNs, human cells infected with protoparvovirus H-1PV produce very little of these antiviral cytokines (65, 92). The Seneca Valley virus actively inhibits the production of type I IFN by cleaving adaptor proteins necessary for this process (93). Nevertheless, these various OVs all show promising oncosuppressive activity in preclinical and clinical studies, indicating that type I IFNs

represent one of several factors that OVs can mobilize to heat up TMEs and activate immune responses against cancer cells.

In addition to the type I IFN induction resulting from some OV/tumor cell interactions, the way by which OVs kill cancer cells can stimulate an antitumor immune response before or during cancer cell lysis. Indeed, OVs (alone or in combination with cytotoxic agents) provoke various intracellular disturbances at the expense of cell organelles in particular mitochondria, lysosomes, endoplasmic reticulum and Golgi apparatus, which eventually results in the lysis of the cancer cell and the release of progeny viruses (94). Several OVs have been reported to induce oxidative stress with the production of ROS and reactive nitrogen species (RNS) and ER stress accompanied by Ca2+ release from ER with consequent Ca2+ dyshomeostasis and unfolded protein response (94). ROS/RNS may themselves induce ER stress with consequent Ca2+ release, while Ca2+ potentiates oxidative stress with enhanced production and release of ROS/RNS, thereby generating a positive amplification loop that results in the induction of apoptosis or other modes of cell death (95). Remarkably, OV-mediated cancer cell death is often immunogenic and associated with the expression, release, and/or exposure of DAMPs including ATP, high mobility group box 1 (HMGB1), and calreticulin (CRT). In particular, extracellular ATP acts as a "find me" signal promoting the recruitment of DCs (96), while HMGB1 functions as a danger signal ligand for Toll-like receptor 4 and can directly activate DCs (97). CRT exposure on the cell surface acts as an "eat me" signal neutralizing CD47 on tumor cells and promoting phagocytosis (98). DAMPs attract APCs, in particular DCs, into the TME and induce them to secrete inflammatory cytokines, present TAAs, and prime cytotoxic T-cells. The temporally concomitant release of type I IFNs and DAMPs from OV-infected tumor cells leads to the consideration of type I IFNs as DAMPs, because they trigger similar immunogenic effects and also because the expression of some DAMPs can most likely be activated by IFNs. While ATP, CRT, and HMGB1 represent the classical hallmarks of immunogenic cell death, other molecules behave as DAMPs, for instance annexin A1 (ANXA1) and cancer cell-derived nucleic acid (99). It would be interesting to analyze these molecules in the context of OV-induced cell death. Furthermore, it is most likely that other DAMPs involved in the completion of immunogenic cell death remain to be identified. Information about OV-mediated (immunogenic) tumor cell death is often incomplete and fragmentary (99) warranting further studies of this essential parameter of virotherapy. These studies will not only improve our understanding of the mode of action of OVs, but also provide clues to improve the efficacy of OV-based treatments.

It should also be stated that in addition to the above-mentioned effects on tumor and immune cells, some OVs are able to infect and replicate in endothelial cells. By causing disruption of tumor vessels, these OVs can thus contribute to the necrosis of tumor cells irrespective of their infection, through oxygen and nutrients deprivation (100–104). Furthermore, these OVs may also promote in this way the infiltration of immune cells into the TME.

In summary, the great interest raised by OVs in the field of cancer therapy relies on their abilities (i) to specifically replicate, multiply, and spread in a lytic manner in tumor cells (oncolysis), (ii) to trigger the release of PAMPs and TAAs/TANs from dying tumor cells, leading to the activation of innate as well as adaptive immune responses, (iii) to directly induce the expression of pro-inflammatory and immuno-stimulatory cytokines, in particular type I IFNs, in some tumor and immune cells, and (iv) to kill tumor cells via immunogenic mechanisms (immunogenic cell death) involving the production of DAMPs that are able to further stimulate immune cells, and (v) for some OVs, to break-down tumor vasculature, causing tumor cell starvation and facilitating immune cell infiltration. Based on these considerations, OVs are attractive candidates to activate innate and adaptive immune responses in TMEs and turn immune-excluded or immune-deserted tumors into inflamed ones (**Figure 1**).

STRATEGIES TO IMPROVE OVs AND OPTIMIZE THEIR IMMUNE STIMULATORY ACTIVITIES

Despite promising results obtained at the preclinical level, only a small proportion of cancer patients seems to benefit from OV-mediated therapy in clinical studies. A number of reasons account for these disappointing results. OVs, especially when delivered systemically, need to overcome several physiological and physical barriers to reach the tumor target and be effective [reviewed in Marchini et al. (105)]. For instance, sequestration, and neutralization by the mononuclear phagocyte system can dramatically restrict the systemic delivery of OVs. The presence of specific neutralizing antibodies (NAb), can also severely hamper OV systemic delivery and effectiveness, especially in the case of OVs based on human pathogens to which patients may have been previously exposed. For instance, seroprevalence is high against Ad type 5 (60 and 70% in Europe and USA) (106) and HSV (50–80% worldwide) (107), two viruses commonly used as OVs. NAb recognize and coat the virus particles, signaling them for destruction by competent cells. Virus clearance can occur very rapidly, eliminating the virus before its anticancer potential is expressed. Optimal use of these OVs therefore requires consideration of the counteracting effect of pre-existing anti-viral immunity. However, recent results obtained with reovirus (against which about 80% of the human population has developed immunity) indicate that the presence of anti-viral NAb is not always a negative event and paradoxically, can even enhance the delivery of systemically administrated reovirus into the tumor bed. Indeed, NAb-reovirus complexes were found to be taken up and delivered to the tumor by the monocytes present in the blood (108, 109). In most cases, NAb still limit OV activity, as exemplified by a phase I trial demonstrating the greater efficiency of MV in myeloma patients devoid of pre-existing NAbs (110). Several strategies have been developed to overcome anti-viral host immune responses and improve delivery, e.g., virus capsid engineering (111), chemical modification of virus capsid [e.g., PEGylation (112)] and use of cell carriers (e.g., DCs) (113). For instance, Ad vector PEGylation was shown to reduce liver uptake,

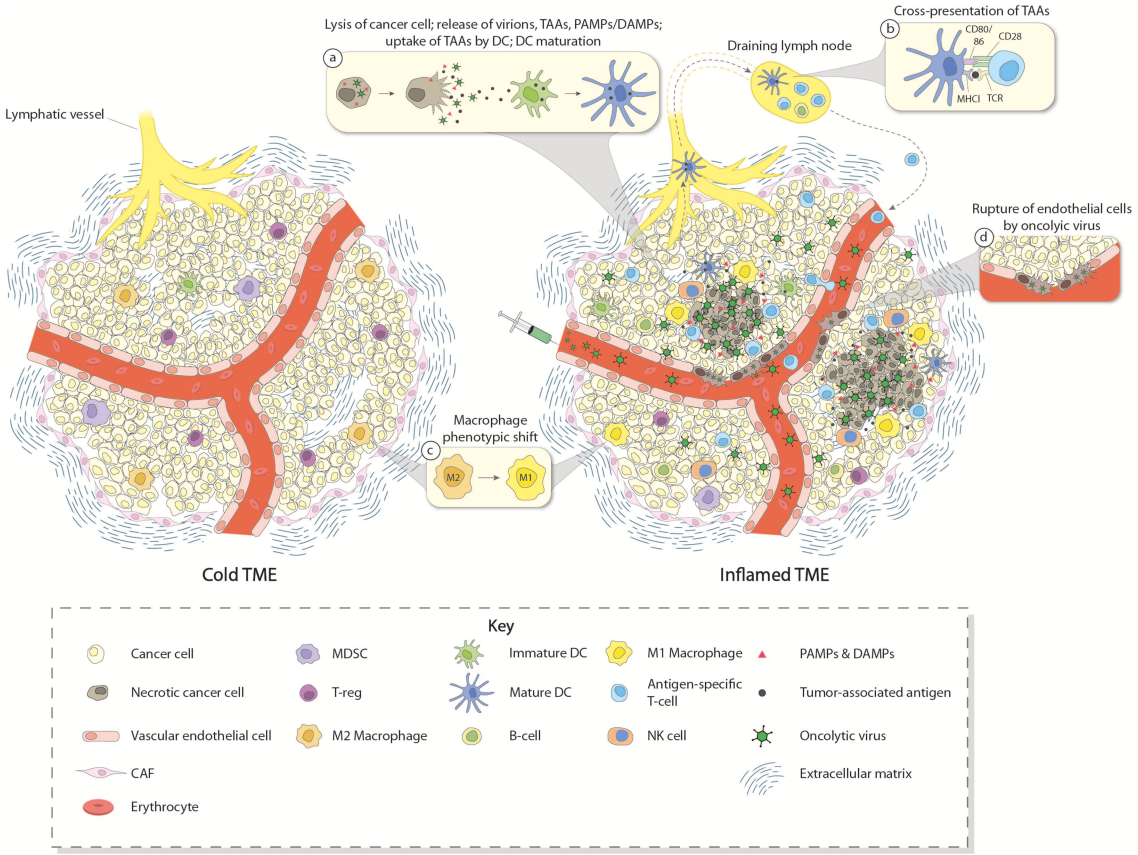

FIGURE 1 | Induction of immune conversion of tumor microenvironment by OVs. The left panel depicts a cold tumor microenvironment (TME). In addition to tumor cells, some other components of the TME are shown, i.e., blood vessel with endothelial cells, CAFs, ECM, and few infiltrating immune cells. These immune cells (mainly Treg, MDSC, and TAM having a M2 immunosuppressive status), together with other cells of TME (e.g., CAF and tumor cells themselves), produce and secrete chemo/cytokines, growth factors, and other molecules which contribute to create an immunosuppressive TME. This "cold" TME supports tumor development and metastasis, and confers resistance to (immuno) therapies. The right panel depicts an inflamed TME after intravenous OV treatment. OVs reach the tumor through the blood stream and act in a multimodal fashion to eliminate cancer cells. OVs specifically replicate in and kill cancer cells by inducing immunogenic cell death. Virus-induced cancer cell lysis is associated with the release of progeny virus particles, TAAs, DAMPs, PAMPs, and pro-inflammatory/immunostimulatory cytokines which contribute to recruiting immune cells in the TME and inducing maturation of DCs, thereby triggering innate as well as adaptive immune responses (inset a). DCs migrate to the draining lymph nodes where they cross-present TAAs to T cells (inset b). After expansion, T cells infiltrate the TME and participate in the destruction of cancer cells together with other effector cells such as NK cells and M1-converted macrophages (inset c). Some OVs may also infect endothelial cells and induce disruption of tumor vasculature, potentially facilitating immune cell migration into the TME (inset d).

prevent NAb binding, and thereby improve Ad half-life in blood and infection of tumors (112).

Potentiation of OVs can be achieved by inserting (a) therapeutic transgene(s) into the viral genome. Notable examples are OVs armed with payloads that have immune stimulatory activity, such as pro-inflammatory cytokine (e.g., GM-CSF, IFN-γ, IL-2, IL-12, or IL-15) or chemokine (e.g., CCL2, CCL5, CCL19, CXCL11) transgenes. OV arming with cytokine and chemokine genes is aimed at providing additional stimuli for turning immune-excluded and deserted tumors into hot inflamed ones by induction of immune cell migration and activation. The impact of different arming strategies on tumor heating up has been recently reviewed by de Graaf et al. (114). The success of this approach is exemplified by the HSV-based T-Vec recombinant expressing GM-CSF, a cytokine that stimulates DC migration and maturation, thereby conferring the virus with enhanced capacity for inducing antigen presentation and T-cell priming (68). Intratumoral administration of T-Vec was found to induce the regression of not only injected tumors but also of non-injected distant tumors, including visceral metastases, indicating virus ability to trigger a systemic antitumor immune response (68, 115, 116). Furthermore, treated mice were protected from re-challenging with the same tumor cells, which indicate a durable antitumor memory response (117). However, it should be stated that cytokine arming, in the context of OV therapy, needs to be carefully evaluated on a case-by-case basis, considering that OVs are replication-competent and transgene expression may thus get amplified. Cytokine overexpression can be deleterious, as illustrated by the severe side effects and hepatic toxicity associated with high dose regimes of recombinant IL-2.

Another promising approach makes use of OVs expressing bi-specific T-cell engagers (BiTEs). BiTEs represent a new class of immunotherapeutic molecules which consist of two single-chain variable fragments (scFv) connected by a flexible linker. One scFv recognizes a T-cell-specific molecule, e.g., CD3, while the second scFv is directed against a TAA expressed on the surface of tumor cells. In this way, BiTEs lead T-cells to target tumor cells, ultimately stimulating T-cell activation, tumor cell killing, and cytokine production. In addition to exert their intrinsic anticancer activity, OVs expressing BITEs are thus able to mobilize T-cells at tumor sites, resulting in an increased oncosuppressive potential (118, 119).

Important improvements of anticancer efficacy have been achieved by inserting genes encoding for scFv targeting immune checkpoint molecules (e.g., PD-1 or CTLA-4) into the viral genome. This approach has been applied successfully with myxoma virus, Ad, MV, and VV (120–123). By achieving intra-tumoral delivery and expression of checkpoint blockade, these recombinant OVs alleviate the risk of systemic unspecific side effects often encountered when the antibody blockers are administered by intravenous infusion. As the PD-1/PD-L1 checkpoint control may be triggered by tumor cells, i.e., at the site of action of OVs, the synergism between the latter and PD-1/PD-L1 checkpoint blockade is expected to be most efficient for this particular immune checkpoint. Indeed, the oncosuppressive activity of MV was found to be reinforced to a greater extent by anti-PD-1/PD-L1 than anti-CTLA-4 transgenes (122). However, intratumorally produced CTLA-4-specific antibodies may still enhance the adaptive antitumor response triggered by OV-activated APCs by getting transported to draining lymph nodes or intratumoral tertiary lymphoid structures where priming takes place. Indeed, growing evidence supports the assumption that local delivery of CTLA-4 blockade can trigger T-cell priming in the periphery (124) and release local effector cells by depleting intratumoral Tregs (125).

Combining multiple OVs also opens up interesting prospects, as demonstrated by a recent study in which Ad treatment was followed by VV administration in a Syrian hamster model. The first line OV treatment was found to protect the second virus from the attack of the immune system, enlarging its therapeutic window, and enhancing efficacy (126). An especially intriguing approach consists in a prime-boost protocol involving the sequential application of two distinct oncolytic viruses (vectors): a first one for priming the immune-system to recognize TAAs and a second one for boosting this response through virus-mediated TAA expression after systemic OV administration [reviewed in (127, 128)]. Also in this case, the use of a different virus vector in the priming phase may reduce the insurgence of NAb against the second virus used during the boosting phase. This strategy has also the potential to sensitize tumors to checkpoint blockade (129).

OV therapy is also compatible with other anticancer modalities, and investigation of OV-based combination treatments is actively being pursued with all OVs under clinical development.

(i) For the sake of expediting clinical translation, OV administration has been combined with conventional chemotherapy and radiotherapy, resulting in a number of cases in synergistic anticancer effects at the preclinical level (67, 130, 131). Some of these combinations are currently being tested in clinical studies.

(ii) In addition to combining with standard treatments, OVs are being tested together with immunomodulators, including drugs that induce immunogenic cell death [reviewed in (94)] or dampen the antiviral innate response (3).

(iii) A particularly promising area of active research involves the combination of OVs with adoptive immune cell therapy. In particular, OV can improve the efficacy of CAR-modified T cell transfer therapy [as recently reviewed in (3, 132)].

(iv) Owing to their ability to induce immune conversion of TMEs, a number of OVs have been clinically tested in combination with checkpoint blockade against a broad range of malignancies [for a recent review see (133)]. The joint application of these therapies is anticipated to improve the clinical outcome in cancer patients by eliciting more robust anticancer immune responses. This concept is supported by multiple preclinical evidence showing that OV treatment sensitizes tumors to checkpoint blockade, resulting in synergistic anticancer activity in animal models (122, 134–139). Recent studies by Samson et al. (138) provided further appealing clinical evidence of OV ability to convert a cold tumor previously resistant to immune checkpoint blockade therapy, into a hot tumor sensitive to immune therapy. This conversion may be due in part to the OV-induced local expression of type I IFNs and type II IFN-γ resulting in the up-regulation of inhibitory ligands (PD-L1 and PD-L2) on tumor cells (140) and thereby making "cold" tumors susceptible to immune checkpoint blockade. Upon intravenous treatment with reovirus, patients with high-grade glioma, or brain metastases, showed increased intratumoral leukocyte infiltration and type I and II IFN-dependent induction of PD-L1 expression (138).

OV-mediated potentiation of checkpoint blockade immunotherapy was also demonstrated in clinical studies using T-Vec in combination with ipilimumab (anti CTLA-4) or pembrolizumab (anti PD-1) (68). In particular, in a randomized open-label phase II trial involving 198 patients with advanced melanoma, ipilimubab/T-Vec co-treatment produced higher objective response rates (ORR) (39 vs. 18%), with 89% of all co-treated patients experiencing durable responses at a median follow up time of 16 months. Furthermore, 52% of patients presented reduced visceral lesions, providing evidence that T-Vec enhanced systemic antitumor immune responses (141). Promising results were also obtained by combining T-Vec with pembrolizumab in a trial's phase Ib arm involving 21 patients with advanced melanoma. In this trial, co-treated patients showed a higher ORR (62%), compared with patients treated with pembrolizumab (34%) or T-Vec (26%) alone. Immune

conversion of the TME was observed in co-treated patients, including CD8+ T cell infiltration and both elevated PD-L1 protein expression levels and IFN-γ production in tumor cells (142). It should also be stated that co-treatment was not associated with additional toxicity. Extension of this trial is ongoing and involves a total of 660 patients receiving either combination treatment or pembrolizumab alone (68).

The pros and cons of oncolytic virotherapy and the current attempts at improving this strategy will be exemplified in the following section with one of the OVs in clinical development, the rodent protoparvovirus H-1PV.

THE RAT PROTOPARVOVIRUS H-1PV

The Virus

The oncolytic protoparvovirus (PV) H-1PV is a non-enveloped single-stranded DNA virus (143, 144). With an icosahedral capsid of 25 nm, H-1PV is the smallest OV presently under clinical development. H-1PV belongs to the *Parvoviridae* family, genus *Protoparvovirus*, species *Rodent protoparvovirus 1*. The *Parvoviridae* family also includes adeno-associated viruses (AAV) that are commonly used in gene therapy for the delivery of therapeutic transgenes (143, 144). However, in contrast to AAVs which need a helper virus for their replication, H-1PV as other protoparvoviruses can replicate autonomously. The *Rodent protoparvovirus 1* also includes the Kiham rat virus, LuIII virus, mouse parvovirus, minute virus of mice (MVM), tumor virus X, and rat minute virus. Some of these viruses are under evaluation at the preclinical level as oncolytic agents.

The H-1PV genome comprises ∼5,100 nucleotides. Small deletions and point mutations can naturally occur in the parvoviral genome, reflecting genetic adaptation to the molecular characteristics of the host cell. The genome consists of two transcription units, termed NS and VP, whose expression is controlled by the early (P4) and late (P38) promoters, respectively. The NS gene unit encodes the non-structural proteins NS1, NS2, and NS3 while the VP unit encodes the VP1, VP2/VP3 capsid proteins and the non-structural SAT protein.

The natural host of H-1PV is the rat; the virus is not pathogenic to humans. H-1PV is unable to replicate in normal tissues, but it can productively infect and kill a broad range of human cancer cell lines from different origins including glioma, breast cancer, hepatoma, pancreatic carcinoma, melanoma, colorectal carcinoma, nasopharyngeal carcinoma, and lymphoma (143). H-1PV oncosuppression has been demonstrated in a number of preclinical animal models (143).

The reasons for H-1PV intrinsic oncotropism and tumor selectivity have been elucidated only in part and are discussed in detail elsewhere (143–146). In brief, the virus has the ability to exploit some of the molecular features that distinguish the cancer cell, such as (i) fast proliferation associated with the overexpression and/or activation of specific cellular factors needed for virus DNA replication and gene transcription belonging to the E2F, ATF/CREB, ETS, NFY families and cyclin A, and (ii) altered signaling pathways accompanied by upregulation of factors controlling viral functions (e.g., the PDK1/PKB/PKC pathway involved in the phosphorylation of the oncotoxic viral protein NS1); (iii) impairment of the innate antiviral immune response in many tumor cells, although the sensitivity of rodent PVs to type I IFN is presently a matter of controversy (92, 144, 147–150).

H-1PV, an Oncolytic Virus Case in Point

Although underlying mechanisms may be at least partly different, H-1PV, and various other OVs share a number of properties that illustrate well the pros and cons of cancer virotherapy. Pros comprise safety, oncotropism, oncosuppressive ability resulting from both oncolytic and immune adjuvant properties, and the possibility of systemic administration. Cons include limited tumor capacity for virus production in cancer patients, and inter/intratumoral heterogeneity of cancer cell permissiveness for virus infection. H-1PV can thus be used to exemplify the prospects and drawbacks of oncolytic virotherapy. PVs still have a number of unique properties distinguishing them from several other OVs [for a review, see Geletneky et al. (151)]. On the one hand, their lack of natural infectiousness and pathogenicity for humans, and the synthetic oncotoxicity of the viral protein NS1 are worth mentioning. On the other hand, PVs are taken up by most normal cells, which leads to typically harmless abortive infections, but results in the sequestration of a major fraction of administered/produced virions in normal tissues. This trapping limits the capacity of H-1PV to work at a distance from the inoculation site, making viral remote activity especially dependent on a bystander immune adjuvant effect, which is the focus of the present review.

H-1PV Oncolytic Activity

Besides being the key regulator of H-1PV replication, the NS1 protein is the major effector of virus oncotoxicity. The molecular mechanisms underlying H-1PV-mediated cell death are not fully understood. It was demonstrated that through NS1, H-1PV has the ability to induce oxidative stress associated with elevated levels of intracellular ROS, RNS, and DNA damage resulting in the activation of the intrinsic pathway of apoptosis (152).

In addition to apoptosis, the virus can activate a range of other cell death programs, including necrosis and cathepsin-mediated cell death in glioma cells (143). The latter mechanism involves relocation of active cathepsins from lysosomes into the cytoplasm accompanied by the downregulation of cystatin B and C, two cathepsin inhibitors (153). In support of the capacity of H-1PV to induce lysosomal-mediated necrosis in glioma cells, we recently obtained evidence of the occurrence of lysosomal membrane permeabilization and ER stress after infection of these cells (Marchini et al. unpublished results). The SAT protein may have a role in H-1PV-mediated ER stress as observed for porcine parvovirus (PPV). A PPV mutant with a deletion in the SAT region had less lytic activity than the wild-type virus and consequently less spreading (154). It is therefore possible that SAT together with NS1 participates in H-1PV-mediated cell death, in agreement with recent results from our laboratory (Bretscher et al. unpublished results).

Depending on the characteristics of the target tumor and on the amount of virus penetrating the tumor cell (i.e., input virus dose), it is possible that multiple cell death pathways are activated in parallel. It is important to point out that some forms of cell death may be more immunogenic than others and may therefore

influence the outcome of H-1PV-based therapies by engaging the immune system at different levels. This needs to be carefully considered in the design of therapeutic protocols (e.g., effective viral dose and treatment fractionation), especially in the context of combination regimens.

The extent of H-1PV-induced oxidative stress may account in part for the capacity of the virus to induce different types of cell death. Indeed, it has been demonstrated that intracellular ROS/RNS levels are pivotal for the determination of cell fate by fine-tuning cell stress responses. While physiological levels of ROS promote cell proliferation, excess production/accumulation of these toxic compounds has been associated with DNA damage and major disturbances such as activation of the inflammasome, induction of TNF-mediated inflammatory pathways, lipid peroxidation, lysosomal dysfunction, ER stress, and calcium and iron dyshomeostasis. Depending on the genetic background of the cell, different ROS/RNS levels can activate distinct forms of cell death, such as apoptosis, pyroptosis, necroptosis, ferroptosis, authophagy, and necrosis (95, 99). Some cancer cells may have more efficient antioxidant mechanisms to counteract H-1PV-induced oxidative stress and therefore be less susceptible to virus oncotoxicity. However, through its ability to activate different cell death pathways, H-1PV may compensate for cancer cell resistance to apoptotic stimuli or DNA damage-inducing agents by engaging the immune system to act against the tumor. Indeed, as briefly summarized in the next section, there is accumulating evidence supporting a role for H-1PV as an activator of immune-mediated anticancer responses.

H-1PV-Mediated Immune Modulation

Preclinical studies of H-1PV demonstrated the involvement of multiple immune cell populations in the anti-neoplastic activity of this virus. Distinct immune cells proved to be activated by H-1PV as a result of both their direct infection with the virus and their exposure to virus-induced tumor cell lysates.

H-1PV can infect a wide panel of human immune cells, namely DCs, macrophages, NK cells, and T-lymphocytes. Infection is abortive and does not result in the production of progeny viral particles. More importantly, no or little direct toxicity of H-1PV for human immunocytes has been observed, while the induced release of cytokines may cause cytopathic effects under *in vitro* conditions (155, 156). H-1PV has been shown to be harmless for rat immune cells as well. Rats treated with repeated high doses of H-1PV showed normal activity of B-cells and developed NAbs against H-1PV. Serum concentrations of IL-6 and TNF-α were normal in these animals, and isolated PBMCs showed proliferative response similar to control (157).

H-1PV infection of human PMBCs results in their maturation and activation, which are associated with the release of IFN-γ and TNF-α. Furthermore, a type I IFN production mediated at least in part by TLR-9 was observed and assigned to infected plasmacytoid DCs (156). Interestingly, H-1PV infection proved able to stimulate CD4+ T-cells, as revealed by the enhanced expression of activation markers (CD69 and CD30) and release of both Th1 and Th2 cytokines (IL-2, IFN-γ, and IL-4) (158).

In addition to its direct impact on human immune cells, H-1PV indirectly causes major immune stimulatory effects which are apparently induced by infected cancer cells. Indeed, while failing to induce type I IFN in these cells, H-1PV can indirectly upregulate both innate and adaptive immune responses through its effects on tumor cells.

On the innate side, H-1PV infection of human pancreas and colon carcinoma cells was shown to enhance their ability to stimulate NK cells, as a result of the downregulation of MHC-I molecules and upregulation of NK-activating ligands on the surface of infected tumor cells. This stimulation is reflected in an increase of both the release of cyto/chemokines (IFN-γ, TNF-α, and MIP-1), and the killing of tumor cells by NK cells (159).

On the adaptive side, effector Th cells (with a Th1 bias) were found to be stimulated in the presence of H-1PV-infected tumor cells, at least in part through the enhanced capacity of the latter for activating APCs. Infection of pancreatic ductal adenocarcinoma (PDAC) cells with H-1PV leads to the release of HMGB1 but apparently not CRT or ATP (160). As mentioned above, HMGB1 interacts with TLR4 and can directly activate DCs. Furthermore, infection of human melanoma cells with H-1PV induces them to release HSP72. Extracellular HSP72 has potent adjuvant properties and can induce migration and activation of DCs as well as activation of NK cells (155, 161). H-1PV-infected melanoma cell lysates are indeed able to induce maturation of DCs, as revealed by the upregulation of co-stimulatory molecules (CD86) and the production of pro-inflammatory cytokines (TNF-α and IL-6) (162). The maturation of DCs resulting from their incubation with H-1PV-induced melanoma cell lysates correlates with the up-regulation of TLR3 and TLR9 expression and the activation of the NFκB signaling pathway (163). DCs pulsed with lysates of H-1PV-infected tumor cells not only mature and produce pro-inflammatory cytokines, but also show the ability to cross-present TAAs to specific CTLs, linking the stimulation of innate immunity to the activation of an adaptive immune response (164).

H-1PV requires functional adaptive immunity to fully express its therapeutic potential. CD8+ cells are essential to suppress metastases of Morris hepatoma cells in rats treated with a therapeutic vaccine based on H-1PV-infected autologous tumor cells (165). Similarly, antibody depletion of CD8+ cells in an immunocompetent rat model of glioma, strongly diminished H-1PV oncosuppressive activity (166). Immune reconstitution of NOD SCID mice bearing human PDAC transplants with autologous DC and T-cells primed *ex vivo* with H-1PV-induced tumor cell lysates resulted in a strong suppression of tumor development (167). These data directly demonstrate the adjuvant effect of H-1PV on the efficacy of a cancer vaccine. It is noteworthy that the vaccination potential of H-1PV can be further improved by combination therapy with IFN-γ (168). The involvement of CTLs in H-1PV anti-cancer activity was further demonstrated in a rat syngeneic bilateral PDAC model. Rats engrafted with tumors in both flanks and injected with virus in only one site, experienced significant reduction in tumor size at both the injected and the distal uninjected sites, arguing for an involvement of the immune system in the regression of untreated lesions. H-1PV particles were not detected in uninjected tumors

which instead showed increased expression of IFN-γ, granzyme B and perforin (169). As additional proof of the role of CTLs in the therapeutic activity of H-1PV, adoptive transfer of splenocytes from H-1PV-treated donors into naïve recipients was shown to significantly prolong survival of animals harboring PDAC (167).

H-1PV Clinical Development

H-1PV is one of the OVs that have successfully transitioned from preclinical studies into clinical development. Two clinical trials, in brain and gastrointestinal (pancreatic) tumor patients, have been conducted recently.

Glioblastoma

Glioblastoma is recognized as one of the tumors with the "coldest" TME. Infiltration of immune cells into the glioblastoma bed is generally very limited (170). Furthermore, mutational signature studies in glioblastoma have revealed the presence of only 30–50 non-synonymous mutations (171). As mentioned above, the success of antigen-specific immunotherapies, such as checkpoint blockade, largely depends on tumor mutational load and the presence and phenotype of tumor-infiltrating immune cells. The benefit of this approach for patients with glioblastoma is therefore presently insufficient and badly predictable. In contrast to this intrinsically low responsiveness to checkpoint blockade seen in the majority of glioblastoma patients, H-1PV treatment of the latter is unlikely to be compromised by the "cold" TME. Moreover, H-1PV-induced tumor cell killing, DAMP/PAMP release and increased neoantigen exposure [recently reviewed in Angelova and Rommelaere (150)] may contribute to TME "warming up" and not only trigger antitumor immune responses *per se*, but also alleviate glioblastoma resistance to checkpoint inhibition. Based on the above, H-1PV deserves consideration also as partner drug in combinatorial immune checkpoint blockade treatments directed against glioblastoma and other tumors with low mutational load.

Similar concerns apply to the applicability of CAR T-cell therapy in glioblastoma. Currently, three CAR T-cell trials have been published which reported promising signs of efficacy in selected glioblastoma patients (172). However, also here the immunosuppressive glioblastoma TME presents obstacles and poses barriers to CAR T-cell proliferation and responses. Whether administered as preceding treatment or simultaneously, in combination with CAR T-cells, H-1PV-mediated TME immune stimulation holds the promise for synergizing with CAR T-cell efficiency. H-1PV-based combinatorial approaches which have yielded encouraging evidence of preclinical and clinical efficacy were recently reviewed in Bretscher and Marchini (144) and are briefly listed below (see Future perspectives in PV therapeutic development).

Yet another advantage of H-1PV as anticancer immunomodulator lies in the gentle way in which the virus reshapes the TME and boosts the immune system. Contrary to immune checkpoint blockade- and CAR T-cell therapy-associated organ toxicities and immune-related adverse events, H-1PV administration to glioblastoma patients is not accompanied by any signs of immune system overstimulation and does not exert any negative impact on laboratory safety parameters. Furthermore, no dose-limiting toxicity could be reached in the first parvovirus glioblastoma clinical trial, as described in more detail below.

The preclinical proof of concept for H-1PV-based virotherapy of brain tumors was provided by *in vivo* experimental evidence demonstrating efficient H-1PV-induced suppression of both rat and human gliomas in syngeneic or immunodeficient animal models, respectively (173). Progressive reduction of tumor size, complete remission in 50% of the responding animals and significant survival prolongation were observed, while no H-1PV treatment-associated side effects could be detected. These data paved the way for the launch of the first-in-man PV clinical trial (ParvOryx01), a phase I/IIa study in patients with recurrent glioblastoma (174). Notably, ParvOryx01 was also the first OV trial in Germany (175). Within the frame of the trial, 18 patients with a history of one previous glioblastoma resection were treated with escalating H-1PV (GMP-grade, ParvOryx) doses. Half of the corresponding dose was applied either intratumorally or intravenously before tumor resection. After tumor resection, at day 10 after treatment, the second half of the planned virus dose was injected into the wall of the resection cavity. The primary trial endpoints were safety, tolerability, pharmacokinetics, and maximum tolerated dose (MTD) estimation. In addition, tumor tissue samples were acquired during resection, allowing for the analysis of markers of intratumoral virus expression and TME immunological landscape. ParvOryx01 convincingly proved H-1PV safety and tolerability (176). MTD could not be reached. Risk assessment ruled out virus transmission from study patients to third persons, since no infectious H-1PV particles were found in fecal and urine samples. Analysis of post-treatment tumor tissues detected virus expression in a subset of glioblastoma cells and remarkably, also in those patients who received systemic ParvOryx treatment. This was in line with preclinical reports showing H-1PV ability to cross the blood-brain/tumor barrier after intravenous administration. Furthermore, TME immune conversion was observed (176). ParvOryx treatment promoted tumor infiltration with immune cells. Most of the infiltrate consisted of Th cells and perforin- and granzyme B-expressing CTLs. Of note, only scarce Treg cells were seen scattered within the tumor. Activation of glioblastoma-associated microglia/macrophages and detection of pro-inflammatory cytokine production in treated tumors hinted at the induction of an inflamed microenvironment and increased immunological visibility of the tumor. Interestingly, in some of the study patients, formation of not only virus-specific but also glioma-specific T-cell responses was demonstrated, raising the hope that H-1PV treatment may contribute to the circumvention of tumor immune evasion mechanisms in glioblastoma and other poorly immunogenic human tumors.

Pancreatic Ductal Adenocarcinoma (PDAC)

The second H-1PV clinical trial (ParvOryx02) was launched in 2015 in patients with metastatic inoperable pancreatic cancer (177). ParvOryx02 was recently successfully completed and clinical and research findings are currently awaiting publication.

Future Perspectives in PV Therapeutic Development

The first clinical evidence of H-1PV capacity to induce an inflamed TME in glioblastoma patients together with favorable survival data (176, 178), prompted further efforts to develop strategies to increase the efficiency of PV-based cancer viro(immuno)therapy. Several approaches hold particular promise and are currently under investigation.

H-1PV-based combinatorial treatments have been evaluated in both preclinical and clinical settings (144). H-1PV combinations with chemotherapeutics (160, 179), histone deacetylase (HDAC) inhibitors such as valproic acid (VPA) (180) and immune checkpoint blockade (181) have been demonstrated to synergistically potentiate the double-faceted anticancer activity of the virus by both inducing enhanced virus replication, oxidative stress and tumor cell lysis (180), and exerting immune stimulatory effects (160, 181) in tumor cell and animal models. Notably, some H-1PV-based combinatorial approaches have also been tested in the clinic. The ParvOryx02 trial combined systemic and intramethastatic H-1PV administration with gemcitabine, the gold standard first-line therapy for PDAC patients. Within the frame of a compassionate use program, favorable response was achieved in glioblastoma patients treated with H-1PV and bevacizumab, an anti-angiogenic agent with still underappreciated immunomodulating properties (182). Some of the patients were also co-treated with the PD-1 inhibitor nivolumab and based on the positive results obtained at the preclinical level, with VPA. This multimodal treatment resulted in partial or complete objective responses in 7 of 9 cases (183, 184). These encouraging results strongly support further (pre)clinical development of PV-based viro(immuno)/chemotherapies for glioblastoma and other cancers treatment.

H-1PV Genetic Engineering

Another intriguing approach to PV efficacy potentiation is arming the PV genome with immunostimulatory CpG motifs (185) or therapeutic transgenes encoding for angiostatic/immunostimulatory molecules (186). However, in the latter example, due to the limited packaging capacity of H-1PV, the therapeutic transgene replaces part of the viral genomic region encoding for the capsid proteins, rendering the virus replication deficient. Production of these recombinant PVs requires the use of helper plasmids (187, 188).

The limited packaging capacity of H-1PV can be overcome through an original strategy proposed by El-Andaloussi et al. (189). An engineered H-1PV genome is inserted into the genome of a replication-defective Ad5 vector. The resulting chimera not only allows H-1PV genome delivery to cancer cells with subsequent production and release of infectious replication-competent viral particles but, importantly, also offers new prospects for reinforcing the anticancer activity of H-1PV by inserting a therapeutic gene into the adenovirus component of the Ad-PV hybrid genome. This chimera provides a unique platform to carry out, by means of a single agent, cancer gene therapy (through the replication-deficient transgene-armed adenovirus carrier) and oncolytic virotherapy (through the released replication-competent H-1PV particles).

As for every OV under investigation and more generally for any other anticancer agents, further development of H-1PV-based therapies would certainly benefit from the establishment of novel models (e.g., use of patient-derived spheroids/organoids, syngeneic, or humanized animal models) that more closely recapitulate human disease and better predict the outcome of the novel therapies once transferred to the clinic.

AUTHOR CONTRIBUTIONS

AM, LD, VP, AA, and JR have contributed in the writing of the manuscript, read and approved the final manuscript. AM designed the figure.

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A Phase Ib Study of the Combination of Personalized Autologous Dendritic Cell Vaccine, Aspirin, and Standard of Care Adjuvant Chemotherapy Followed by Nivolumab for Resected Pancreatic Adenocarcinoma—A Proof of Antigen Discovery Feasibility in Three Patients

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Despite the promising therapeutic effects of immune checkpoint blockade (ICB), most patients with solid tumors treated with anti-PD-1/PD-L1 monotherapy do not achieve objective responses, with most tumor regressions being partial rather than complete. It is hypothesized that the absence of pre-existing antitumor immunity and/or the presence of additional tumor immune suppressive factors at the tumor microenvironment are responsible for such therapeutic failures. It is therefore clear that in order to fully exploit the potential of PD-1 blockade therapy, antitumor immune response should be amplified, while tumor immune suppression should be further attenuated. Cancer vaccines may prime patients for treatments with ICB by inducing effective anti-tumor immunity, especially in patients lacking tumor-infiltrating T-cells. These "non-inflamed" non-permissive tumors that are resistant to ICB could be rendered sensitive and transformed into "inflamed" tumor by vaccination. In this article we describe a clinical study where we use pancreatic cancer as a model, and we hypothesize that effective vaccination in pancreatic cancer patients, along with interventions that can reprogram important immunosuppressive factors in the tumor microenvironment, can enhance tumor immune recognition, thus enhancing response to PD-1/PD-L1 blockade. We incorporate into the schedule of standard of care (SOC) chemotherapy adjuvant setting a vaccine platform comprised of autologous dendritic cells loaded with personalized neoantigen peptides (PEP-DC) identified through our own proteo-genomics antigen discovery pipeline. Furthermore, we add nivolumab, an antibody against PD-1, to boost and maintain the vaccine's effect.

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We also demonstrate the feasibility of identifying personalized neoantigens in three pancreatic ductal adenocarcinoma (PDAC) patients, and we describe their optimal incorporation into long peptides for manufacturing into vaccine products. We finally discuss the advantages as well as the scientific and logistic challenges of such an exploratory vaccine clinical trial, and we highlight its novelty.

Keywords: pancreatic adenocarcinoma, dendritic cell vaccine, antigen discovery, neoantigen, cancer immunotherapy

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the seventh leading cause of cancer-related death in the world in 2018 (1), with an overall 5-year survival rate of ∼5% (2). Approximately 70% of deaths are due to widespread metastasis and the remaining cases have limited metastasis but extensive primary tumors which eventually lead to mortality (3). Surgery is the only potential hope of cure for PDAC, but tumors are resectable only in 20% of patients at the time of diagnosis. Therapeutic research efforts have mainly focused on improvements in radio/chemo treatments and to date, there are only a few chemotherapeutic agents that have shown to be effective against advanced pancreatic cancer, including gemcitabine with or without abraxane (4). At present, it is difficult to conclude that there is a definite SOC adjuvant chemotherapy for all patients with PDAC. However, multiagent adjuvant therapy (modified folforinox) has been demonstrated to be more effective than gemcitabine alone in the adjuvant setting, but its use is limited only to patients with excellent performance status (5). Recently it has suggested that gemcitabine plus capecitabine is a valid option for these patients since it has been shown that it is more efficient than gemcitabine alone (6).

One of the most promising new cancer treatment approaches is immunotherapy. Recent studies have shown that PDAC is an immunogenic tumor. Antigens expressed on pancreatic tumor cells able to induce specific B and T cells comprise (7): Wilms' tumor gene 1 (WT1) (75%) (8), mucin 1 (MUC1) (over 85%) (9), human telomerase reverse transcriptase (hTERT) (88%) (10), mutated K-RAS (nearly 100%), survivin (77%), carcinoembryonic antigen (CEA) (over 90%) (11), HER-2/neu (over 60%) (12), p53 (over 65%) (13), and α-enolase (ENO1) (14). Several studies have reported that dysfunction of the immune system is one of the key contributors for the development of PDAC (15, 16). Moreover, PDAC is known to have an immunosuppressive tumor microenvironment characterized by (i) the absence of intratumoral effector T-cells (17, 18), (ii) the presence of an inflammatory tumor microenvironment led by the RAS oncogene (19), and (iii) massive infiltration of immunosuppressive leukocytes into the tumor microenvironment, which predicts poor survival (18, 20, 21). Additionally, the analysis of immune infiltrates in human tumors has demonstrated a positive correlation between prognosis and the presence of humoral response to pancreatic antigens (MUC-1 and mesothelin) (22, 23) or of tumor-infiltrating T cells (20, 24). Therefore, cancer immunotherapy can be a promising alternative treatment for PDAC patients.

A major mechanism of immune resistance engaged by tumors is the enforcement of immune checkpoint pathways, aiming to shutdown T cells specific for tumor antigens. An important immune checkpoint is mediated by the programmed cell death protein 1 (PD-1) expressed on the surface of activated T cells during initial activation (25, 26). The major role of PD-1 is to limit the activity of T cells in peripheral tissues at the time of an inflammatory response to infection and to restrict autoimmunity (27, 28). Cancer immunotherapy targeting anti-PD-1 (e.g., nivolumab, pembrolizumab), as well as anti-cytotoxic T-lymphocyte-associated antigen 4 (anti-CTLA-4, ipilimumab), has changed the treatment landscape of several tumors (29). Yet the success of immunotherapy has not been proven effective for the treatment of metastatic pancreatic cancer patients (30), who have been shown unresponsive except for the population with mismatch-repair deficiency which comprises only 0.8% (31). A broad array of clinical trials in pancreatic cancer have been completed or are ongoing using different combinations with ICB (32, 33). However, the most adequate combination for PDAC patients is not clear so far.

Dendritic cell (DC)-based vaccines for cancer immunotherapy have been studied and tested for more than a decade and proven clinically safe and efficient to induce tumor-specific immune responses, however only limited efficacy was observed in patients with advanced recurrent disease after DC vaccination (34, 35). Several groups have attempted to test safety and efficacy of DC-based vaccines against pancreatic cancer in early phase clinical trials, loading DCs with tumor associated antigens (TAAs) *ex vivo*, and subsequently re-infusing them in patients, yet with low clinical benefit so far (36–39). One possible reason for reduced vaccine efficacy could be that most cancer vaccines tested to date were targeted against defined non-mutated self-antigens. Tumors express two major kinds of antigens that can be recognized by T cells: non-mutated self-antigens and mutated neoantigens, generated in tumor cells due to their inherent genetic instability (40). Tumor cells usually harbor between 10 and few thousands private somatic mutations, as identified by deep sequencing analysis, and even among tumors of the same histotype, most mutations are different (41, 42). Thus, neoantigens are mostly "private" and patient-specific (43) and trigger a higher more robust T-cell response. Indeed, increasing evidence associates clinical benefit from immunotherapy with specific responses to private tumor epitopes (44–48), leading to increased interest in neoantigen vaccination (40).

Several clinical trials describing vaccines designed to harness neoantigen-specific immunity have been recently reported

mainly in melanoma patients: The first study reported the feasibility, safety and efficacy of a DC vaccine pulsed with neoantigen peptides (49). Another phase I study has evaluated a peptide vaccine targeting up to 20 predicted personal tumor neoantigens and demonstrated an expansion of the repertoire of neoantigen-specific T cells which correlated with clinical benefit (50). A second group performed a phase I study using RNA vaccines that contained up to 10 mutations per patient and demonstrated that these vaccines can mobilize specific anti-tumor immunity against these cancers (51). These studies provide proof-of-principle that a personalized vaccine can be produced and administered to a patient to generate highly specific immune responses against that individual's tumor, showing that a personalized neoantigen vaccine broadens the repertoire of neoantigen-specific T cells substantially beyond what is induced by existing immunotherapeutics.

To determine whether targetable mutations and neoantigens exist in PDAC, several studies have been performed using genomic profiles of PDAC tumor samples. A whole-genome sequencing and copy number variation (CNV) analysis was performed on 100 pancreatic ductal adenocarcinomas (PDACs) and found in total 11,868 somatic structural variants at an average of 119 per individual (range 15–558) (52). Furthermore, the genomic profile of 221 PDAC tumors were analyzed and the findings revealed that nearly all PDAC samples harbor potentially targetable neoantigens (53). To define the importance of neoantigens in PDAC, one study compared stage-matched cohorts of treatment-naive, surgically resected, rare long-term survivors to short-term survivors with a more typical poor outcome. The authors detected a median of 38 predicted neoantigens per tumor, and showed that the association of higher neoantigen quantity and $CD8^+$ T-cell infiltrate with survival was independent of adjuvant chemotherapy, suggesting that neoantigen quality, and not purely quantity, correlates with survival (54).

We hypothesize that effective vaccination in PDAC patients along with interventions that can reprogram important immunosuppressive factors in the tumor microenvironment can enhance tumor immune recognition, thus enhancing response to PD-1/PD-L1 blockade. To this end, we designed a phase 1b trial where we incorporated a vaccination schedule of a novel autologous DC pulsed with personalized neoantigen peptides (PEP-DC) identified through our own proteo-genomics antigen discovery pipeline in the SOC chemotherapy adjuvant setting followed by nivolumab. We hereby set the objectives and design of our study, and we demonstrate the feasibility of identifying personalized neoantigens in three PDAC patients, and their optimal incorporation into long peptides for manufacturing into vaccine products.

MATERIALS AND METHODS

Clinical Study Design

This is a phase Ib trial (CHUV-DO-0017_PC-PEPDC_2017) to evaluate the feasibility, safety, immunogenicity, and efficacy of subcutaneous DC vaccine loaded with personalized peptides (PEP-DC), in combination with SOC chemotherapy (gemcitabine/capecitabine) and enteric-coated aspirin, followed by the anti-PD-1 antibody nivolumab to boost and maintain the vaccine's effect in patients with surgically resected PDAC. The components of the vaccine to be investigated in this study include agents for which safety has been previously demonstrated to be acceptable. This trial has been approved by Swissmedic and the competent Ethics Committee. Before any study-specific procedure is performed, a signed and dated informed consent is obtained. In order to be eligible, patients must present: (a) histologically confirmed resected adenocarcinoma of the pancreas (T1–T4, N 0–1, minimum 2 cm–AJCC 8th ed.) and (b) appropriate amount of tumoral tissue collected from the cytoreductive surgery, allowing the identification of top 10 personalized peptides (PEP) for preparation of PEP-DC vaccine.

Objectives

The primary objectives of the trial are to determine: (1) the feasibility of producing and administering PEP-DC vaccine in the indicated patient population; (2) the safety and tolerability of the study treatment vaccine and aspirin given together with SOC chemotherapy, and followed by nivolumab; (3) the immunogenicity by measuring acquired T cell mediated immune activation events post vaccination. This study has also a secondary objective, which is to evaluate relapse free survival at 6, 12, 18, 24, and 36 months and overall survival in the indicated population of patients.

Statistical Methods

We hypothesize that the delivery of the PEP-DC vaccine through the subcutaneous route in combination with aspirin, nivolumab, and adjuvant chemotherapy in advanced pancreatic cancer patients is feasible, safe without additional toxicity, and immunogenic. Based on study feasibility and anticipated accrual rate, a total of 12 evaluable patients is expected to enter this Phase Ib study if treatment limiting toxicities (TLTs) are in the acceptable range.

The feasibility hypothesis for PEP-DC vaccine will be assessed by (a) the number of patients in which vaccine production is successful (at least 6 doses are manufactured and released), and (b) the number of patients who receive at least one dose of PEP-DC vaccine (since the mainstay of the therapeutic approach here is PEP-DC) and the corresponding percentages in the ITT population (i.e., all registered patients). Exact binomial confidence intervals for the corresponding rates will be estimated.

The safety and tolerability of the PEP-DC vaccine in combination with other protocol drugs will be evaluated by the occurrence of TLTs and adverse events (AEs) in both the "TLT evaluation" and the safety population. The severity of toxicities will be classified according to the NCI CTCAE Version 4.03 and will be presented in tabular as well as graphical format. For each patient, each AE will be presented considering the highest (worst) grade of toxicity observed over the whole treatment period according to CTCAE version 4.03. Although the safety of the vaccination backbone has been already established, a continuous monitoring rule will be followed, to allow for early termination of the study. Any patient who receives at least one vaccination will be included in the toxicity (safety) analysis. A Bayesian rule will

be employed to monitor TLTs after groups of 4 patients have been treated and complete the final TLT evaluation.

As only 5 weeks are allocated for target prioritization, assessment of pre-vaccination immune responses against the predicted neoantigens will not be performed to assist their selection. Therefore, the selection of long peptides is done *in silico* by the NeoDisc pipeline. The immunogenicity of PEP-DC vaccine will be assessed (based on ITT population as well as the safety population) by measuring acquired, T cell-mediated immune activating events post vaccination compared to pre-vaccination levels. Descriptive statistics of absolute and relative differences will be calculated overall and for subgroups of interest.

Regimen

The study was designed so that eligible subjects with PDAC who undertook cytoreductive surgery followed by chemotherapy may plan to enroll in the vaccine study. Should the subject wishes, and upon informed consent, tissue can be harvested at the time of surgery for identification of personalized targets for vaccination. Screening of patients may be completed after the collection of tumor was performed during the surgery. Upon registration for the trial, all patients would receive 8 cycles of 21 day cycle of gemcitabine/capecitabine. Eligible patients will undergo apheresis during the last week of the third cycle of gemcitabine/capecitabine to collect peripheral blood mononuclear cells for DC vaccine production. Patients will receive at least six PEP-DC vaccinations starting concomitant with the 5th cycle of chemotherapy. PEP-DC vaccine of $5–10 \times 10^6$ autologous DC in 1 ml volume/treatment will be delivered subcutaneously every 3 weeks. Patients will receive oral enteric-coated aspirin daily for the duration of the study starting from the day of first vaccination until the end of study. Nivolumab will be administered starting 3 weeks after last chemotherapy cycle and will be given during the vaccination period until the last vaccine dose. Afterwards, it will be given as a maintenance therapy until appearance of new lesion(s) or unacceptable toxicity for maximum 2 years.

To verify that the combination of PEP-DC vaccine and enteric-coated aspirin during and following standard adjuvant chemotherapy, followed by nivolumab, will significantly enhance tumor immunogenicity, and allow tumor response, the translational objectives of the study are the following: (a) to deeply characterize the tumor microenvironment of pancreatic adenocarcinoma patients; (b) to assess the overall effects of the combined PEP-DC vaccine during and following standard adjuvant chemotherapy, followed by nivolumab on peripheral blood and plasma; (c) to determine tumor antigens against which the treatment elicits a response.

Identification of Personalized Targets for Vaccination With NeoDisc

Processing of Patients' Material for PEP-DC Vaccine Preparation

Informed consent of the participants was obtained following requirements of the institutional review board (Ethics Commission, CHUV). The translational research has been approved by the CHUV ethics committee (protocols 2017-00305).

DNA Extraction and Sequencing

DNA was extracted for HLA typing and exome sequencing with the commercially available DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following manufacturers' protocols. Five hundred nanograms of gDNA were used to amplify HLA genes by PCR. High resolution 4-digit HLA typing was performed with the TruSight HLA v2 Sequencing Panel from Illumina on a MiniSeq instrument (Illumina) (**Supplementary Table 1**). Sequencing data were analyzed with the Assign TruSight HLA v2.1 software (Illumina). For exome sequencing, SureSelect Exome V5 library type (Sureselect v5 capture, Agilent Technologies, Santa Clara, CA, USA), and paired end reads were chosen, with at least 100x coverage for the tumor and PBMCs.

LC-MS/MS Analyses of Eluted HLA Peptides

For immunoaffinity purification of HLA peptides from tissues, we applied a previously published protocol (55, 56). Briefly, anti-HLA-I and anti-HLA-II monoclonal antibodies were purified from the supernatant of HB95 (ATCC® HB-95™) and HB145 cells (ATCC® HB-145™) using protein-A sepharose 4B beads (Invitrogen, Carlsbad, California), and cross-linked to the beads. Snap-frozen PDAC tissue samples were homogenized in lysis buffer on ice in 3–5 short intervals of 5 s each using an Ultra Turrax homogenizer (IKA, T10 standard, Staufen, Germany) at maximum speed, as previously described (55, 56). Lysates were cleared by centrifugation at 25,000 rpm (Beckman Coulter, JSS15314, Nyon, Switzerland) at 4°C for 50 min. The Waters Positive Pressure-96 Processor (Waters, Milford, Massachusetts) was used with 96-well, 3 μm glass fiber and 10 μm polypropylene membranes micro-plates (Seahorse Bioscience, North Billerica, Massachusetts). A depletion step of endogenous antibodies was performed with plates containing Protein-A beads, and then the lysates were passed through a plate containing beads cross-linked to anti-HLA-I, and then sequentially through a plate with the anti-HLA-II cross-linked beads. After washing with varying concentrations of salts, the beads were washed twice 2 mL of 20 mM Tris-HCl pH 8. HLA complexes and the bound peptides were eluted directly into pre-conditioned Sep-Pak tC18 100 mg plates (ref number: 186002321, Waters) with 1% TFA. After washing the C18 wells with 2 mL of 0.1% TFA, HLA-I peptides were eluted with 28% ACN in 0.1% TFA, and HLA-II peptides were eluted from the class II C18 plate with 500 μL of 32% ACN in 0.1% TFA. HLA-I, and HLA-II peptide samples were dried using vacuum centrifugation (Concentrator plus Eppendorf) and stored at −20°C.

We measured the peptides with LC-MS/MS system consisting of an Easy-nLC 1200 (Thermo Fisher Scientific, Bremen, Germany) and the Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were separated on a 450 mm analytical column of 75 μm inner diameter for 120 min using a gradient of H2O/FA 99.9/0.1% (A) and ACN/FA 80/0.1% (B). The gradient was run as follows: 0 min 2% B, then to 5% B at

5 min, 35% B at 85 min, 60% B at 100 min, and 95% B at 105 min at a flow rate of 250 nL/min.

MS spectra were acquired in the Orbitrap from m/z = 300–1,650 with a resolution of 60,000 (m/z = 200), ion accumulation time of 80 ms. The AGC was set to 3e6 ions. MS/MS spectra were acquired in a data-dependent manner, and 10 most abundant precursor ions were selected for fragmentation, with a resolution of 15,000 (m/z = 200), ion accumulation time of 120 ms and an isolation window of 1.2 m/z. The AGC was set to 2e5 ions, dynamic exclusion to 20 s, and a normalized collision energy (NCE) of 27 was used for fragmentation.

NeoDisc Pipeline

Alignment

Exome sequence reads were aligned to the Genome Reference Consortium Human Build 37 assembly (GRCh37) with BWA-MEM version 0.7.17 (57). The resulting SAM format was sorted by chromosomal coordinate and converted into a BAM file, then PCR duplicates were flagged, using the Picard AddOrReplaceReadGroups and MarkDuplicates utilities, respectively (from http://broadinstitute.github.io/picard). Various quality metrics were assessed with the Picard MarkDuplicates, CollectAlignmentSummaryMetrics, and CalculateHsMetrics utilities. Following GATK best practices, GATK BaseRecalibrator (within GATK v3.7-0) was used to recalibrate base quality scores (BSQR) prior to variant calling (58, 59). BQSR corrects base quality scores based on an estimation of empirical error frequencies in the alignments. The recalibrated tumor and germline BAM files were then used as input for each of three variant callers: GATK HaplotypeCaller; MuTect v1; and VarScan 2.

Caller 1: GATK HaplotypeCaller

The GATK HaplotypeCaller algorithm improves variant calling by incorporating *de-novo* assembly of haplotypes in variable regions, thus reducing the overall false-positive variant call rate (58, 59). HaplotypeCaller was run in GVCF mode on each tumor and germline recalibrated BAM file to detect SNV and Indel variants. The resultant gVCF files were combined using GATK GenotypeGVCF to produce raw variant calls for tumor and germline within in a single VCF. Subsequent variant quality score recalibration, following GATK best practices, was performed separately for SNVs and Indels (insertions/deletions) using the GATK variant Recalibrator tool to identify high-confidence calls. Variant quality was assessed by the GATK VariantEval tool. Patient-specific SNPs were defined as variants present in both tumor and germline, while variants present only in tumor were defined as somatic mutations.

Caller2: MuTect v1

The MuTect variant calling algorithm predicts somatic mutations based on log odds scores of two Bayesian classifiers (from https://github.com/broadinstitute/mutect). The first classifier identifies non-reference variants in the tumor sample while the second detects whether those variants are tumor specific. Candidate somatic mutations are then filtered based on read support, for example by ensuring that supporting reads map to both DNA strands, in order to reduce next-generation sequencing artifacts. Identified somatic mutations are exported in VCF format.

Caller3: VarScan 2

The VarScan2 algorithm, unlike GATK and MuTect, relies on hard filtering of calls rather than Bayesian statistics (60). This has the advantage of being less sensitive to bias such as extreme read coverage and sample contamination. VarScan 2 filters reads based on parameters such as read quality, strand bias, minimum coverage, and variant frequency. The multisample pileup file required for VarScan 2 input was generated with SAMtools (61, 62). VarScan 2 was run using default parameters and generated a VCF containing SNVs and Indels for both somatic mutations and SNPs.

Non-redundant call set

Variant calls from GATK, MuTect v1, and VarScan 2 were combined into a single VCF that contains the union of the variants of all three callers. Ambiguous calls (i.e., different calls at the same genomic coordinate) were resolved by a simple majority rule. If there was no majority, the call was rejected. GATK ReadBackedPhasing was used to retrieve the phasing information of all variants in the combined VCF (58, 59). The functional effect of the variants was annotated by SnpEff which predicts the effects of variants on genes based on reference databases. To maximize variant annotation we used annotations from the hg19 (Refseq) and GRCH37.75 (Ensembl) databases (63–65). This non-redundant, annotated VCF file was used for further genomics and proteogenomics analyses.

Prediction and Prioritization of Neoantigens

For the identification of neoantigens, only "high confidence" calls were selected, defined as the set of variants containing all somatic mutations plus linked SNPs (i.e., those SNPs present on the same allele as the somatic mutation) detected by MuTect v1 alone or by a combination of at least two of the three variant callers described above. The novel amino acid generated by each single nucleotide somatic mutation was placed at the center of a 31mer peptide that also included any amino acid changes resulting from non-synonymous linked SNPs. In the case of a somatic indel mutation, the entire polypeptide encoded by the new open-reading frame plus the upstream 24 amino acids could be subjected to HLA ligand prediction. However, for the described three PDAC samples this option was disabled.

HLA-I and HLA-II ligands were predicted by the MixMHCpred.v2.0.2 and MixMHC2pred.v1 algorithms, respectively (66–68). Both algorithms have been trained on naturally presented peptides and compute the likelihood of a peptide to bind to one of a given set of HLA alleles. Mutant peptides of sizes ranging from 9 to 12 and 12 to 19 amino acids, derived from the 31mer were supplied as input for HLA-I and HLA-II predictions, respectively, using patient-specific allotypes as determined by HLA typing (**Supplementary Table 1**).

Tissue-specific gene expression data was downloaded from The Genotype-Tissue Expression (GTEx) project, a public resource that contains data from 53 non-diseased tissues across nearly 1,000 individuals (69). We used a custom R script to

retrieve gene expression values, based on GTEx v7 publicly available data. The 90th percentile expression of the wild type gene in the tissue-derived tumor was reported from GTEx data, and mutations in genes not expressed (TPM < 1) in pancreas were excluded.

Due to the intrinsic content and properties of protein sequences, HLA ligands are not distributed equally along proteins and tend to cluster in hotspots. We captured this information across dozens of cell types in our ipMSDB database (70). The overlap of the wild-type-form of a mutant peptide with a hotspot in ipMSDB was calculated, as well as the level of presentation of the source protein. Any mutant peptide matching any wild-type sequence in SwissProt (71) or found in the reference GRCh37 (64) proteome was filtered out.

Finally, we used a custom python script to design the best long peptide(s) for every mutation, encompassing the highest possible number of HLA-I and HLA-II binding peptides (MixMHCpred and MixMHC2pred %Rank < 5% or found in ipMSDB). Long peptides were ranked by the minimum *p*-value of the predicted HLA-I neoantigens, and the top 10 long peptides were selected.

Proteogenomics

For every sample, we created a reference fasta file where residue mutation information was added to the header of the affected translated transcripts, in a format compatible with MaxQuant v1.5.9.4i as previously reported (72). We used the GENCODE v24 (73) (GRCh37 human reference assembly, downloaded from https://www.gencodegenes.org/human/release_24lift37.html) as the standard reference dataset (89,543 entries). We parsed the GENCODE comprehensive gene annotation file, in GFF3 format, to extract genomic coordinate information for every exon. These coordinates were compared with sample-specific variant coordinates to derive non-synonymous amino acid changes within each protein.

For every patient, we searched the immunopeptidomics MS data against the patient-specific customized reference database, including a list of 247 frequently observed contaminants. The enzyme specificity was set as unspecific, and peptides with a length between 8 and 25 AA were allowed. The second peptide identification option in Andromeda was enabled. A false discovery rate (FDR) of 5% was required for peptides and no protein FDR was set. The initial allowed mass deviation of the precursor ion was set to 6 ppm and the maximum fragment mass deviation was set to 20 ppm. Methionine oxidation and N-terminal acetylation were set as variable modifications.

PEP-DC Manufacturing

The PEP-DC vaccine is composed of autologous monocyte-derived DC pulsed with personalized peptides (PEP). Monocytes are enriched from a fresh leukapheresis using $CD14^+$ cells selection on the CliniMACS Prodigy (Miltenyi). This process is GMP compliant and allows for a fast and reliable monocyte selection in a closed system. Purified monocytes are differentiated into immature monocyte-derived DC (iDC) by a 5 days culture in the presence of IL-4 and GM-CSF. On day 6, iDC are then loaded overnight with 10 long peptides and matured/activated for 6–8 h using a maturation cocktail composed of MPLA and IFNγ. Cells are finally harvested and cryopreserved as vaccine doses (5–10×10^6 cells per dose). For each injection of PEP-DC vaccine, one dose is thawed, washed and resuspended in NaCl 0.9% supplemented with 1% human albumin before being transferred into syringes and stored at 2–8°C until administration.

Immunogenicity Assessment of PEP-DC Candidates Pre-immunization

The immunogenicity the long peptides was evaluated in cryopreserved peripheral blood mononuclear cells (PBMC) from the three subjects as described (74). PBMC were thawed, rested overnight in RPMI 10% FBS with Penicillin/Streptomycin. For the *in vitro* stimulation (IVS), cells were plated in 24- to 96-well plates at 2×10^6 cells per well in RPMI, 8% human serum supplemented with Penicillin/Streptomycin, 50 μM beta-mercaptoethanol and recombinant human IL-2 at a final concentration of 100 UI/ml. The cells were stimulated with peptide pools containing 1 μg/ml of each candidate peptide. At day 12, intracellular cytokine stainings (ICS) were performed. Each individual well was splitted in two identical fractions and one fraction only was re-challenged with 1 μg/ml of the corresponding peptide for 16–18 h at 37°C and 5% CO2 in presence of 1 μg of brefeldin A (Golgiplug, BD). As a positive control, cells stimulated with staphylococcal enterotoxin B (SEB) at a concentration of 0.25 ng/ml. After 16–18 h of re-stimulation with individual long peptides, cells were harvested and stained with anti-CD3, anti-CD8, anti-CD4, anti-IL-2, anti-TNF-α, anti-IFN-γ (BD biosciences), and with viability dye (Life technologies). Flow cytometry was performed using a four-lasers Fortessa (BD biosciences) and analyzed with FlowJo v10 (TreeStar).

RESULTS

We here present a novel study were a vaccination schedule is incorporated in the SOC chemotherapy adjuvant setting in patients with non-metastatic resectable pancreatic adenocarcinoma followed by nivolumab (an antibody against PD-1), to boost and maintain the vaccine's effect (**Figure 1**). The study was optimally designed to offer innovative cancer vaccines for a PDAC patient population that on one hand would fit the course of standard of care, and on the other hand will be feasible in terms of the time required for the process of antigen discovery and the manufacturing of the vaccine.

It has been correctly pointed out that putting a mutanome-based individualized treatment concept into practice requires both highly interdisciplinary research and an innovative drug development process (75). To fit the tight schedule of the clinical trial, a period of 5 weeks was dedicated for antigen discovery. Upon reception of a pair of tumor tissue sample and matched PBMCs, DNA extraction is performed for whole exome sequencing and HLA typing, and the tumor tissue sample is processed for purification of HLA-I and HLA-II peptides for MS analyses. Within the 2 weeks required for sequencing the DNA samples, HLA typing and MS analyses are completed. The following 2 weeks are dedicated for executing the NeoDisc

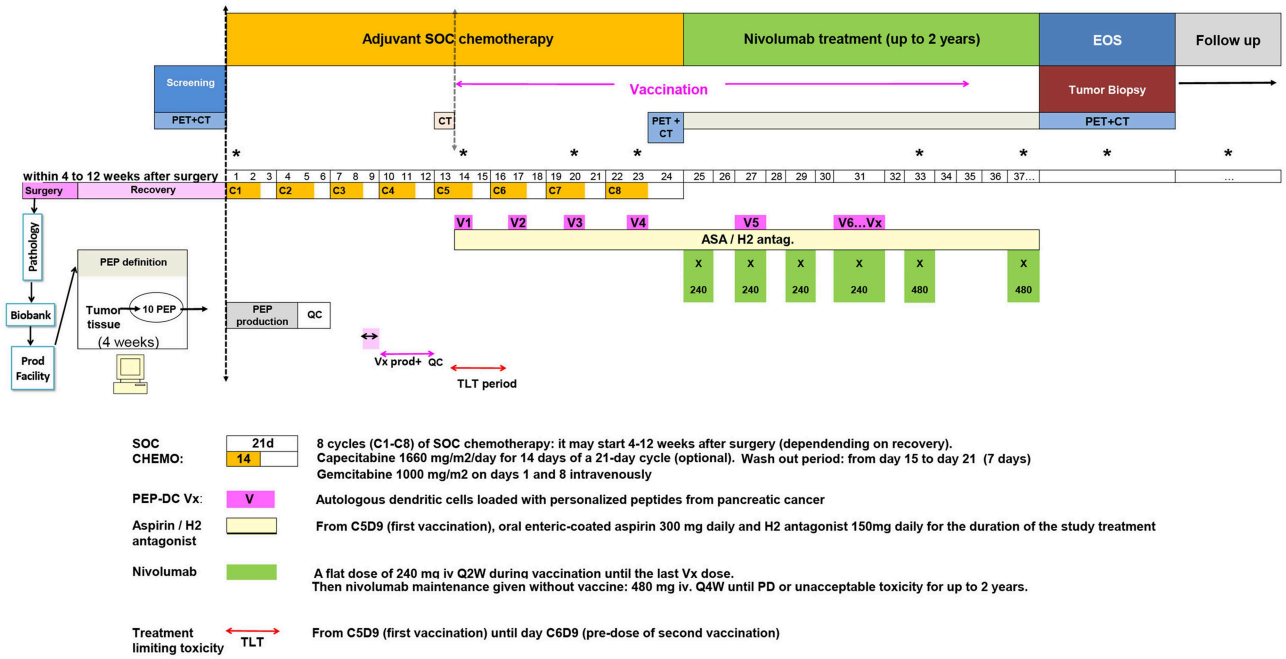

FIGURE 1 | Clinical trial study design.

pipeline, for data mining and for manual inspection of the data and results, leading to the selection of 10 long optimally designed neoantigens. Finally, the production needs to be "on demand," cost-effective, rapid, and compliant with Good Manufacturing Practice (GMP).

NeoDisc Pipeline for Neoantigen Discovery in PDAC

We here tested the feasibility of prioritizing neoantigens in PDAC as targets for our PEP-DC vaccine in three PDAC patients, 14JQ, 154H, and 16AY. The NeoDisc pipeline integrates multiple types of data input from next generation sequencing data, MS immunopeptidomics datasets, and publicly available resources (**Figure 2**). First, the NeoDisc pipeline requires a list of non-synonymous somatic mutations that affect protein-coding regions as identified by three different mutation-calling algorithms: MuTect, VarScan2, and GATK. A combined VCF file is generated and annotated with amino acid changes and transcript information. To increase accuracy, only "high confidence" calls were selected, defined as the set of somatic mutations detected by MuTect alone or by a combination of at least two of the three variant callers described above. As expected, the mutational load in the three PDAC patients was low, with 60, 39, and 23, non-synonymous somatic mutations in 14JQ, 154H, and 16AY, respectively, which is within the range previously reported (53). Among them, we detected mutations in predicted driver genes, the MLLT4 (Ser1708Ala) and PTPN12 (Gly532Glu) (76–78). We then attempted to identify personalized neoantigens using two different approaches; direct identification with mass spectrometry and by prediction of HLA ligands encompassing any of these mutations.

We first performed MS immunopeptidomics analyses on exactly the same tumor tissue used for the genomics analysis from the three PDAC patients, and applied a proteogenomics pipeline as previously described (72) in order to identify neoantigens naturally presented in the PDAC tissues. We have identified 11,437, 4,437, and 6,158 HLA-I and 1,569, 448, and 3,319 HLA-II peptides, from the 14JQ, 154H, and 16AY tumor tissues, respectively (**Supplementary Table 2**). However, no neoantigens could be identified by MS. Either many of the potential neoantigens remain undetected in the MS-based analyses because of the lack of sensitivity, or they might not be naturally presented. The likelihood of detecting neoantigens by discovery MS increases with the overall depth of ligandomic data available and with the mutational load. Here, both aspects were not sufficient to successfully detect neoantigens.

Additional tumor-associated antigen (TAAs) derived HLA ligands are frequently identified by MS, such as "normal" (wild-type) proteins overexpressed or restricted to tumors (e.g., MelanA, Tyrosinase, PMEL in melanoma; NY-ESO in multiple cancer types). Such targets have been exploited in innovative personalized vaccines and T cell based therapies (79, 80). We have identified multiple TAAs in the immunopeptidome for each of the three PDAC patients, including the testis-specific protein bromodomain testis-specific protein (BRDT), L-lactate dehydrogenase C chain (LDHC), outer dense fiber protein 2 (ODF2), coiled-coil domain-containing protein 110 (CCDC110), tumor associated antigens mesothelin (MSLN), mucin-1 (MUC1), prolyl endopeptidase FAP (FAP), and the cellular tumor antigen p53 (TP53) (**Supplementary Table 2**). Nevertheless, after thorough data mining, we estimated that these

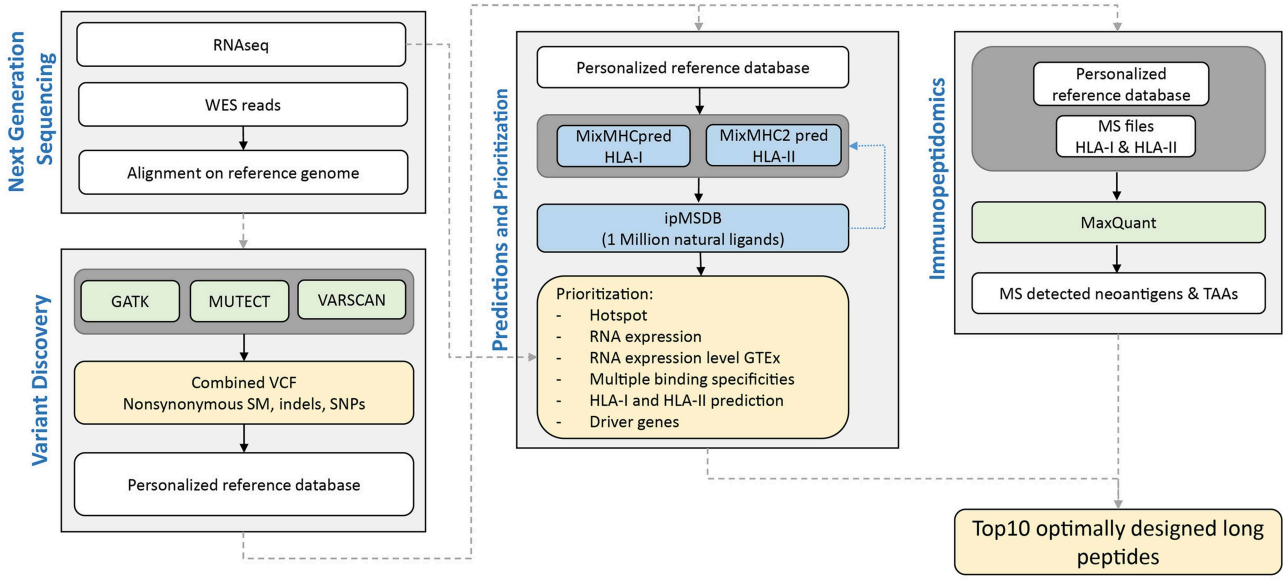

FIGURE 2 | Schematic overview of NeoDisc pipeline for prioritization of neoantigens in PDAC for the design of optimally long peptides for vaccination.

ligands were unlikely to be immunogenic and therefore have decided not to include them in the vaccine.

Prioritization of Neoantigens and Design of Long Peptides

Consequently, in the three PDAC patients, the selection of targets was based exclusively on prediction of neoantigens. To increase accuracy, the "high confidence" calls were selected, and a list of 31 mer peptides with mutation in the middle position was then generated and subjected to binding predictions of HLA class I (9–12 mers) and class II (12–19 mers) with the MixMHCpred.v2.0.2 and MixMHC2pred.v1 algorithms, respectively (66, 68, 81). Both algorithms have been trained on naturally presented peptides and compute the likelihood of a peptide to bind to one of the given set of the patient HLA alleles. We have previously showed that large scale immunopeptidomics dataset may help in prioritizing predicted neoantigens (70, 82). Therefore, we mapped the list of predicted neoantigens on our ipMSDB ligandomic database that contains a million of HLA-I and HLA-II ligands. The overlap between the predicted neoantigen and the wild-type (WT) form present in ipMSDB was determined, as well as the level of presentation of the source genes. These values were considered for prioritization; neoantigens matching exactly WT counterparts in ipMSDB were prioritized (**Table 1**). Mutated source genes that were underrepresented in ipMSDB were excluded. In addition, we excluded predicted neoantigens that are identical to other WT sequences in the human proteome (GRCh37 Genome assembly and UniProt database) and all predicted neoantigens derived from highly mutated genes, which are likely to be false positives. Finally, we excluded genes that are known not to be expressed in pancreas (TPM $<$ 1 in GTEx). For each mutation, we designed a few long peptides covering as many predicted HLA-I and HLA-II neoantigens as possible. We ranked the mutations in the format of long peptides according to the best predicted binding affinity to HLA-I alleles (%Rank $\leq$ 5% MixMHCpred.v2.0.2), the number of HLA-I and HLA-II predicted neoantigens harboring the mutation, and the number of represented HLA alleles. Finally, for each mutation we selected the shortest long peptide covering as many predicted HLA-I and HLA-II neoantigens (**Figure 3**) and completed the list of ten long peptides (PEP).

DC Vaccine Production

The DC-vaccine used in this study is a frozen suspension of patient-specific, *ex vivo* cultured autologous monocyte derived DCs loaded with synthetic neoantigen (personalized) peptides. The proprietary name for the biological product comprising this substance is PEP-DC, which refers to Personalized Peptides loaded onto autologous DCs. Manufacturing of PEP-DC consists of five main steps described in **Figure 4**, starting with peptides identification and manufacturing. We have tested and validated this production process with healthy donor leukapheresis and peptides mixes of up to 9 peptides. Indeed, because of the peptide length, some synthesis failure should be expected even after sequence optimization and careful peptide selection. Based on three experimental batches, we can evaluate that PEP-DC process leads to the production of $2.8 \pm 2.1 \times 10^8$ PEP-DC cells, corresponding to 56 ± 41 PEP-DC cryopreserved vaccine doses (based on 5.0×10^6 cells per dose) per manufacturing run. Specifications of the final product ensure safety (sterility, mycoplasma, endotoxin), viability (Trypan blue exclusion), identity (phenotype), and functionality (IL12p70 secretion upon maturation) of the PEP-DC vaccine (**Table 2**). For each vaccine injection, a PEP-DC dose is thawed, washed and reconstituted NaCl-Albumin before injection. Viability is checked on each reconstituted dose with a target of $\geq$60.0% viable cells. All

TABLE 1 | Basic clinical information and detailed information about the 10 optimally designed long peptides for each patient.

Rank	Chromosome position	Gene	Expression in pancreas, GTEx [TPM]	Mutation	Gene driver and mutation status	Long peptide sequence	ipMSDB HLA-I	ipMSDB HLA-II	Lowest HLA-I binding pval	Lowest HLA-II binding pval	# predicted peptides	# HLA-I alleles	# HLA-II alleles
14JQ, PDAC, 60 NON-SYNONYMOUS SOMATIC MUTATIONS													
1	16_8994451	USP7	11.509	p.Tyr749Asp		LYEEVKPNLTERIQDDDVSLDKALDE	EXACT		0.002	0.01209	31	4	3
2	7_27169740	HOXA4	1.06	p.Ala205Thr		VVYPWMKKIHVSTVNPSYNGGEPKRSRT	EXACT	EXACT	0.004	0.0005	58	3	7
3	9_33797978	PRSS3	13983	p.Val175Ile		TLDNDILLIKLSSPAIINSRVSAISLPT	EXACT	INCLUDED	0.02	0.00096	39	4	7
4	14_105415346	AHNAK2	1.162	p.Thr2148Ala		AHLQGDLTLANKDLTAKDSRFKM	EXACT	PARTIAL	0.002	0.00914	31	3	2
5	1_17083776	MST1L	34.35	p.Arg674Leu		ARSRWPAVFTLVSVFVDWIHKVMRLG			0.0001	0.00578	54	3	6
6	6_168366581	MLLT4	11.744	p.Ser1708Ala	Driver	LPRDYEPPSPAPAPGAPPPPPQRNAS			0.0001	0.00054	80	3	3
7	8_52732961	PCMTD1	16.471	p.Pro342Thr		EPPQNLLREKIMKLTLPESLKAYLT	PARTIAL	PARTIAL	0.0008	0.00098	66	4	3
8	6_150001239	LATS1	3.182	p.Asp789Asn		KDNLYFVMDYIPGGNMMSLLIRMGIFPE	PARTIAL		0.0009	0.00126	58	3	7
9	3_123419461	MYLK	3.273	p.Asp952Asn	Passenger	RKVHSPQQVNFRSVLAKKGTSKT			0.001	0.01599	24	4	3
10	1_155697428	DAP3	12.728	p.Leu168Phe		IPDAHLWVKNCRDFLQSSYNKQRFD			0.002	0.00521	45	4	2
154H, PDAC, 39 NON-SYNONYMOUS SOMATIC MUTATIONS													
1	2_85576579	RETSAT	17.724	p.Arg309Trp		IAFHTIPVIQWAGGAVLTKATVQSVL	EXACT	EXACT	0.0004	0.00026	65	5	4
2	12_51453191	LETMD1	14.494	p.Asn367Asp		AELSLLLHNVVLLSTDYLGTRR	EXACT	EXACT	0.006	0.00298	50	4	4
3	20_34457413	PHF20	2.4	p.Arg288Gly		NSQTLQPITLELRRGKISKGCEVPL		EXACT	0.02	0.03246	19	4	2
4	3_57908703	SLMAP	3.553	p.Lys783Gln		KQSITDELQQCKNNLKLLREK			0.0007	0.00357	40	3	2
5	2_241700220	KIF1A	11.548	p.Ser769Phe		KKVQFQFVLLTDTLYFPLPPDLLPPEAA			0.0008	0.00201	72	5	5
6	2_238253286	COL6A3	16.075	p.Arg2459Trp		VAVVTYNNEVTTEIWFADSKRKSVLLDK			0.0009	0.00133	61	5	5
7	13_96592287	UGGT2	3.986	p.Val579Gly		KKDQNILTVDNVKSGLQNTF			0.002	0.01697	24	6	3
8	7_77256591	PTPN12	12.375	p.Gly532Glu	Driver	DRLPLDEKEHVTWSFHGPENAIPI	PARTIAL	PARTIAL	0.003	0.0391	12	6	1
9	18_55352319	ATP8B1	10.673	p.Asn486Lys		DHRDASQHKHNKIEQVDFSWNTYA			0.003	0.0339	14	5	2
10	8_9627645	TNKS	3.91	p.Gly1257Glu		HRQMLFCRVTLEKSFLQFSTMKMAHA	PARTIAL	PARTIAL	0.003	0.00039	39	6	5
16AY, PDAC, 23 NON-SYNONYMOUS SOMATIC MUTATIONS													
1	17_15134320	PMP22	11.978	p.Gly133Ser		HPEWHLNSDYSYSFAYILAWVAFPLALL	EXACT		0.0004	0.00018	86	4	8
2	X_54014354	PHF8	5.113	p.Ser621Tyr		LLMSNGSTKRVKSLYKSRRTKIAKKVDK			0.0001	0.0075	48	5	2
3	9_33794809	PRSS3	13983	p.Ser5Asn		MRETNVFTLKKGRSAPLVF			0.0004	0.00558	10	4	3
4	12_9085452	PHC1	9.639	p.Gln467Lys		TQQVPPSQSQQKAQTLVVQPMLQSSPL	PARTIAL		0.0004	0.0044	42	5	6
5	19_1037681	CNN2	20.083	p.Asp259Asn		APGTRRHIYDTKLGTNKCDNSSMSLQMG			0.0008	0.01696	27	5	5
6	20_50704942	ZFP64	1.539	p.Arg187Leu		YASRNSSQLTVHLLSHTGDTPFQ			0.002	0.00077	40	3	4
7	12_31254897	DDX11	5.459	p.Arg728His		LRQVHAHWEKGGLLGHLAARKKIFQE			0.003	0.00764	37	3	2
8	11_32954416	QSER1	3.78	p.Asn409Asp		SSNQQEVLSSVTNEDYPAQTRDLSSVSQ	PARTIAL		0.003	0.00478	39	3	4
9	1_27177681	ZDHHC18	4.114	p.His299Tyr		FFSIWSILGLSGFYTYLVASNLTTNEDI	PARTIAL		0.005	0.00012	106	4	8
10	20_60892518	LAMA5	23.906	p.Arg2465Gln		AKEELERLAASLDGAQTPLLQRMQT	PARTIAL	PARTIAL	0.008	0.00763	41	5	6

The position of the mutation in the long peptide is indicated in red. ipMSDB HLA-I and ipMSDB HLA-II columns show the matching of the WT counterpart of the predicted neoantigen in the ipMSDB. Prediction of driver genes and mutation status annotations are derived from IntOGen database.

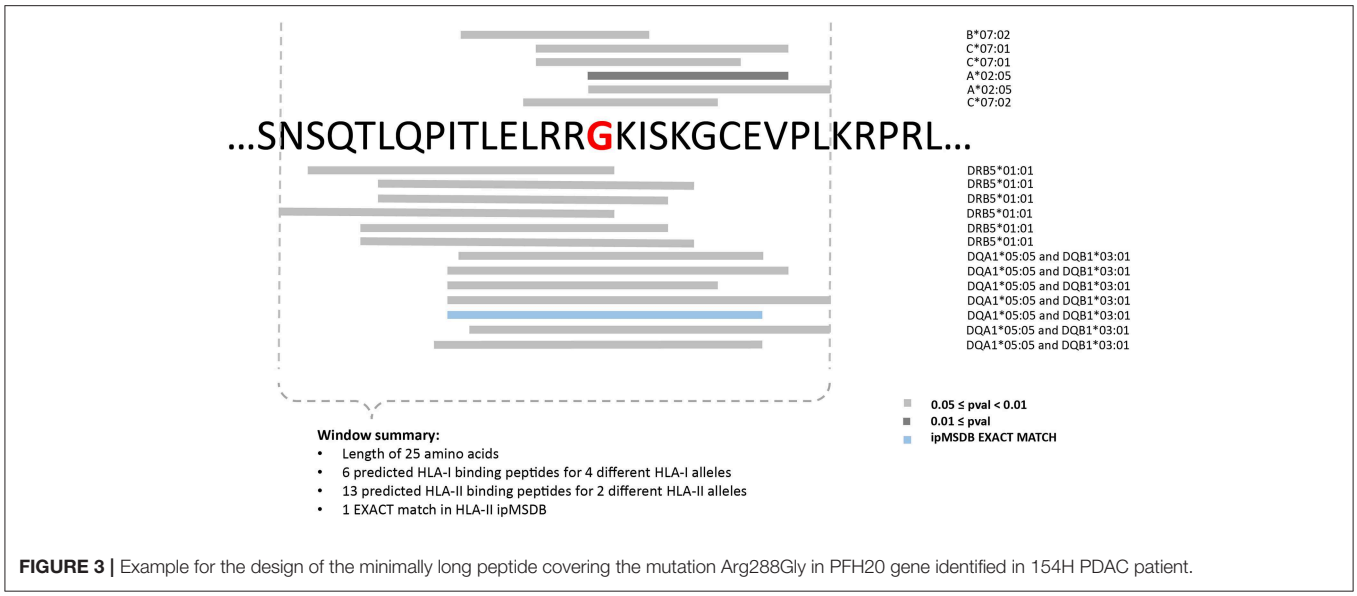

FIGURE 3 | Example for the design of the minimally long peptide covering the mutation Arg288Gly in PFH20 gene identified in 154H PDAC patient.

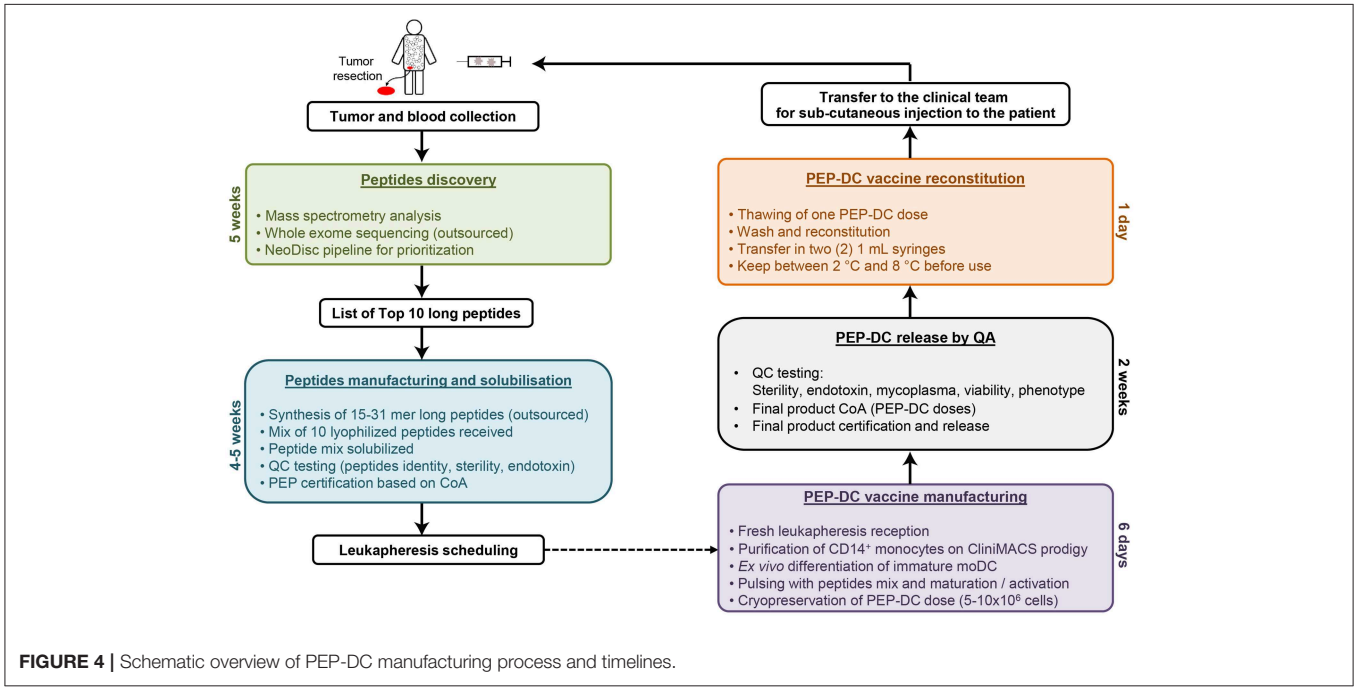

FIGURE 4 | Schematic overview of PEP-DC manufacturing process and timelines.

PEP-DC batches prepared in this pilot study met specification for product release as described in **Table 2**. This confirms that our GMP-compliant manufacturing process is suitable for the production PEP-DC.

Pre-immunization Immunogenicity of PEP Candidates

Even though immune responses against neoantigens prior to vaccination are typically rare, we decided to test if any of the 10 PEP long peptides may be recognized by autologous T cells from peripheral blood. Pre-immunization immunogenicity was tested for the three PDAC patients. Although some of the long peptides failed the synthesis quality control and could not be tested, CD4+ T-cell responses against PEP candidates were detected against at least one long peptide in all three donors, while no CD8+ T cell responses could be detected (**Figure 5**).

DISCUSSION AND INNOVATION

Over the last 10 years immunotherapy has changed the treatment landscape of several tumor types in metastatic setting. Management of patients with non-metastatic cancer relies

on multimodality treatment that includes surgical resection depending on tumor type and peri-operative chemotherapy. In PDAC, despite these aggressive measures, the high propensity of relapse has a detrimental effect on survival. The high metastatic potential is due to the presence of micrometastasis at systemic sites in patients with early-stage pancreatic cancer (83). In this proof of concept trial, our aim is to demonstrate that in such very aggressive diseases, there is a need to act without any delay, and early immunomodulation may be the key response.

TABLE 2 | Specification for release for the final product of PEP-DC doses.

	Test	Analytical procedure	Specification
PEP-DC at day 6 (final product)	Sterility	BacTEC (aerobic and anaerobic)	No growth
	Mycoplasma	MycoSeq	Negative
	Endotoxin	Endosafe	≤10.0 EU/mL
	Cell count	Manual cell count by Trypan blue exclusion	≥45.0×10^6 viable cells
	Viability		≥60.0% viability
	Cell purity/identity	Flow cytometry	≥60.0% live HLA-DR$^+$CD86$^+$ cells ≤20.0% CD14$^+$ cells
Culture supernatant at day 6 after maturation	Functionality IL-12p70	ELISA	≥50.0 pg/mL

Although pancreatic cancer patients present high frequencies of functional tumor-reactive T cells in the bone-marrow and blood (84), and show an average mutation burden similar to other solid tumors (85), parsing tumor immune microenvironment (TME) of pancreatic cancer seems to be a challenge. In PDAC, tumor-specific CTLs become "trapped" in the peritumoral tissue and in the tumor stroma, not reaching pancreatic tumor cells in sufficient amounts (86, 87). Additionally, exhaustion of effector CD8+ T-cells by the TME as well as hampered recruitment of cDC1s by downregulating CCL4 signaling upon constitutively active β-catenin signaling may explain the ineffective antitumoral response, which underscores the importance of endogenous DCs for initiating anti-tumor immunity.

Rationale for Combination Immunotherapy in PDAC

Currently, clinical benefit using different agents in monotherapy is very limited in PDAC. Therefore, combination strategies are required, in order to obtain a synergistic effect on potential efficacy, yet keeping expected adverse events under manageable conditions. Therefore, it is important to establish both the scientific rationale of the proposed combination, as well as the best timing for introducing each component.

Treatment of metastatic cancer essentially relies on cytotoxic drugs that kill tumor cells or hinder their proliferation. Although a primary goal of anti-cancer chemotherapy is the tumor mass reduction, it is now clear that off-target effects, especially directed to the host immune system, may reduce the

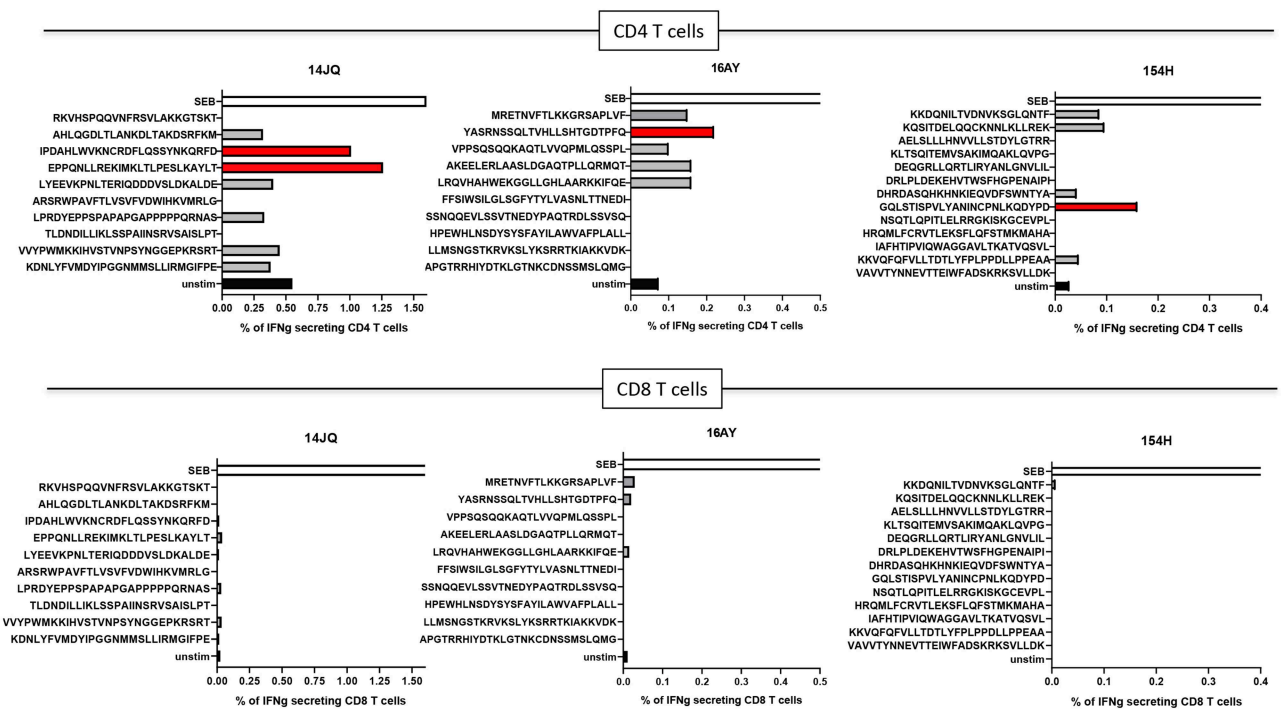

FIGURE 5 | T-cell responses in donors 14JQ, 16AY, and 154H against long peptides. The percentage of IFN-γ-producing T cells are shown. Black (unstim) and white (SEB) bars represents negative and positive controls, respectively. Positive peptides are identified in red.

immunosuppressive activity of malignant cells and cooperate for successful tumor eradication (88). Gemcitabine (GEM) is a chemotherapeutic agent acting as a nucleoside analog that also targets ribonucleotide reductase by inactivating the enzyme irreversibly. It is used in various carcinomas such as non-small cell lung cancer, pancreatic cancer, bladder cancer, and breast cancer, and it represents the primary systemic agent for the treatment of pancreatic cancer. On standard dose schedules in patients with pancreatic cancer, the drug is associated with manageable toxicity, and its administration has led to a survival benefit both in the primary and adjuvant settings (89, 90). In advanced pancreatic cancer patients, GEM therapy may decrease memory T-cells, promote naive T-cell activation (91), and induce the proliferation of $CD14^+$ monocytes and $CD11c^+$ DC (92). GEM is also able to induce apoptotic destruction of tumor cells and potentially load the immune system with large amounts of tumor antigen, but this is not enough to initiate a protective antitumor response and adjuvant immunotherapy is required (93).

A peptide cocktail vaccine OCV-C01 containing epitope peptides [coding for vascular epithelial growth factor receptor (VEGFR1 ad VEGFR2)] was investigated in combination with GEM in the adjuvant treatment for resected pancreatic cancer patients ($n = 30$) in a single arm multicenter Phase II study. OCV-C01 combined with GEM was tolerable with a median DFS of 15.8 months (and a DFS rate at 18 months of 34.6%), which was favorable compared with previous data for resected pancreatic cancer (94). In another phase I pilot study, a Wilms tumor gene-1 peptide-pulsed DC vaccination was evaluated in combination with GEM as a first-line of treatment in 10 patients with advanced pancreatic cancer. WT1 peptide-pulsed DCGEM is feasible, well-tolerated, and effective for inducing anti-tumor T-cell responses (95). Kimura et al. evaluated a DC-based vaccine alone or in combination with lymphokine-activated killer (LAK) cells, along with gemcitabine and/or S-1 in 49 patients with inoperable pancreatic cancer (96). Of these patients, two manifested a complete remission, five a partial remission, and 10 had stable disease. The median survival of these individuals was 360 days, which appeared to be longer than what could be achieved with gemcitabine and/or S-1. Thus, the combination of DC-based immunotherapy and chemotherapy seems well-tolerated by advanced PDAC patients but warrants further investigation through combination with ICB or other immunotherapies. In our study, we build on the gemcitabine/capecitabine backbone for not fit pancreatic cancer population (ECOG PS 1 or 2) and explore the additive benefits of DC-vaccination from the 5th cycle of chemotherapy, followed by nivolumab treatment.

In pancreatic cancer, a possible explanation for the therapeutic failure of PD-1/PD-L1 blockade therapy is the lack of natural infiltration of effector immune cells in most cases (17, 18, 20). Vaccine-based immunotherapy is a potential strategy to activate effector T cell trafficking into the TME. Additionally, it has been shown that the repertoire of clonally expanded tumor antigen-reactive cells within TILs expresses PD-1 (97), either in spontaneous responses or vaccine-mediated. Furthermore, vaccination induces intratumoral PD-L1 expression (98), suggesting a role for PD-1 blockade in enhancing vaccine efficacy (98, 99). Consistently, in a preclinical model for pancreatic cancer, GVAX administration (a cancer vaccine composed of allogeneic pancreatic tumor cell line engineered to secrete GM-CSF) induced upregulation of PD-L1 expression when compared to untreated human and mouse pancreatic tumors. Combination therapy with GVAX and PD-1/PD-L1 blockade improved survival, and correlated with increased CD8+ T infiltration into pancreatic tumors (100).

Currently very few clinical trials combining cancer vaccines and PD-1/PD-L1 blockade have been reported in the setting of pancreatic cancer. Combination strategies using DC vaccines with ICB should generate an additive effect (98, 99, 101), with low additional toxicity due to DC vaccination (102, 103). Nesselhut et al. demonstrated that the efficacy of DC based therapy can be improved by blockade of PD-L1, enhancing the T-cell specific response (104). Dose and schedule for anti-PD-1 therapy and vaccines have been minimally studied; however, both PD-1 on activated T cells and PD-L1 on tumors appear rapidly following exposure to interferon (105), suggesting that early application of PD-1 blockade may be important. For this reason, we have decided to start nivolumab treatment 3 weeks after the end of SOC treatment, aiming also to avoid potential toxicities due to combined chemo-ICB.

Because Treg may persist despite checkpoint blockade, Treg depletion in conjunction with checkpoint blockade and vaccination may enhance clinical anti-tumor efficacy. Systematic reviews of the results of aspirin in cardiovascular studies have suggested that low-dose aspirin reduces overall cancer incidence and mortality including in pancreatic cancer (106, 107). In terms of its mechanism (108), it has been shown that non-steroidal anti-inflammatory drugs may limit carcinogenesis and enhance the immune response by (a) preventing prostaglandin E_2 (PGE_2)-mediated inhibition of DCs and reducing the transition of monocytes to immunosuppressive MDSCs (109); (b) reducing the inhibitory potential of Tregs induced by PGE2 (110); and (c) abrogating the PGE2 induced suppression of effector T-cell proliferation by regulatory T cells (111), therefore contributing to enhanced immune surveillance. Furthermore, PGE2 inhibitors like aspirin can counteract the FasL mediated elimination of activated lymphocytes by the tumor endothelial cells, as well as reduce the immunosuppressive conditions, thus enhancing the immune response against the tumor. We therefore consider that blockade of PGE2 in cancers using aspirin can reverse the endothelial barrier and synergize with vaccination allowing T cell infiltration. Consequently, we will use aspirin all along our study, which we expect to synergize with T cell activation by PD-1/PD-L1 blockade.

Neoantigen Prediction and Selection for PEP-DC

Identification and selection of targets for neoantigen based vaccines is challenging. Mass spectrometry has been instrumental for the identification of cancer-associated antigens among the endogenously presented peptides. In recent years, dedicated computational pipelines for proteogenomic applications facilitated the direct identification of neoantigens by MS in

murine and human cancer cell line models (49, 112–115), B cell lymphomas (116), and melanoma tissues (72) as well as other cryptic peptides resulting from unconventional coding sequences in the genome (117, 118). However, only a handful of neoantigens have been identified by MS in a given sample, and typically in high mutational load tumors such as melanoma (72). Indeed, we could not identify with discovery MS-based immunopeptidomics neoantigens in the three investigated PDAC samples. While several tumor-associated antigens were identified, after literature mining we concluded that these antigens might be poorly immunogenic, and in these three cases we decided to exclude non-mutated targets.

The prioritization and selection of neoantigens for personalized vaccines in low mutational load tumors like PDAC is largely performed with HLA ligand interaction prediction algorithms. The performance of such tools has improved significantly with the incorporation of MS HLA ligand elution data in the training of the algorithms, both for HLA-I (81, 119–121), and more recently for HLA-II (68). Furthermore, interrogation of properties of the thousands of different source-proteins has revealed biological determinants that correlate with presentation, such as level of translation and expression, turnover rate, proteasomal cleavage specificities, hotspots, and biological functions. Integrating such variables into a single predictor further improves prediction of neoantigens (70, 72, 120, 122). Because predictors of immunogenicity are still immature (123) false positives are inevitably included among the predicted neoantigens, which may eventually be included in a vaccine.

A main innovative aspect of our study is the identification of PDAC mutated neoantigens. We have designed NeoDisc, a novel proteogenomics antigen discovery pipeline for identification and selection of neoantigens, and we apply it for the first time in PDAC. NeoDisc integrates multiple state of the art prediction tools, large-scale ligandomic database, and a unique personalized and optimized design of long peptides that maximizes the likelihood that the selected mutations will eventually be presented by the HLA-I and HLA-II complexes on the loaded DCs. While in this PEP-DC study the existence of pre-existing immune responses against the long peptides is not a prerequisite for inclusion in the vaccine, such analysis is performed as part of a large translational program that aims to provide extensive immunogenicity training data that will allow future development to improve the performance of NeoDisc.

This proof of concept study aimed to assess specifically the feasibility of prioritizing immunogenic neoantigens with NeoDisc. Indeed, we were able to confirm for the three patients pre-existing immune responses against in total four long neoantigen peptides with autologous peripheral CD4+ T cells. No CD8+ T cell responses could be detected. This might be related to the low frequency of neoantigen specific CD8+ T cells. Alternative strategies could have been more sensitive to detect CD8+ T-cell responses, such as peptide-MHC multimers screening. However, unfortunately, there were no PBMC left to test this hypothesis. The clinical trial has not started yet, and therefore the investigation of immune responses post-vaccine could not be performed. This trial will give us the opportunity to (1) better understand PDAC TME since we will be able to evaluate the mutational rate in PDAC and predict the presentation of neoantigens; (2) assess the frequency of specific T cells to such mutant epitopes in PDAC patients, before and after treatment with ICB; (3) validate the immunogenicity of neoantigens and their therapeutic effect.

In conclusion, PDAC in early-stage remains a deadly disease with limited treatment options and the development of novel strategies tailored to individual patients is the key. Our approach is focused particularly on patients with a borderline performance status or a comorbidity profile that precludes multiagent adjuvant therapy (type folforinox). In this context, we give the opportunity even in patients with the worst prognosis to have access to innovative therapies.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors. To respect patient confidentiality, access to the data will be obtained by formal application to a Data Access Committee that requires researchers to sign a Data Access Agreement (DAA).

AUTHOR CONTRIBUTIONS

MB-S is responsible for the development of NeoDisc and for clinical antigen discovery, and wrote the manuscript. BS and FH developed NeoDisc. JM and HP performed Bristol-Myers Squibb (BMS) immunopeptidomics experiments. JR and DG developed MixMHC2perd. CS, DW, and ND provided the pancreatic cancer specimens and reviewed the manuscript. AD, KB, and SM contributed to the clinical trial design and manuscript writing. GC contributed to the clinical trial design. A-CT and AH developed the immunogenicity validation data. CB developed the vaccine clinical grade data. LK developed the clinical grade vaccine, conceived the clinical study, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01832/full#supplementary-material

Table S1 | General clinical information and high resolution HLA typing.

Table S2 | List of HLA-I and HLA-II peptides identified by MS-based immunopeptidomics in the three PDAC tumor samples, including MS intensity, peptide length and mass, identification score and posterior error probability (PEP), source proteins and gene names.

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A Non-interventional Clinical Trial Assessing Immune Responses After Radiofrequency Ablation of Liver Metastases From Colorectal Cancer

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Background: Radiofrequency ablation (RFA) is an established treatment option for malignancies located in the liver. RFA-induced irreversible coagulation necrosis leads to the release of danger signals and cellular content. Hence, RFA may constitute an endogenous *in situ* tumor vaccination, stimulating innate and adaptive immune responses, including tumor-antigen specific T cells. This may explain a phenomenon termed abscopal effect, namely tumor regression in untreated lesions evidenced after distant thermal ablation or irradiation. In this study, we therefore assessed systemic and local immune responses in individual patients treated with RFA.

Methods: For this prospective clinical trial, patients with liver metastasis from colorectal carcinoma (mCRC) receiving RFA and undergoing metachronous liver surgery for another lesion were recruited ($n = 9$) during a 5-year period. Tumor and non-malignant liver tissue samples from six patients were investigated by whole transcriptome sequencing and tandem-mass spectrometry, characterizing naturally presented HLA ligands. Tumor antigen-derived HLA-restricted peptides were selected by different predefined approaches. Further, candidate HLA ligands were manually curated. Peripheral blood mononuclear cells were stimulated *in vitro* with epitope candidate peptides, and functional T cell responses were assessed by intracellular cytokine staining.

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Immunohistochemical markers were additionally investigated in surgically resected mCRC from patients treated with ($n = 9$) or without RFA ($n = 7$).

Results: In all six investigated patients, either induced immune responses and/or pre-existing T cell immunity against the selected targets were observed. Multi-cytokine responses were *inter alia* directed against known tumor antigens such as cyclin D1 but also against a (predicted) mutation contained in ERBB3. Immunohistochemistry did not show a relevant influx of immune cells into distant malignant lesions after RFA treatment ($n = 9$) as compared to the surgery only mCRC group ($n = 7$).

Conclusions: Using an individualized approach for target selection, RFA induced and/or boosted T cell responses specific for individual tumor antigens were more frequently detectable as compared to previously published observations with well-characterized tumor antigens. However, the witnessed modest RFA-induced immunological effects alone may not be sufficient for the rejection of established tumors. Therefore, these findings warrant further clinical investigation including the assessment of RFA combination therapies e.g., with immune stimulatory agents, cancer vaccination, and/or immune checkpoint inhibitors.

Keywords: colorectal cancer, radiofrequency ablation, liver metastasis, HLA ligandome, T cells, tumor-associated antigens, neoepitopes, abscopal effect

INTRODUCTION

Percutaneous radiofrequency ablation (RFA) has initially been established as a therapeutic modality enabling the physical destruction of malignant tissue by heat. During RFA, an alternating electric current is generated within the tissue leading to ion agitation and frictional heat, resulting in coagulative necrosis of cells due to local heating of tissues (>60°C) (1, 2). This minimally invasive technique is an additional therapeutic option or alternative to surgical treatment, mainly applied for patients for whom a complete surgical tumor resection cannot be achieved or who do not qualify for surgery due to other reasons.

Besides various other malignancies, RFA is frequently used to reach tumor control in colorectal cancers (CRC) metastasized to the liver (mCRC), where it has been established as a safe and effective procedure (3, 4). Since recurrence rates surpass 50% for patients undergoing potentially curative liver resection for mCRC (5), RFA may not only constitute a promising adjunct treatment approach, but also have beneficial effects beyond local tumor control. Nevertheless, the definite benefit of RFA treatment in mCRC of the liver remains to be established (6), and respective randomized controlled trials are still ongoing (7).

It has only been appreciated recently that RFA treatment may also have profound immunological implications and that there are effects occurring beyond mere local tumor destruction (8). Like radiotherapy and cryoablation, RFA may also induce so-called abscopal effects, where subsequent to the treatment of one malignant lesion, another untreated distant lesion responds to treatment. The phenomenon is still insufficiently understood (9) and even in mouse models no robust effects are observed (10). However, particularly in mouse models there is convincing evidence that the involvement of the immune system represents the most plausible mode of action, since RFA and comparable treatment approaches may constitute a form of *in situ* whole cell vaccination comparable to lysates from tumor cells. Such tumor cell lysates have been proposed to contribute a wide array of immunogens that may induce tumor rejection (11–13). Abscopal effects have been ascribed to the stimulation of tumor-specific T cells recognizing tumor antigen-derived HLA-restricted peptides. Interestingly, these effects were shown to occur with disproportionally high frequency in malignancies considered as immunogenic such as malignant melanoma, renal cell carcinoma, and lymphomas (12) but they still remain rare and cannot be regularly reproduced (14–16). In this context, it can be assumed that single T cell targets such as mutated HLA ligands bear great potential for tumor rejection and may even hold the key for patient cure, in case they can be specifically

Abbreviations: APC, allophycocyanin; BSC, best supportive care; BV, brilliant violet; CD, cluster of differentiation; CID, collision-induced dissociation; CPM, counts per million mapped reads; CRC, colorectal cancer; DMSO, dimethylsulfoxide; ERBB3, human epidermal growth factor receptor 3; FCS, fetal calf serum; FFPE, formalin-fixed, paraffin embedded; FN1, fibronectin 1; FSC, forward scatter; H&E, hematoxylin and eosin; HLA, human leucocyte antigen; HSP, heat shock protein; ICI, immune checkpoint inhibition; ICS, intracellular cytokine staining; IFI6, interferon alpha-inducible protein 6; IFN, interferon; IL, interleukin; IRISS, Interventional Radiology, Immunology, Surgery Study; LC, liquid chromatography; LTQ, linear trap quadrupole; mAB, monoclonal antibody; mCRC, metastasized colorectal cancer (to the liver; unless stated otherwise); mRNA, messenger ribonucleic acid; MHC, major histocompatibility complex; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MSI, microsatellite instable; NML, non-malignant liver; NMT, non-malignant tissue; PGCA, aggrecan core protein; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PMA, phorbol myristate acetate; RFA, radiofrequency ablation; RNA, ribonucleic acid; TCR, T cell receptor; TNF, tumor necrosis factor; uHPLC, ultra-high-performance liquid chromatography; WTS, whole transcriptome sequencing.

exploited for therapy (17). Nonetheless, based on the current state-of-the-art in characterizing HLA-presented ligands by mass spectrometry (MS), mutated HLA ligands are probably very rare. This aspect is of particular relevance for malignancies with very few mutations (18). Only a small fraction of predicted mutated gene products was detectable by MS on tumors (19) or shown as immunogenic and may therefore mediate tumor rejection (20), a notion that may also help to explain the sporadic nature of abscopal effects.

In CRC for instance, highly mutated (e.g., microsatellite instable) cancers were shown to respond to immune checkpoint inhibition (ICI) immunotherapies, whereas sporadic CRC with low mutation rates did not (21). Further, it is becoming clearer, that not only mutated HLA ligands may drive the immune response against cancers, but also alterations beyond exome-derived mutations may prove relevant for the rejection of malignant cells, such as (non-mutated) neoantigens, originating from tumor-specific alterations, protein modifications, RNA-editing and alterations in non-coding regions (22–25). Excluding some exceptions, most of these alterations are patient-individual. In addition, there have been recent reports that ICI in combination with radiotherapy can increase the occurrence of clinically significant abscopal effects (26).

In a previous study, we have shown that tumor antigen-specific antibodies and T cells can be induced in a fraction (<10%) of patients following RFA treatment (27). In this study, we aimed at studying patient-individual anti-tumor T cell responses occurring in the context of RFA in patients with metastasized colorectal cancer (mCRC), as well as assessing immune infiltrates that may arise in distant metastases following RFA treatment.

MATERIALS AND METHODS

Ethics Approval and Informed Consent

This trial using the acronym IRISS (Interventional Radiology, Immunology, Surgery Study) was conducted in accordance with the principles of the Declaration of Helsinki and approved by the local institutional review board of the University Hospital Tübingen (Reference No. 169/2005V and 638/2014BO2). All participants provided written informed consent before study inclusion.

Study Design and Patients

Sixteen patients were recruited for this study. Patient characteristics are provided in **Supplementary Table 1**. The first group (**Figure 1**) included all consenting patients with metastases from CRC in different liver segments, scheduled for treatment with RFA and subsequent liver surgery at Tübingen University Hospital, recruited in the course of a 5-year period ($n = 9$). This RFA + surgery group [all men; mean age 64 years (range, 45–79 years) at initial diagnosis] included six patients with sufficient sample materials for in-depth analyses (mCRC and non-malignant liver (NML) tissues, as well as PBMCs for immunological evaluation). Patients in this group were treated with one session of RFA for one of the tumor lesions, followed by subsequent surgical resection (on average 4 weeks after RFA; range 1–8 weeks) of the non-RFA-pretreated, distant liver metastases.

A corresponding control group included patients ($n = 7$) with liver metastases of mCRC scheduled for surgery only (five males; mean age 58 years (range, 45–77 years) at initial diagnosis). Of note, no fresh frozen tumor/NML tissue or PBMCs was available for this group and only paraffin embedded tumor tissue was accessible for immunohistochemical evaluation.

All patients were treated with curative intent according to institutional standards and presented with a median number of two mCRC lesions (min. – max.: 1–7).

Sample Materials

For patients included in the RFA + surgery group and evaluated in immunological experiments ($n = 6$), blood samples were collected before RFA treatment, at surgery (∼1 month later), and at several follow-up visits thereafter, at intervals of 1–4 months (**Figure 1**). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation and cryopreserved in freezing medium [fetal calf serum (FCS) with 10% dimethylsulfoxide (DMSO)] until subsequent analysis.

Additionally, during elective liver surgery for mCRC, scheduled after RFA treatment, resected tissue was obtained from both mCRC as well as NML tissue. Tissue samples without diagnostic relevance were divided and snap frozen in liquid nitrogen or else stored in RNA later (ThermoFisher Scientific, Waltham, MA) and kept at −80°C for long-term cryopreservation until analysis.

For all patients from both groups ($n = 16$), mCRC tissue samples were paraffin embedded, and diagnosis was confirmed by expert pathologist review. Paraffin embedded tissue was used for immunohistochemical evaluation.

HLA Typing

For the patients included in the RFA + surgery group ($n = 6$), high-resolution HLA typing from peripheral blood (LUMINEX and sequence-based typing according to implemented validated institutional clinical routines) was performed for HLA-A and HLA-B (**Table 1**).

Isolation of HLA Ligands From Surgical Specimens

Immunoaffinity purification was used for parallel isolation of HLA class I and II molecules from tissue lysates, employing the pan HLA class I monoclonal antibody W6/32 (28) as well as the HLA-DR monoclonal antibody L243 (29) together with the pan HLA class II monoclonal antibody Tü39 (30) (all produced in-house at the Department of Immunology, University of Tübingen, Germany) as previously described (31). HLA class I and II-bound peptides were separately eluted using 0.2% trifluoroacetic acid.

Analysis of HLA Ligands by LC-MS/MS

Purified HLA-bound peptides from HLA class I and II immunoprecipitates were analyzed in up to six technical replicates of each sample, as previously described (32). Briefly,

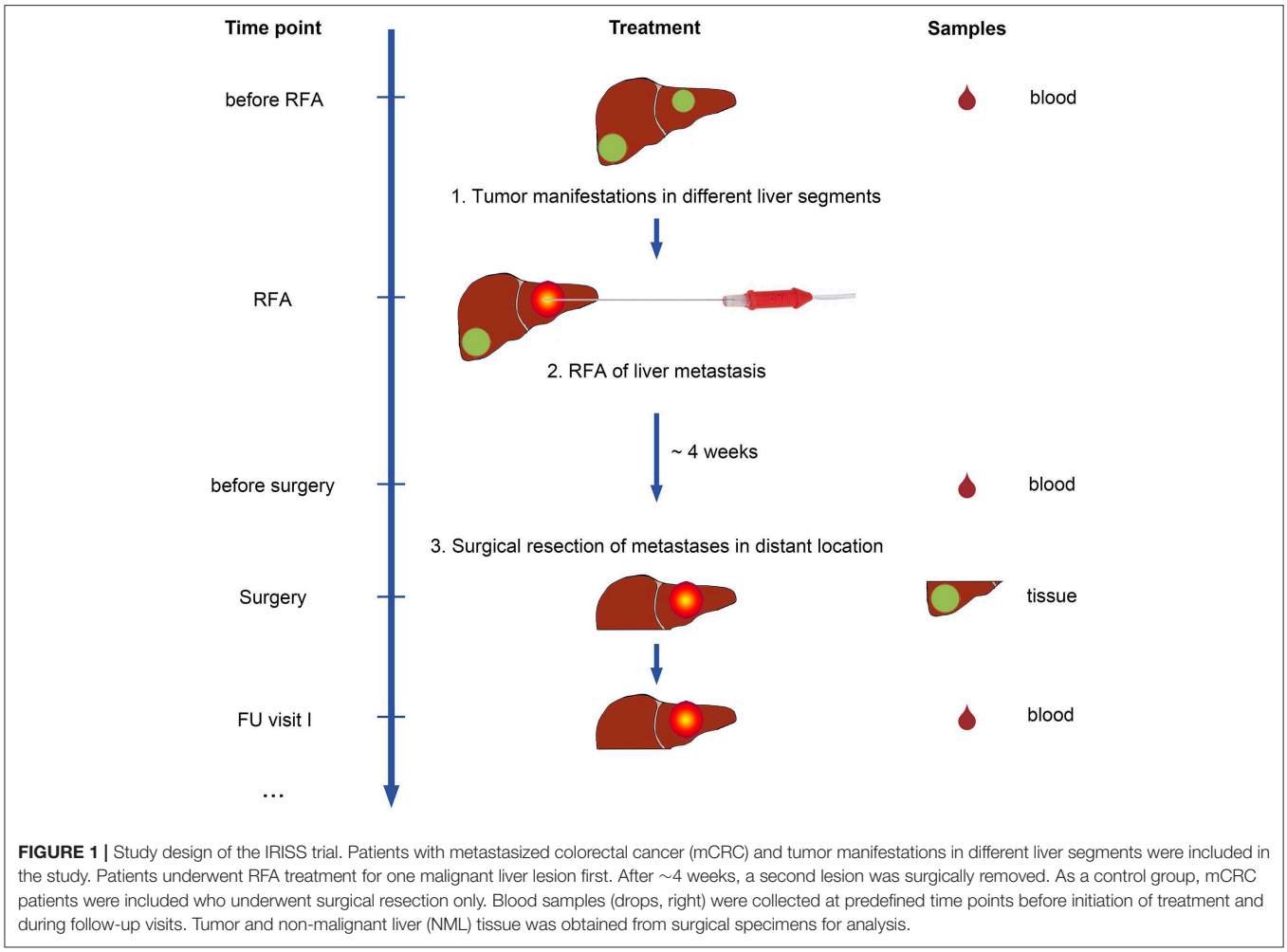


FIGURE 1 | Study design of the IRISS trial. Patients with metastasized colorectal cancer (mCRC) and tumor manifestations in different liver segments were included in the study. Patients underwent RFA treatment for one malignant liver lesion first. After ~4 weeks, a second lesion was surgically removed. As a control group, mCRC patients were included who underwent surgical resection only. Blood samples (drops, right) were collected at predefined time points before initiation of treatment and during follow-up visits. Tumor and non-malignant liver (NML) tissue was obtained from surgical specimens for analysis.

purified peptides were separated by nanoflow ultra-high-performance liquid chromatography (uHPLC; UltiMate 3000 RSLCnano System, ThermoFisher) using a 50 µm × 25 cm column (PepMap RSLC, ThermoFisher) and an acetonitrile gradient ranging from 2.4 to 32.0% over the course of 90 min. uHPLC eluting peptides were analyzed in an online coupled linear trap quadrupole (LTQ) Orbitrap XL mass spectrometer (ThermoFisher), equipped with a nanoelectron spray ion source employing a top 5 collision-induced dissociation (CID) fragmentation method.

Database Search and Spectral Annotation

The Mascot search engine (Mascot 2.2.04, Matrix Science, Boston, MA) was used to search the human proteome contained in the Swiss-Prot database (20,279 reviewed protein sequences, as of September 2013) without any enzymatic restriction (required Mascot ion score ≥20; search engine rank: 1). As a dynamic modification oxidized methionine was allowed. The false discovery rate was estimated with the Percolator algorithm (33) and set to 5%. Peptide lengths for HLA class I-eluted peptides were limited from 8 to 12 amino acids (required charge state: 2–3) and for HLA class II-eluted peptides from 9 to 25 amino acids (required charge state: 2–5). Protein inference was disabled, allowing for multiple protein annotations of peptides. HLA class I annotation was performed using SYFPEITHI (34), and NetMHC (vers. 3.4) (35).

Whole Transcriptome Sequencing (WTS) and Data Analysis

Whole transcriptome sequencing (WTS) was performed after isolation of mRNA from the patient's tissue samples (mCRC vs. NML) using 100 ng of total RNA and the TruSeq Stranded mRNA Kit (Illumina, San Diego, CA) with 14 cycles of PCR. Tissue sample from patient IRISS06 were processed using 40 ng of total RNA and the TruSeq RNA Access Kit (Illumina) with 15 cycles of amplification. All samples were sequenced on a HiSeq 2500 device (Illumina) as paired-end sequencing. Sequencing depth was 20–40 million cluster/ sample with 68 cycles per read.

Data Preprocessing

Adapters were trimmed using SeqPurge [v. 0.1 (36), https://github.com/marc-sturm/ngs-bits]. Trimmed reads were mapped to hg19 using STAR (v. 2.4.2a). Duplicates were removed

TABLE 1 | Patient characteristics and results of HLA ligandomics performed by tandem mass spectrometry.

UPN	Diagnosis	HLA-A*		HLA-B*		Tissue	Sample weight	HLA class I			HLA class II	RIN
							[mg]	Peptides (n=)	Binders (n=)	Binders [%]	Peptides (n=)	
IRISS01	mCRC	24	66	27	44	Tumor	160	1,785	1,508	84.5	850	7.2
						NMT	710	1,917	1,507	78.6	1490	7.1
IRISS05	mCRC	01	02	08	18	Tumor	46	260	198	76.2	556	8.4
						NMT	290	922	820	88.9	803	7.1
IRISS06	mCRC	02	24	15	35	Tumor	54	711	666	93.7	n.d.	7.4
						NMT	n.d.	n.d.	n.d.	n.d.	n.d.	3.3
IRISS08	mCRC	02	33	14	18	Tumor	24	231	175	75.8	220	8.6
						NMT	280	1101	923	83.8	631	7.5
IRISS09	mCRC	01	08	Tumor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
						NMT	130	560	341	60.9	445	8.3
IRISS12	mCRC	01	02	08	27	Tumor	920	1887	1714	90.8	1461	6.9
						NMT	840	1372	1244	90.7	1307	8.3

CRC, colorectal cancer; HLA, human leukocyte antigen; m, metastasized; n.d., not determined; NMT, non-malignant tissue; RIN, RNA integrity number; RNA, ribonucleic acid; UPN, uniform patient number. Binders were defined as HLA-eluted peptides predicted to bind to the respective HLA alleles of the patient above the thresholds given in Materials and Methods determined by suitable software.

by picard tools (MarkDuplicates v. 1.85, http://broadinstitute.github.io/picard/).

Expression Analysis: Read counts were calculated using the HTSeq count based method implemented in STAR and Ensembl gene annotations (GRCh37 v. 75). Read counts were normalized using CPM (counts per million mapped reads) and $\log_2$ fold-changes (FC) were calculated to filter genes with high expression differences.

Variant Calling: Strelka (v. 1.0.11; in matched tumor/normal mode) was used for variant calling and called variants were annotated based on several different databases including among others dbSNP, ExAC, COSMIC, ClinVar and HGMD. SNPeff, Sift, MetaLR, and Polyphen were used to predict effects on gene function. For detection of gene fusions deFuse (v. 0.6.1) was used.

Peptide Selection

Selection of HLA Class I-Restricted Tumor-Specific Peptide Candidates

For the six patients of the RFA + surgery group, the multi-step selection approach used included the reassessment of MS/MS detected HLA class I-eluted peptides (a representative example for this approach is provided in **Figure 2** for patient IRISS12) regarding their HLA binding affinity by dedicated software [SYFPEITHI >50% max. score (34) and NetMHC v. 3.4 (IC50 <500 nM) (37)]—step 1 (see binders in **Table 1** and counts in **Supplementary Table 3**), subtraction of HLA ligands eluted from non-malignant liver tissue (NML) from those of corresponding mCRC tissue—step 2, as well as the subtraction of all HLA ligands identified on all available non-malignant colon tissue (NMT) samples from the mCRC cohort (32)—step 3. Since the target pool remained extensive at this stage, the strategy was extended to filter out HLA-eluted peptides from non-malignant colon samples available from previous studies—step 4, and subsequently expanded to all HLA class I ligands included in an in-house database comprising 132 non-malignant human tissues from different organs, as already used previously in CRC (32)—step 5. To avoid the selection of peptides presented on HLA class II, any HLA class I-eluted peptides presumably representing shorter length variants of longer HLA class II ligands were discarded—step 6. Finally, to enhance the stringency of selection, HLA class I-eluted peptides were only retained when surpassing a relative SYFPEITHI score of >60% of the maximal allelic score—step 7. For patient IRISS06, step 2 was omitted, because autologous NML tissue was not available. In addition, for patient IRISS09, the HLA-peptide elution resulted unsuccessful for tumor tissue.

Selection of HLA Class II-Restricted Tumor-Specific Peptide Candidates

For HLA class II (**Figure 3**, **Supplementary Table 4**), peptides eluted from mCRC were initially compared to the peptides characterized by MS/MS on corresponding NML, discarding the overlap—step 1. Subsequently, all HLA class II-eluted peptides of NMT of the entire mCRC cohort were deducted—step 2, as well as all HLA class II-eluted peptides detected in non-malignant colon tissue from previous studies—step 3, then all HLA class II ligands included in an in-house HLA class II peptide database comprising 82 non-malignant human tissues from different organs (32) were eliminated—step 4. Finally, for stringency, all HLA class I peptides comprised in a comprehensive database of benign tissues ($n = 132$) and in the NML tissue from the mCRC cohort were subtracted—step 5. A representative HLA class II selection approach (for patient IRISS12) is provided in **Figure 3**. For patient IRISS06, step 1 was omitted because autologous NML tissue was not available. Again, for patient IRISS09, the HLA-peptide elution remained unsuccessful for mCRC.

Selection of (Predicted) Mutated HLA Ligands

For prediction of mutation-derived HLA ligands, only non-synonymous somatic variants [single nucleotide variants (SNVs)

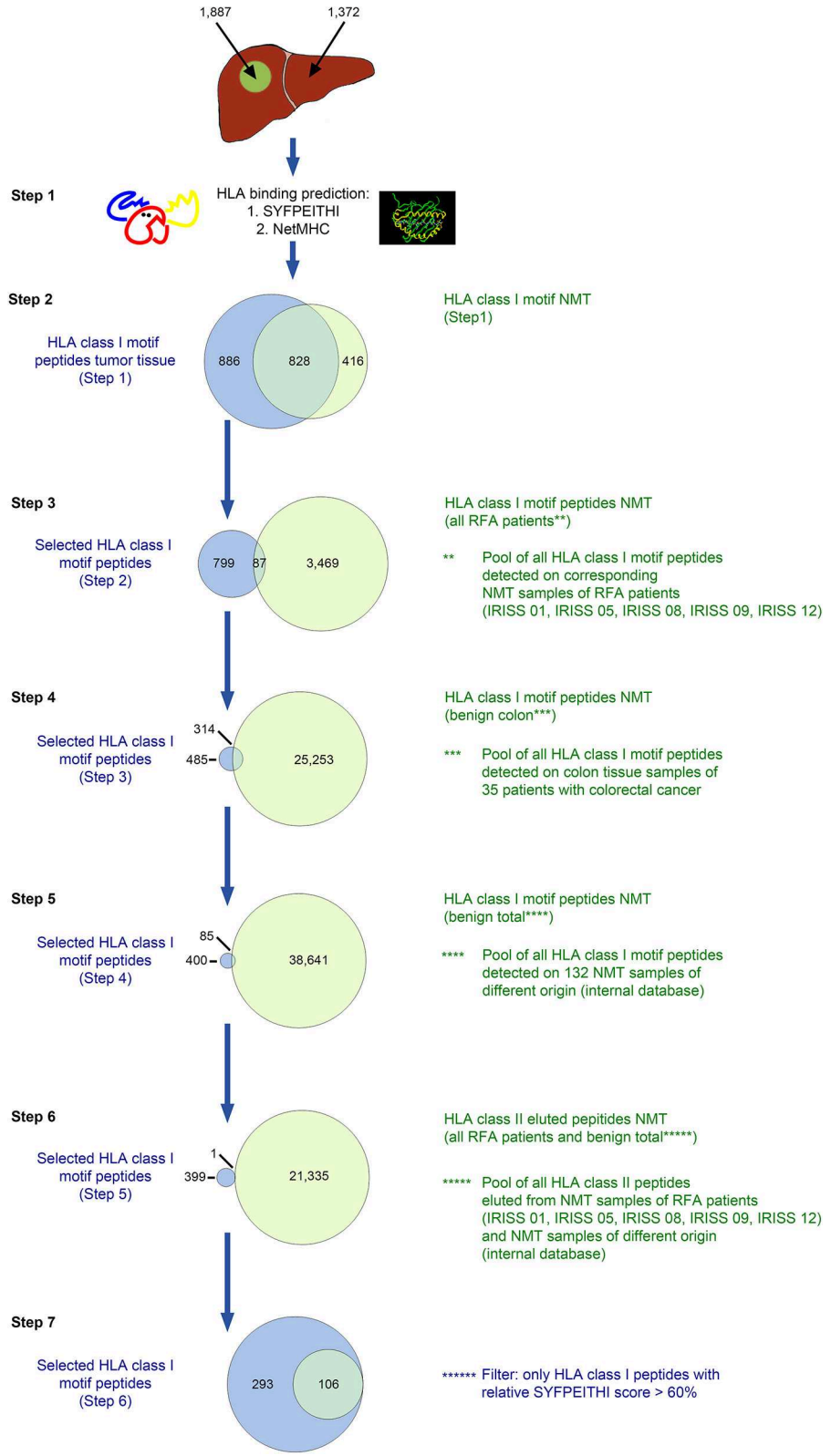


FIGURE 2 | *In silico* selection strategy for candidate HLA class I-presented antigens (exemplified for patient IRISS12). HLA class I-restricted peptides were eluted from mCRC tissue (n = 1,887) and corresponding non-malignant liver (NML) tissue (n = 1,372) by HLA immunoprecipitation using suitable antibodies followed by

(Continued)

FIGURE 2 | uHPLC tandem mass spectrometry (MS/MS). Spectra were annotated using the MASCOT search engine. All peptides eluted were evaluated for their HLA binding affinity using SYFPEITHI and NetMHC version 4.0 (step 1, $n = 1{,}714$ and n=1,244 from malignant and non-malignant tissues, respectively). For further selection, only peptides were included with appropriate HLA class I binding motifs (step 2, $n = 886$). In the following step, the peptides were excluded that were also present on corresponding NML in all included RFA patients (step 3, $n = 799$), 35 non-malignant colon tissues (NMT; step 4, $n = 485$) or on any of 132 non-malignant tissues of different origins (step 5, $n = 400$), as previously reported in Löffler et al. (32). Peptides were further cross-matched with all HLA class II-restricted peptides eluted from NML samples from all included RFA patients (step 6, $n = 399$). As a final step, only peptides which exhibited a SYFPEITHI binding score >60% of the respective maximal allelic score were considered suitable candidate antigens for further manual curation (step 7, $n = 293$). Specific data for all analyzed samples are provided in **Supplementary Table 3**.

and Insertion/Deletions (InDels)] were selected, when being sequenced with >25 reads in mCRC and simultaneously remaining undetectable in corresponding NML. Additionally, all ambiguous gene transcripts mapping to more than one genetic locus were discarded. Gene fusions were chosen in case >10 split-reads were detectable in mCRC with a probability value >0.8%. Only known driver mutations and variants affecting genes with established relevance for malignant development were selected. For gene fusions the latter was required for at least one of the involved genes.

Non-synonymous somatic variants and gene fusions were translated into the corresponding protein containing the amino acid altered by mutation. The protein sequence flanking the altered amino acid sequence was then disaggregated and screened for HLA class I peptide sequences with a SYFPEITHI score >60% of the maximal allelic score. Mutation containing peptides predicted to bind to the respective patient's HLA class I alleles were extended at the N- and C-terminus to produce a 15 mer peptide, covering both the predicted binding HLA class I peptide sequence as well as peptide sequences showing HLA class II binding properties. Finally, two predicted potential mutated neoantigens were selected, a mutated sequence in the ERRB3 protein for IRISS06 (mERBB3) and a fusion-derived peptide between the two proteins Malic enzyme 2 and SMAD family member 4 (MAOM-SMADA4) for patient IRISS12.

None of the predicted mutation-derived HLA ligands could be confirmed in MS/MS data of HLA ligands eluted from respective mCRC tissue.

Selection of Candidate Peptides for Immunological Analyses

Candidate tumor antigen-derived peptides were collated and manually curated for each patient, selecting a manageable set of short and/or long peptides for immunological testing. Criteria for non-mutated peptide selection included increased expression of the source antigen in the tumor as compared to autologous normal tissue [fold change (log_2); FC], frequency of identification among RFA and CRC (32) cohorts (for HLA class II ligands, length variants were considered), tumor association (e.g., involvement of the source protein in cancerogenesis according to the literature, representation in tumor-associated pathways…). Representation in cancer-associated pathways (**Supplementary Table 2**) was established by literature research (www.pubmed.gov), as well as through the human protein atlas (www.proteinatlas.org).

The two mentioned predicted mutated peptides were prioritized. Altogether, a ranking list of peptides to be tested was established for each individual patient, and the final number of peptides tested was adjusted to the numbers of available PBMCs (between 6 and 9 peptides/patient, **Supplementary Table 2**).

Peptide and HLA-Peptide Monomer Synthesis

Peptides required for T cell stimulation assays (**Supplementary Table 2**) were synthesized in house (Department of Immunology, University of Tübingen, Germany) by solid-phase synthesis with the 9-fluorenylmethyl-oxycarbonyl/tert-butyl (Fmoc/tBu) strategy (38) in an automated peptide synthesizer (EPS 221, Abimed; ABI 433A, Applied Biosystems). Lyophilized peptides were diluted at 1 mg/ml in distilled water with 10% DMSO and stored at −80°C.

In vitro Stimulation of T Cells and Functional Assays

PBMCs from six patients (RFA + surgery group) were thawed, washed and seeded at ∼3–6 × 10^6 cells per well in a 48-well-plate in IMDM (Lonza, Verviers, Belgium) with 10% heat-inactivated human serum containing 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) and 50 μM β-mercaptoethanol (Roth, Karlsruhe, Germany) (culture medium). After overnight resting, pooled synthetic peptides were added at 2.5 or 5 μg/ml, for HLA class I and HLA class II peptide stimulations, respectively. Cell culture was performed for 12 days and medium supplemented with recombinant IL-2 (2 ng/ml, R&D Systems, Minneapolis, MN) on days 3, 5, 7, and 9.

Peptide-specific T cells were quantified by intracellular cytokine staining (ICS) for both $CD8^+$ and $CD4^+$ cells. Directly after 12-day pre-sensitization, cultivated cells were washed and stimulated with the relevant individual peptides (10 μg/ml; in pools or individually) and pre-incubated for 1 h (37°C; 7.5% CO_2) in the presence of the monoclonal antibody (mAb) CD107a-FITC (clone H4A3, BD Biosciences, Heidelberg, Germany). Phorbol myristate acetate (PMA) (5 ng/ml) plus ionomycin (1 μM) (both Sigma-Aldrich) served as positive control and 10% DMSO was used as negative control. Subsequently, secretion of intracellularly produced cytokines was prevented by adding GolgiSTOP (BD Biosciences) and Brefeldin A (10 μg/ml, Sigma-Aldrich). After a 12 h stimulation period, cells were washed and stained as previously described (39) with mAbs CD3-BV711 (clone OKT3, Biolegend, San Diego, CA), CD8-PE-Cy7 (clone SFCI21Thy2D3, Beckman Coulter, Brea, CA), CD4-APC-Cy7 (clone RPA-T4, BD Biosciences), anti-IFNγ-BV421

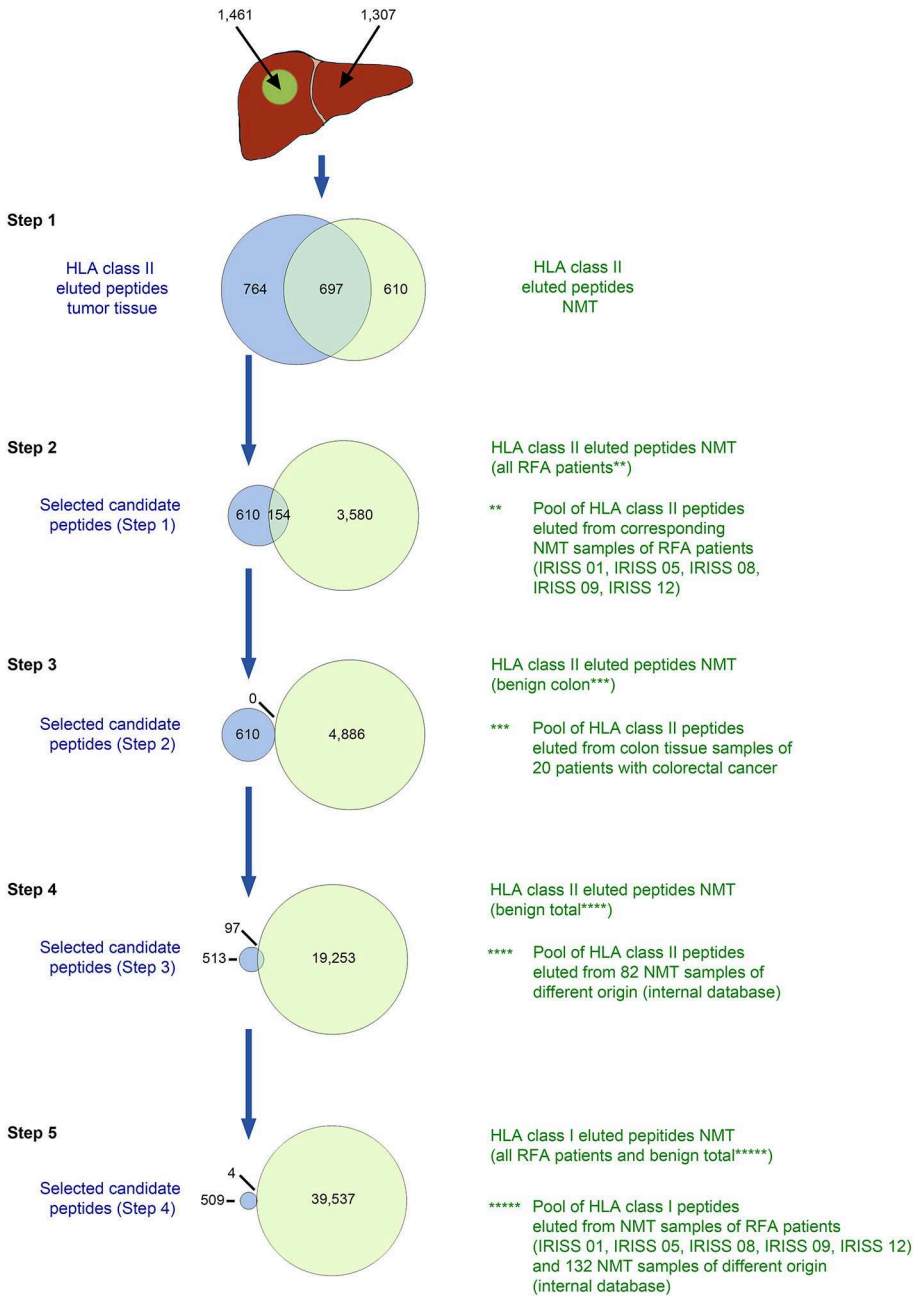


FIGURE 3 | *in silico* selection strategy for candidate HLA class II-presented antigens (exemplified for patient IRISS12). HLA class II-restricted peptides were eluted from mCRC tissue ($n = 1,461$) and corresponding non-malignant liver (NML) tissue ($n = 1,307$) by HLA immunoprecipitation using suitable antibodies followed by tandem mass spectrometry (MS/MS). HLA-eluted peptides were compared between corresponding mCRC and autologous NML tissue and only peptides exclusively found on mCRC were included (step 1, $n = 764$). Further, peptides which were presented on NML of any of the other RFA patients were excluded (step 2, remaining peptides $n = 610$). In the next step, cross-evaluation with a database of 20 non-malignant colon tissues (NMT) (32) could not restrict peptides further (step 3, $n = 610$). Peptides were additionally compared to peptides eluted from 82 non-malignant tissue samples of different origins (32) (step 4, $n = 513$). Before manual assessment, further peptides were excluded when presented as HLA class I antigens on any NML of all RFA patients and 132 tissues of different origins (step 5, $n = 509$). Specific data for all analyzed samples are provided in **Supplementary Table 4**.

(clone 4S.B3, Biolegend), anti-TNF-BV605 (clone Mab11, Biolegend), anti-IL-2-PE and anti-CD154-APC (clone MQ1-17H12 and clone TRAP1, respectively, both BD Biosciences). LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (ThermoFisher) was included in the stainings. Samples were acquired on a flow cytometer (LSR Fortessa, BD Biosciences) equipped with the DIVA software and analyzed with FlowJo software (TreeStar, Ashland, OR).

The following gating strategy was applied: time gate (histogram)/singlet cells (FSC-H/FSC-A), living cells (FSC-A/ Live/Dead® Fixable Aqua), lymphocytes (FSC-A/SSC-A), $CD3^+$ (FSC-A/CD3) $CD4^{neg}$ and $CD8^{neg}$ cells (CD4/CD8); T cell activation (cytokine production, CD107a and CD154 of $CD8^+/CD4^+$ subsets was assessed within the $CD4^{neg}$ and $CD8^{neg}$ lymphocytes, respectively). Results are expressed as % of marker-positive cells within $CD4^+$ or $CD8^+$ subsets.

Immune responses were considered positive if (I.) the percentage of cytokine producing cells within the sample was 2-fold above the percentage of cytokine producing cells within the corresponding negative control (10% DMSO; no stimulation, as described above), (II.) the number of cytokine producing cells within the sample was ≥20 cells after subtraction of the number of cytokine producing cells within the corresponding negative control (10% DMSO; no stimulation), and (III.) at least two of the five investigated parameters (IFNγ, TNF, IL-2 cytokine production or CD107a, CD154 upregulation) were positive according to the criteria under (I.) and (II.). All dot-plots were audited.

Immunohistochemistry

Formalin-fixed, paraffin embedded (FFPE) tissue from all 16 patients of both groups was cut in 3–5 μm-thick sections and stained with haematoxylin and eosin (H&E). Immunohistochemistry was performed by an automated immunostainer (Roche Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions for open procedures with slight modifications. Samples were stained with antibodies against CD4 (clone SP35, Zytomed Systems, Berlin, Germany), CD8 (clone C8/144B, DAKO, Glostrup, Denmark), CD14 (clone EPR3653, MEDAC Diagnostika, Wedel, Germany), CD19 (clone LE-CD19, Zytomed), CD45RO (clone UCH-L1, Abcam, Cambridge, UK), CD68 (clone KP1, DAKO), Granzyme B (clone 11F1, Novocastra, Wetzlar, Germany), HLA class I (polyclonal, Santa Cruz Biotechnology, Dallas, TX), HLA-DR, -DP, and –DQ (clone CR3-43, DAKO), HSP70 (clone W27, Santa Cruz Biotechnology), IL-10 (polyclonal, Abcam), and LAMP3 (polyclonal, Sigma-Aldrich). Appropriate positive and negative controls were employed to confirm the adequacy of the staining.

Stained slides were digitalized using a Hamamatsu NanoZoomer (C9600-12) using NDP.scan (v. 2.5.88) and NPD.view (v. 2.6.13) software (all from Hamamatsu Photonics, Hamamatsu City, Japan).

Slides were first counted using automated digital slide analysis. For each marker, five representative high-power fields (HPF) were captured using a 200-fold magnification. The number of positive cells was enumerated and the mean for every case was calculated. CD4, CD8, CD19, and CD68 stainings were evaluated using the CD4Quantifier software, which is part of the CognitionMaster Professional Suite (VMscope GmbH, Germany) (40). CD14, CD45RO, HLA-DR, HSP70, IL-10, HLA class I and LAMP3 were evaluated manually. For CD14 and CD45RO, the mean number of positive cells per five HPF was assessed by manual counting. Concerning HLA-DR, we calculated the percentage of positive tumor cells (41). For HSP70, IL-10, and MHC I, we used the immunoreactive score (IRS) (42). In brief, the IRS is calculated by multiplying the number of positive cells (0 = 0%, 1 = 1–10%, 2 = 11–50%, 3 = 51–80%, 4 = >80%) with the staining intensity (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining), resulting in a score ranging from 0 to 12.

Slides were also counted manually using the count tool of Adobe Photoshop (v. CC 2018, Adobe Systems, San José, CA). Areas for manual counting were defined as follows: invasive margins were defined as 500 μm in both directions of the tumor border (inwards/outwards) (43). Counting areas were selected using the hot-spot method (good pathological practice) and were defined as areas with subjective/visually most positive stained cells [3 × 0.2 mm^2 (radius: 252 μm) for each area] (43).

Both automated and manual counting was performed in a blinded fashion by expert pathologists and group assignment was only unblinded to the evaluating pathologists after completion of statistical evaluation.

Evaluation of Microsatellite Instability (MSI)

Genomic DNA was extracted from macrodissected paraffin sections using the Maxwell® RSC FFPE Plus DNA Purification Kit and the Maxwell® 16 Instrument (Promega, Madison, WI), according to the manufacturer's instructions. Microsatellite PCR in duplicates was performed using genomic DNA and AmpliTaq Gold DNA Polymerase (ThermoFisher) as well-fluorescent labeled primers (Sigma-Aldrich). For GeneScan analysis PCR products were mixed with sample loading solution (Beckman Coulter). The products were separated by capillary electrophoresis on the GenomeLab GeXP Genetic Analysis System and analyzed by the GenomeLab GeXP software 10.2 (Beckman Coulter).

Statistical Analyses

Mann Whitney *U*-Tests were performed using GraphPad Prism Version 6.0 (GraphPad Software, San Diego, CA). Kaplan Meyer, COX regression and log rank analyses were performed using SPSS Version 24 (IBM, Armonk, NY). Significance levels were set to $p < 0.05$ and respective values considered as statistically significant.

RESULTS

Study Design

We recruited two groups of patients with liver metastasis from colorectal carcinoma (mCRC). One group with mCRC was treated merely with surgical resection for their liver lesions ($n = 7$), and another group received RFA first and subsequently a surgical resection ($n = 9$) for the remaining metastases not treated by RFA (**Figure 1**, **Supplementary Table 1**). We obtained blood samples from RFA-treated patients before intervention (i.e., RFA treatment followed by surgery) as well as in the course of clinical follow-up ($n = 6$) to obtain peripheral blood mononuclear cells (PBMCs) for immunomonitoring. Further, we obtained tissue samples, encompassing mCRC as well as NML, enabling mRNA analysis by whole transcriptome sequencing (WTS) and the immunoprecipitation and characterization of naturally presented HLA ligands using tandem mass

spectrometry (MS/MS). The mean sample weight was 340 mg (range 24–920 mg), yielding on average 835 peptides identified by MS/MS for mCRC and 1,174 peptides for NML (**Table 1**). On average >75% of HLA class I-eluted peptides from the included samples showed HLA binding properties as corroborated with dedicated software; RNA integrity (RIN) was >6.5 in all cases, except for NML of IRISS06 (RIN = 3.3), which was processed with a high fidelity kit for this reason, to enable the generation of suitable data. Samples with insufficient yields were excluded from downstream analyses.

Selection of Individual Candidate Antigens

A key challenge for our study was the choice of relevant candidate antigens for testing of T cell recognition. In principle, RFA resembles a whole cell *in situ* vaccination approach, whereby both the priming of novel target-specific T cells as well as boosting of pre-existing T cell responses may occur. Therefore, an extensive spectrum of potential targets prevails that may comprise tumor-specific targets, such as mutated HLA ligands, but also tumor-associated antigens (TAA), a class of tumor antigens that was already previously tested in this setting (27, 44).

In this study, we aimed at a patient-individual selection strategy for candidate HLA class I and II ligands, including MS/MS detected natural HLA ligands exclusive to each patient's own malignant tumor tissue by incorporating information from HLA ligandome as well as from WTS. The approach was complemented by complementary information available from our in house HLA ligand database, which contains an array of natural HLA ligands presented on various different tumor entities (including CRCs) and benign tissues ($n = 132$ and $n = 82$ for HLA class I and HLA class II ligands, respectively, including NML and NMT).

Hence, we used a comprehensive HLA ligand-based multi-step selection strategy (strategies are described in the Materials and Methods section and visualized exemplarily in **Figures 2**, **3**), which was followed for each patient when feasible, aiming at the identification of natural HLA ligands presented by the individual patient's mCRC, ideally derived from tumor-specific proteins, and including both non-mutated as well as selecting mutated peptides, when available. Of note, upon testing all mCRC included in the study were tested as non-MSI high tumors.

An example of the target selection procedure (patient IRISS12) is presented for HLA class I binding candidate peptides in **Figure 2**, resulting in a decrease of the initial target peptide pool by 84%. The selection procedure for HLA class II binding candidate peptides for the same patient is provided in **Figure 3**. Respective data for the other included mCRCs disaggregated according to the described steps is presented for HLA class I and class II in **Supplementary Tables 3**, **4**, respectively. The remaining candidate peptides (ranging between 3 and 293 HLA-eluted peptides) encompassed between 1 and 16% of the initially available peptide pool.

High confidence somatic variants identified by WTS were used to predict mutated HLA ligands, selecting only peptides with the required patient-specific HLA class I binding properties. Peptide sequences were elongated to 15 mers, aiming to increase chances for the verification of $CD8^+$ and/ or $CD4^+$ mediated T cell responses.

Peptides identified through these different procedures were merged and manually curated individually for each patient, selecting a manageable set of short and/or long peptides for immunological testing (6–9 peptides per patient, **Supplementary Table 2**). Peptides predicted from gene fusions or mutations were preferentially selected, when available ($n = 2$).

TABLE 2 | Overview of T cell reactivity measured by ICS.

Patient (UPN)	Pre-RFA (d0)	Post-RFA	Effector cells
IRISS01	$CCND1^{198-212}$	$CCND1^{198-212}$ (1 M, 4 M) [not enhanced]	$CD4^+$
IRISS05	Pool: $AREG^{93-106}$, $FN1^{1789-1804}$, $CCND1^{198-212}$	Pool: $AREG^{93-106}$, $FN1^{1789-1804}$, $CCND1^{198-212}$ (1 M) [not enhanced]	$CD4^+$
IRISS06		$mERBB3^{96-110}$ (4 M, 7 M) **[induced]**	$CD4^+$
IRISS08	$IFI6^{106-114}$	$IFI6^{106-114}$ (6 M) [not enhanced]	$CD8^+$
	$CCND1^{198-212}$	$CCND1^{198-212}$ (1 M, 4 M, 6 M) **[enhanced]**	$CD4^+$
IRISS09	$GPA33^{52-67}$	$GPA33^{52-67}$ (12.5 M) [not enhanced]	$CD4^+$
IRISS12		$FN1^{1797-1811}$ (1.5 M) **[induced]**	$CD4^+$

AREG, amphiregulin; CCND1, cyclin D1; d0, before RFA; FN1, fibronectin 1; GPA33, Glycoprotein A33; IFI6, interferon alpha inducible protein 6; ICS, intracellular cytokine staining; M, month post-RFA; mERBB, (mutated) human epidermal growth factor receptor 3; RFA, radiofrequency ablation; UPN, uniform patient number.

RFA Induces Tumor-Specific T Cell Reactivity

We were able to detect immune responses against various individually selected candidate peptides involving all patients of our small test cohort ($n = 6$); most of these T cell reactivities were directed at long candidate epitopes (presumably HLA class II-restricted). Preexisting antigen-specific T cell responses were confirmed in 4/6 patients, and one of them was assessed as enhanced after RFA treatment (patient IRISS08, $CCND1^{198-212}$). Additionally, *de novo* priming of tumor-specific T cells was observed in two patients, including one immune response against a mutated peptide. These findings can be most probably ascribed to RFA, since the T cell responses could not be measured before treatment in the respective patients (**Table 2**).

In one patient (IRISS06), a $CD4^+$ T cell response was induced after RFA, which was directed against one predicted

mutated peptide derived from the human epidermal growth factor receptor 3 (mERBB3) containing an amino acid exchange from valine to leucine at position 104 (TLPLPNLR**L**VRGTQV). The induced T cell reactivity to mERBB3 was polyfunctional (**Figure 4A**; encompassing CD154, interferon γ (IFNγ), tumor necrosis factor (TNF) and interleukin (IL)-2, but not CD107a) with robust responses (∼1.4% of the $CD4^+$ T cell subset) at 7 months post-RFA treatment (**Figure 4B**). Importantly, further experiments demonstrated that the corresponding wildtype peptide (wtERBB3: TLPLPNLR**V**VRGTQV) induced strongly attenuated cytokine responses in $CD4^+$ T cells, as compared to the mERBB3 peptide (**Figure 4C**). The other tested peptides (**Figure 4A**) derived from amphiregulin (AREG) as well as epithelial cell adhesion molecule (EpCAM) did not elicit any detectable T cell responses.

Another patient (IRISS08) showed an enhancement of a pre-existing immune response directed against the long cyclin D1-derived peptide (CCND1) NPPSMVAAGSVVAAV (**Supplementary Table 2**). This $CD4^+$ T cell response was evidenced before as well as 1, 4, and 6 months after RFA with increased functionality after RFA (encompassing positivity for CD154 and cytokines IFNγ, TNF, and IL-2), which peaked at 4 and 6 months but was no longer measurable subsequently (at 17 months post-RFA) (**Figures 5A,B**). In the same patient, $CD8^+$ T cell reactivity against an interferon alpha-inducible protein 6–derived peptide (IFI6: VVIGNIGAL; HLA-A*02) was detected before and also after RFA treatment, stimulating IFNγ, TNF and CD107a in ICS, however this response was not boosted (**Supplementary Figure 1**) and the aggrecan core protein (PGCA)–derived peptide DEFPGVRTY tested simultaneously showed no reactivity.

In addition to these findings, several pre-existing immune responses could be detected, among them $CD4^+$ cells responding to the long CCND1-derived peptide previously mentioned, which were not found enhanced after RFA treatment at this time (patient IRISS01) but remained detectable after 1 and 4 months following RFA, encompassing positivity for CD154 as well as positive staining for IFNγ, TNF, IL-2 in ICS (**Supplementary Figure 2A**). One further patient (IRISS05) was shown to respond to a three peptide pool of long peptides containing the same CCND1-derived peptide NPPSMVAAGSVVAAV as well as a long FN1- (VSVYALKDTLTSRPA) and an AREG-derived peptide (IPGYIVDDSVRVEQ) before as well as 1 month subsequent to RFA with $CD4^+$ cells positive for CD107a, CD154 as well as cytokines IFNγ, TNF, and IL-2 (**Supplementary Figure 2B**). In this case, due to limited sample material, it was impossible to distinguish, which of the peptides was ultimately responsible for the $CD4^+$ T cell response. Patient IRISS09 showed a preexisting $CD4^+$ T cell response detectable prior to RFA, triggered by the cell surface A33 antigen (GPA33) peptide REGLIQWDKLLLTHTE, which persisted for over 12 months post-RFA (**Supplementary Figure 2C**). Regrettably, from this patient individual data (transcriptome and HLA ligandome) were lacking, which is why peptides identified in other patients of the study cohort matching to the HLA alleles of interest were selected for evaluation in this case.

Further, we detected an immune response against a long fibronectin peptide ($FN1^{1797-1811}$; patient IRISS12), which proved negative before RFA but showed induction of $CD4^+$ cells staining positive for IFNγ, TNF, IL-2 in ICS as well as for CD154, 6 weeks after treatment (**Supplementary Figures 2D,E**). Whereas, analyses of a predicted peptide derived from a MAOM-SMADA4 fusion (**Supplementary Table 2**) remained negative.

Immune Cell Infiltration in Distant Metastases Is Not Increased After RFA

To determine whether RFA impacts immune cell infiltration into distant, non-ablated, tumor lesions, we assessed our expanded mCRC patent cohort, consisting of patients that received RFA first and a liver resection for additional malignant lesions subsequently ($n = 9$ patients; mCRC lesions were surgically removed ∼4 weeks following RFA), as well as a control group of mCRC patients that merely received surgery for their liver metastases ($n = 7$). FFPE tissue was stained by immunohistochemistry for different markers (comprising CD4, CD8, CD14, CD19, CD45RO, CD68, granzyme B, HLA class I, HLA-DR, HLA-DP and HLA–DQ, HSP70, IL-10, and LAMP3). Results were compared between both groups. Overall, no drastic change in the immune cell infiltrate into distant tumor lesions was observed in RFA-pretreated patients, as exemplified by stainings with CD45RO (activated lymphocytes) and granzyme B (cytotoxic lymphocyte effectors) (**Figures 6A,B**). $CD8^+$ cells (potentially cytotoxic T lymphocytes) in the tumor center were generally scarce (<100 cells/HPF) and did not show significant differences between both patient groups neither at the invasive margin nor the tumor center (**Figures 6C,D**). However, numbers of $CD8^+$ cells appeared to be slightly decreased at the invasive margin (**Figure 6C**). In addition, for patients pre-treated with RFA, we observed significantly decreased numbers of $CD4^+$ cells (including cell subsets such as effector T_H, T_{regs}, and possibly also macrophages) both within the tumors and at the invasive margin, when compared to the resection only group (**Supplementary Figures 3A,B**). Further, HSP70 expression [indicating an inflammatory environment (45)] showed significantly decreased staining in the patients treated with RFA, in contrast to those that only received surgery (**Supplementary Figures 3C,D**). All other assessed markers, including the expression of HLA molecules, were not found to be significantly different.

Altogether, these findings suggest that no significant influx of immune effector cells was observed 4 weeks after RFA in non-ablated tumor lesions. It should be noted however, that we assessed only lesions that were not treated directly by RFA but distant and resected at later time points (∼4 weeks) following RFA treatment.

Clinical Course of Study Patients

For clinical follow-up (data from individual patients are provided in **Supplementary Table 1**), the date of surgery was defined as day 0 (d0) for both the RFA + surgery and the surgery only (control) groups for reasons of comparability. Patients were followed in median for 43 months (range, 3–124 months). The clinical course of each patient is depicted in **Figure 7A**. After RFA and surgery, all patients reached complete disease remission

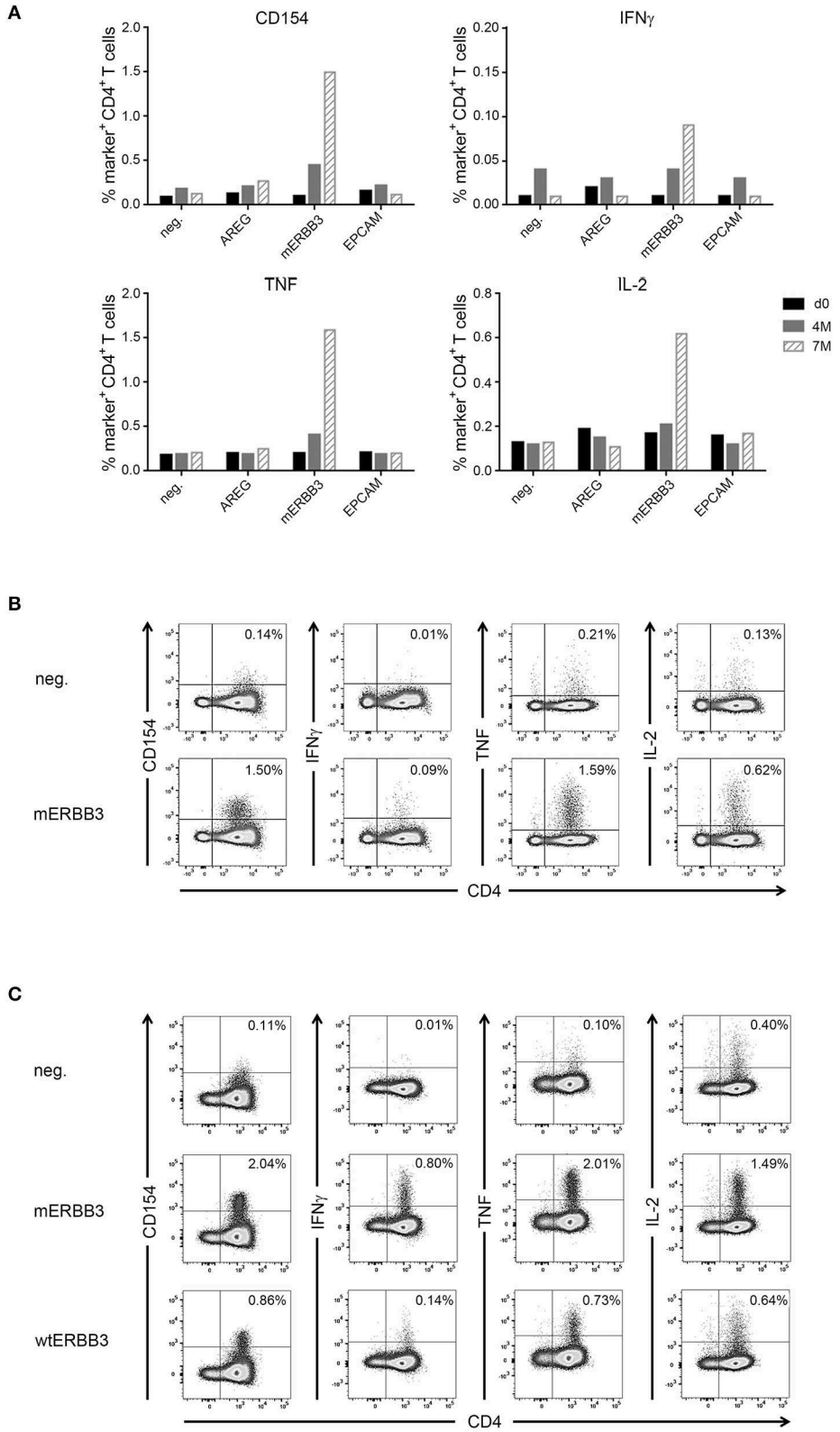


FIGURE 4 | Analysis of antigen- and neoantigen specific CD4+ T cells in patient IRISS06. Reactivity of antigen-specific CD4+ T cells against selected patient-individual tumor peptides was evaluated by flow cytometry. **(A)** Summary of intracellular cytokine staining (ICS) experiments after 12 day prestimulation followed by restimulation

(Continued)

FIGURE 4 | with AREG, mutated ERBB3 (mERBB3) and EpCAM peptides. Patient individual PBMCs obtained before RFA (black bars), as well as 4 months (gray bars) and 7 months (hatched bars) after RFA were assessed. Activation of mERRB3-specific $CD4^+$ T cells is reflected by expression of CD154, as well as production of IFNγ, TNF, and IL-2. **(B)** Examples of ICS dot plots (7 month sample) after stimulation with the mERBB3 peptide (TLPLPNLR**L**VRGTQV) after 12 day-prestimulation. Activation of antigen-specific $CD4^+$ T cells is reflected by positivity for CD154, as well as cytokine production, including IFNγ, TNF, and IL-2. **(C)** Based on the data presented in **(B)**, a new experiment was performed where PBMCs were tested for reactivity against the mutated and wildtype ERBB3 peptides (TLPLPNLR**L**VRGTQV and TLPLPNLRVVRGTQV, respectively). Activation of $CD4^+$ T cells was detected by secretion of IFNγ, TNF, and IL-2, as well as expression of CD154.

(CR) as confirmed by abdominal computed tomography (CT) or magnetic resonance imaging (MRI) scans without signs of active disease.

Median progression free survival (PFS) was 9.6 and 11.3 months for RFA + surgery and surgery only groups, respectively ($p = 0.814$, **Figure 7B**, left panel). Cumulative incidence of tumor recurrence was 75 and 60% at 12 months for patients undergoing RFA + surgery or surgery only, respectively ($p = 0.969$, hazard ratio 0.978). Sites of recurrence comprised the liver ($n = 8$), the liver and the lung ($n = 2$), as well as the lung, the brain or the abdominal wall and retroperitoneal lymph nodes ($n = 1$ each). Altogether, during follow-up 62% of patients showed tumor recurrence within the liver, whereas in only one of the nine patients treated with RFA (∼10%) recurrence was confirmed at the ablation site (for details see **Supplementary Table 1**).

Upon disease recurrence, patients received standard palliative therapies including repeated local treatment, chemotherapy and best supportive care (BSC), according to local institutional standards. The median overall survival (OS) was comparable for both groups (with 43.1 and 41.9 months in the RFA + surgery vs. surgery only group; $p = 0.886$, **Figure 7B**, right panel). At the end of follow-up, in the RFA + surgery group three of nine patients remained alive, two with active disease and one in CR. In the surgery only group, two of seven patients remained alive, one with active disease and one in CR. Cause of death was disease recurrence in all cases ($n = 11$).

DISCUSSION

We and others have previously observed that RFA leads to the induction and release of heat shock proteins (45–47) and is able to induce antigen-specific T cell responses against known tumor-antigens, such as MAGE-A-derived peptides in humans (8, 27). However, so far, these immune responses were only verified at very low frequencies in patients (<5%). Although the patient collective assessed for this study was very limited ($n = 6$), we found T cell responses that were either induced or boosted after RFA (after 1.5–4 months) in 50% of them. Hence, T cell responses were more frequently detected as compared to our previous study (27), which is likely due to the patient-individual strategy of selecting peptides to be assessed as T cell targets. Here we show systemic changes in the immune cell repertoire, encompassing both $CD8^+$ and $CD4^+$ T cells, responding to long as well as short peptides, fulfilling the characteristics required for HLA presentation.

Using a fully individualized selection strategy, based on patient-specific mCRC HLA ligand profiles as well as whole transcriptome sequencing (WTS), complemented with comprehensive knowledge regarding the HLA ligand repertoire in the context of CRC from previous work (32) and from additional benign and malignant tissues, the broad range of candidate peptides could be substantially minimized for each patient. A multistep selection approach was employed to reduce the amount of candidate peptides to numbers manageable for manual curation. We combined different lines of evidence, including both candidate HLA class I and HLA class II-presented peptides as well as complementary predicted mutated HLA ligands. We thereof selected an individual set of target peptides for each RFA patient for immunological testing. We are aware of the limitations of such an approach that introduces potential –in our view limited– bias, precluding full reproducibility, but it was essential to cope with the challenge of an extensive target pool. Of note, this approach proved effective for the successful identification of targets and for enriching an existing T cell repertoire, validated by the numerous antigen-specific T cell responses evidenced. The obtained results indeed suggest that immunomodulation is a rather frequent feature in the context of RFA, whereas without any obvious clinical effects. These findings are generally in line with reports from previous research in humans, where clinical manifestations of induced immune responses triggered by interventional techniques remain anecdotal (48). This notion is also supported by results from animal testing, where such immune responses are observed but do not appear to be robust or consistent (10). In mouse RFA models for instance, it has been shown that although *in situ* tumor ablation does create a suitable antigen source for generating anti-tumor immunity, the induced T cell responses are usually weak and offer protection from malignancy only in a small subset of animals (11). Of note, in those experiments, performed more than a decade ago, it could already be shown that ICI may potentially augment the occurrence of RFA-induced immune responses.

In our study, mainly non-mutated tumor-antigens were evaluated. The antigenic repertoire of tumor cells comprises a vast array of potential targets, which is partly invisible to confirmatory tools like tandem mass spectrometry (MS/MS), due to specific technical limitations. *In silico*, an excessive quantity of potential HLA-restricted targets can be predicted based on NGS data. Numbers of confirmed HLA ligands are substantially lower than expectable by these predictions, which is likely the reason why MS/MS-confirmed mutated neoantigens remain rather anecdotal at present (18, 19, 49, 50), and suggests that the sole prediction of HLA class I ligands yields an array of false positives (51). Nevertheless, it can be stated that responses to ICI based on tumor mutational burden (TMB) or predicted load of mutated neoantigens may indicate which cancers are more likely

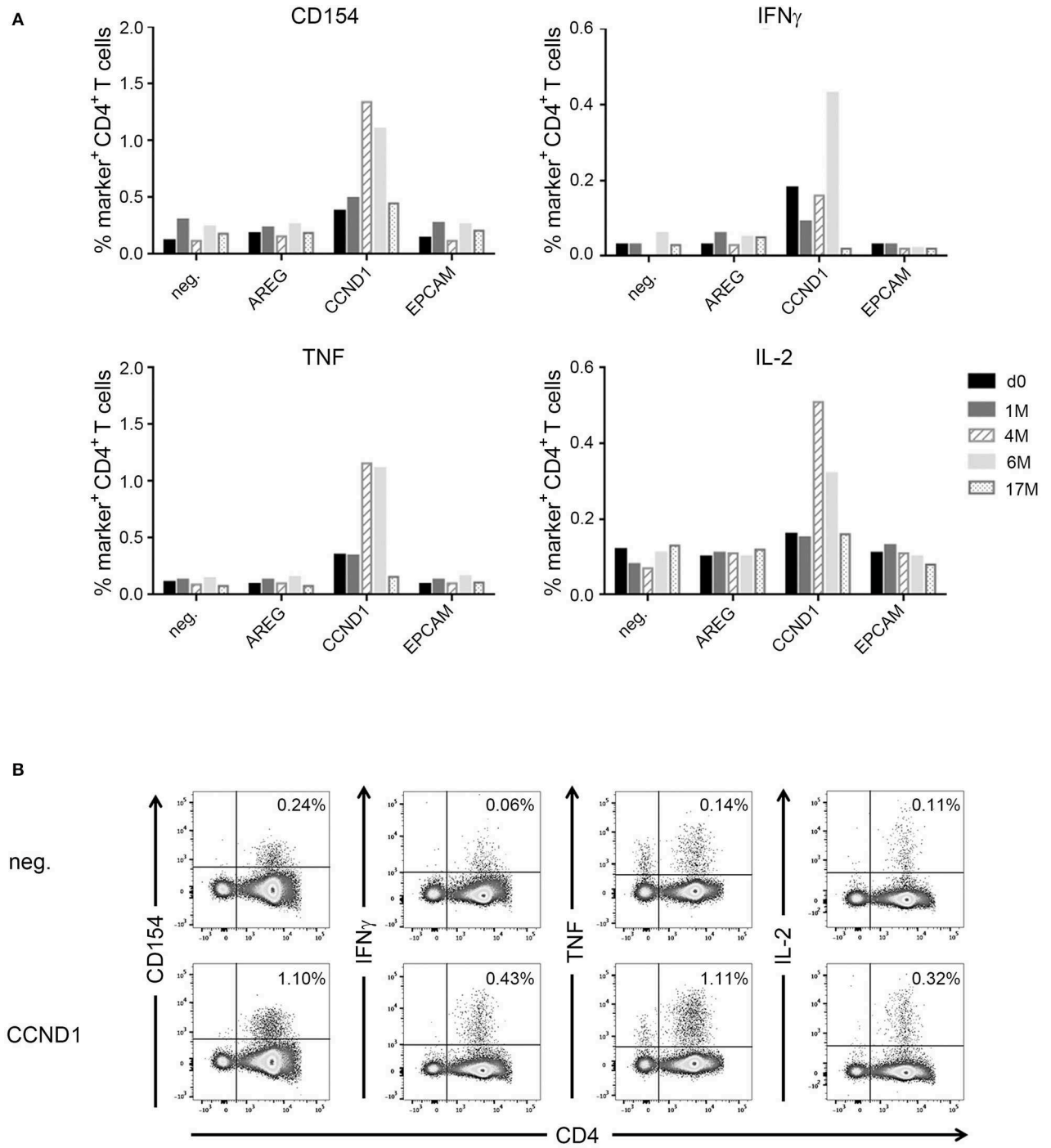


FIGURE 5 | Analysis of antigen-specific CD4$^+$ T cells in patient IRISS08. Reactivity of antigen-specific CD4$^+$ T cells against selected individual tumor-associated peptides was evaluated by flow cytometry. **(A)** Summary of intracellular cytokine staining (ICS) experiments after 12 day prestimulation followed by restimulation with AREG, CCND1, and EpCAM peptides over time. Patient individual PBMCs obtained before RFA (black bars), as well as 1 month (gray bars), 4 months (hatched bars), 7 months (light gray bars), and 17 months (dotted bars) after RFA were evaluated. Activation of antigen-specific T cells is reflected by expression of CD154, as well as by cytokine production (IFNγ, TNF, and IL-2). **(B)** Examples of ICS dot plots (6 month sample) after stimulation with the HLA class II-restricted CCND1 peptide (NPPSMVAAGSVVAAV) after 12 days prestimulation. Activation of CD4$^+$ T cells is reflected by expression of CD154, as well as cytokine production (IFNγ, TNF, and IL-2).

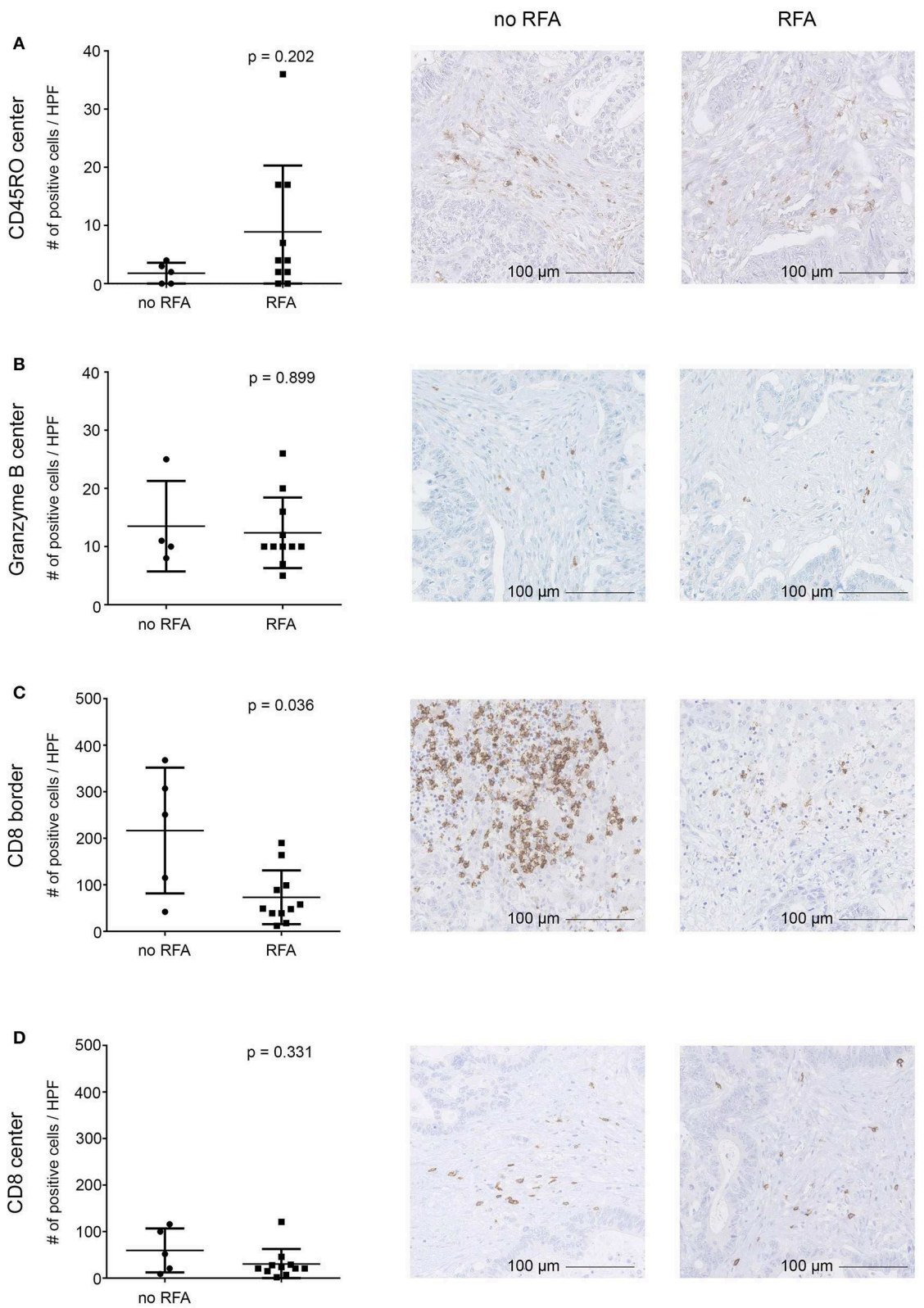


FIGURE 6 | Immunohistochemical evaluation of tumor-infiltrating immune cells into distant CRC liver metastases resected after RFA. Infiltration of immune cells into the tumor center **(A,B,D)** and the invasive tumor margin **(C)** and was assessed by immunohistochemistry revealing comparable infiltration of CD45RO **(A)** and

(Continued)

FIGURE 6 | granzyme B **(B)** positive cells, while infiltration of $CD8^+$ cells **(C,D)** was diminished in the invasive margin **(C)** but not in the tumor center **(D)** in patients who underwent RFA before surgery as compared to patients who solely underwent surgery. Staining of cells was automatically calculated (left) in digitalized slides. Numbers represent absolute cell counts with specific staining per high power field (HPF) by automated counting. Exemplary immunohistochemistry stainings are provided in the middle (patients after surgical resection) and right (patients after both RFA and surgical resection) columns (20-fold magnification). Differences were assessed using the Mann Whitney *U*-Test with $p < 0.05$ considered as significant.

to present respective mutated neoantigens on HLA. The reasons for this are indeed multifactorial. We have recently shown that fundamental differences exist between high and low mutated tumors, suggesting this may be of relevance for the probability of presentation of mutated HLA ligands (18). Further, cancer-related pathways may influence the HLA-presented ligandome (32). These alterations may give rise to tumor-specific HLA ligands with wildtype sequence. When sufficiently vetted these targets may prove as a valid alternative to mutations (44, 52) and might further warrant both *in vitro* and *in vivo* investigations as performed in our study.

Descriptions of abscopal effects in mCRC with liver metastasis however are particularly rare even after radiotherapy (53), since the liver is considered inherently tolerogenic and does not favor the induction of immune responses (54).

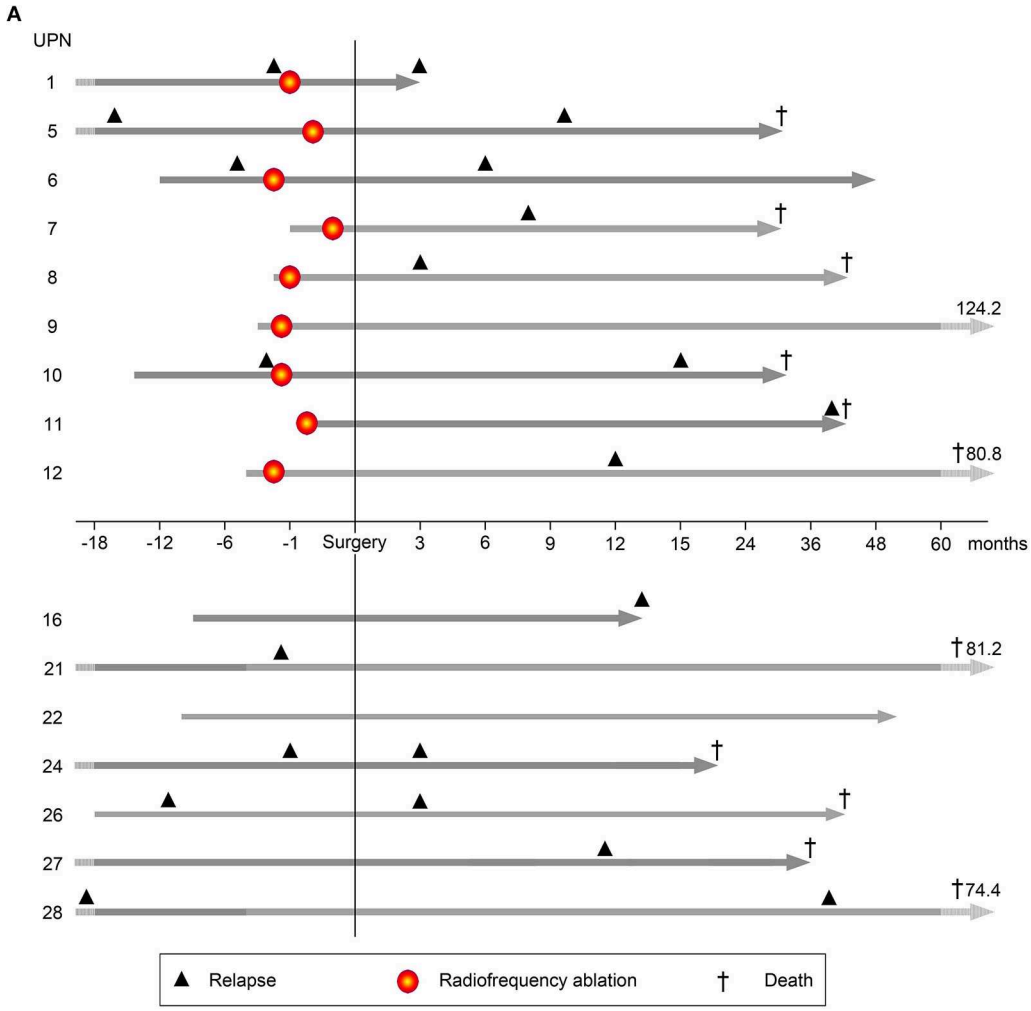
Furthermore, clinically relevant RFA-induced immunity apparent by distinct clinically recognizable effects in humans is hardly known and most insights in this regard have been derived from animal research. It may be indeed relevant how RFA is precisely performed for the generation of immune responses, since immunological effects may result more effective in malignant tissue that is only treated with subtotal RFA, which has been shown to enable induction of tumor-specific $CD8^+$ and $CD4^+$ T cells as well as tumor regression in mice (55).

Further, putative influencing factors are *inter alia* the properties of the ablated tumor tissue and numbers and quality of immunogenic epitopes (56). It is easily conceivable that these properties might influence tumor recognition by the immune system, something that has been impressively shown for CRC treated with ICI, where highly mutated cancers responded, whereas sporadic CRCs with low mutation rates did not (21). Here, we observed immune responses to various antigens, among these established tumor-antigens such as cyclin D1 used already in different vaccination approaches (57, 58), but also in one case the recognition of a predicted mutation-derived peptide. The mutation, which was recognized by $CD4^+$ T cells, was directed against ERBB3 and could be shown to induce multi-cytokine responses (strongly attenuated for the respective wildtype peptide). Further, this immune response was induced only after RFA and shown as generally increased 7 months after treatment. Of note, a mutation in ERBB2 interacting protein, also recognized by $CD4^+$ T cells, exhibiting a T_H1 profile, has been shown effective for mediating tumor regression in a patient with metastatic cholangiocarcinoma treated by adoptive cell transfer (17).

However, in our study immune infiltrates in non-ablated mCRC liver lesions resected after RFA proved generally scarce by immunohistochemistry. Comparing these non-ablated malignant liver lesions removed after RFA to lesions from mCRC patients with surgery only, significantly lower $CD4^+$ cell counts in the tumor center as well as decreased numbers of $CD8^+$ and $CD4^+$ cells at the tumor border were observed for the RFA + surgery group. These findings support the notion that clinically relevant abscopal effects are rare and not clinically robust. It should be noted though, we do only provide a very limited patient cohort and the analyses only give an impression of the effects observed about 4 weeks after RFA in liver lesions. Further, for instance potential dynamics over time remain unknown. Also, in RFA-treated hepatocellular carcinoma, significantly increased responsiveness to tumor antigens and elevated frequencies of circulating tumor antigen-specific T cells were reported, whereas these effects showed insufficient for tumor control (59). Hence, we may conclude that although immunomodulatory effects in the context of RFA seem to constitute rather the norm than an exception, they may still prove largely ineffective for the induction of robust clinical effects.

Concerning the clinical course of our patients, the combined RFA and surgical treatment, proved comparable to the surgery only group assessed in parallel both with regard to progression free survival (PFS) and overall survival (OS). Some patients in both groups even showed long-term survival. That in mCRC metastasized to the liver, both RFA and surgery and surgical treatment alone may yield similar OS results has recently been concluded from a meta-analysis (60). It is important to realize that patients with several CRC liver metastases are usually considered to be in a palliative stage but may still benefit from a combination of RFA and surgery, as also our survival data suggest. Against this background, larger clinical trials to evaluate the combination of both treatment modalities seem warranted.

In summary, our data show that thermal ablation of metastases induced or boosted tumor-antigen specific T cell responses in half of the mCRC patients evaluated by us. These T cell reactivities can be detected on an individual level, supporting the hypothesis that tumor-directed immunity might include mutated neoantigens and tumor-associated antigens with wildtype sequence that are "selected by nature itself" and that most successful immunotherapies remain limited to strategies strictly confined to individualized approaches (61–64). Since ICI unleash T cell-mediated immune responses non-specifically but rely on natural T cell responses that are individual for each patient (65–67), approaches such as RFA for modulating T cell immunity are anticipated to prove beneficial in this context. There is no doubt to us that T cell responses triggered by thermal ablation generate very limited clinical activity [reviewed in (8)], which is also supported by the data presented in this study. However, our data suggest that RFA-induced immune responses are very frequent and might be boosted by adequate combination



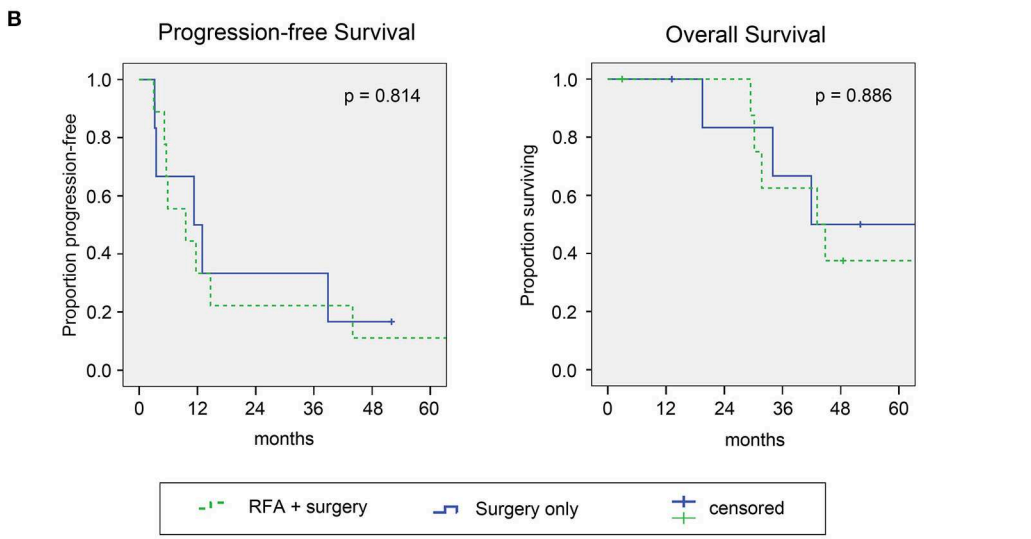


FIGURE 7 | Clinical course and survival of study patients. **(A)** Individual clinical course of patients with colorectal cancer (CRC) metastasized to the liver undergoing RFA followed by surgical resection (top 9 patients, above x-axis) and patients with surgery only (lower 7 patients, below x-axis). Gray arrows indicate time between

(Continued)

FIGURE 7 | initial CRC diagnosis and last follow-up. Light gray parts of the arrows indicate variable time spans not fitted to scale. Numbers shown indicate durations of follow-up after surgical resection. In line, respective time spans are normalized to the date of surgery (for comparability with the control group; here defined as day 0). Time points on the x-axis are relative to the time of surgery. Black triangles indicate disease recurrence before (left of y-axis) and after (right of y-axis) study inclusion. Patients with recurrence before RFA and/or surgery represent individuals with metachronous metastasis, while patients without recurrence before RFA and/or surgery had synchronous metastases. Red circles indicate time points of RFA. Crosses indicate passing of patients. **(B)** Progression free (left; PFS) and overall survival (OS) of the complete patient cohort was estimated using Kaplan Meier Regression analysis ($n = 16$). Survival data are presented for patients undergoing RFA followed by surgical resection (green dashed lines, $n = 9$) and for patients with surgical resection only (blue lines, $n = 7$). Differences were assessed by log rank with $p < 0.05$ considered as significant.

treatments. This needs to be investigated in future trials, combining thermal ablation with established (e.g. immune checkpoint inhibitors) and/or novel adjuvants in order to induce more potent –and presumably clinically relevant– immune responses.

DATA AVAILABILITY STATEMENT

The manuscript datasets, generated, and analyzed during this study have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRoteomics IDEntifications (PRIDE) database partner repository (68) with the dataset identifier PXD015947.

ETHICS STATEMENT

This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the local institutional review board of the University Hospital Tübingen (Reference No. 169/2005V and 638/2014BO2). All participants provided written informed consent before study inclusion.

AUTHOR CONTRIBUTIONS

ML performed *in vitro* experiments, analyzed and interpreted data, wrote the article, supervised the study, and obtained funding. BN performed *in vitro* experiments, analyzed and interpreted data, and wrote the article. DK and LM performed mass spectrometry analyses, analyzed, and interpreted data. GJ and CS performed transcriptome sequencing, analyzed, and interpreted data. PJ, JB, FB, BS, and CD performed analyses of immunohistochemical slides analyzed and interpreted the data. SC, IK, SB, and RL performed patient treatment, managed the clinical part of the study, analyzed, and interpreted the data. SW provided sample material, managed the clinical part of the study, analyzed, and interpreted the data. PP and AK designed the study, performed patient treatment, analyzed, and interpreted the data. TG analyzed and interpreted the data. SS analyzed and interpreted the data and supervised the study. H-GR and CG designed the study, wrote the study protocol, performed *in vitro* experiments, analyzed and interpreted the data, wrote the article, supervised the study, and overall responsibility. SH designed the study, wrote the study protocol, performed *in vitro* experiments, analyzed and interpreted the data, wrote the article, supervised the study, obtained funding, and overall responsibility. All authors revised the article and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02526/full#supplementary-material

Supplementary Figure 1 | Analysis of antigen-specific $CD8^+$ T cells in patient IRISS08. Reactivity of antigen-specific $CD8^+$ T cells against selected individual

tumor-associated peptides was evaluated by ICS over time before RFA **(A)** and after 6 months **(B)**. T cells were prestimulated for 12 days and restimulated with peptides derived from interferon alpha-inducible protein 6 (IFI6) and aggrecan core protein (PGCA). As positive control, PMA and ionomycin were used. As negative control, 10% DMSO was employed. Positivity criteria used throughout this article are provided in the materials and methods section. Activation of $CD8^+$ T cells is reflected by expression of CD107a, as well as cytokine production of IFNγ and TNF.

Supplementary Figure 2 | Analysis of antigen-specific T cells in patients IRISS01, IRISS05, IRISS09, and IRISS12. Reactivity of antigen-specific $CD4^+$ T cells against selected individual tumor-associated peptides was evaluated by ICS over time. Respective time points of sample obtainment for individual patients are indicated. Patients PBMCs were presensitized for 12 days, restimulated with denoted peptides, and tested in ICS as detailed in Material and Methods. **(A–D)** Expression of CD154, IFN-γ, TNF, and IL-2 in the $CD4^+$ subset. For IRISS05 and IRISS12, cell numbers were limited before RFA treatment (day 0). Therefore, peptide pools were used. **(E)** Dot-plots corresponding with tests shown in **(D)** showing FN1-reactive $CD4^+$ T cells 1.5M after RFA. Positive responses were defined as detailed in Material and Methods. Additional negative test results are omitted.

Supplementary Figure 3 | Immunohistochemical evaluation of CD4 and HSP70 in distant CRC liver metastases resected after RFA. **(A,B)** Infiltration of $CD4^+$ cells (including Th, T_{regs}, possibly macrophages) into the invasive tumor margin (**A**; border) and tumor center **(B)** was assessed in immunohistochemistry revealing decreased detection of $CD4^+$ cells in patients who underwent RFA before surgery. **(C,D)** Heat shock protein 70 (HSP70) expression was significantly diminished in the cytoplasm (cyt., **C**) and in the nucleus (nuc., **D**). Staining of cells was automatically calculated (left) in digitalized slides. Numbers represent absolute cell counts with specific staining per high power field (HPF) by automated counting. Exemplary immunohistochemistry stainings are provided in the middle (patients after surgical resection) and right (patients after both RFA and surgical resection) columns (20-fold magnification). Differences were assessed using the Mann Whitney *U*-Test with $p < 0.05$ considered as significant.

Supplementary Table 1 | Patient characteristics.

Supplementary Table 2 | Overview of selected individual peptides for immunological testing.

Supplementary Table 3 | Selection of HLA class I peptides for identification of potential candidate antigens for immune analyses. A detailed description of the different selection steps can be found in **Figure 2** (exemplified for patient IRISS12).

Supplementary Table 4 | Selection of HLA class II peptides for identification of potential candidate antigens for immune analyses. A detailed description of the different selection steps can be found in **Figure 3** (exemplified for patient IRISS12).

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Conflict of Interest: ML, DK, SS, and SH are the inventors of patents owned by Immatics Biotechnologies GmbH. DK is an employee of Immatics biotechnologies GmbH, GJ is employed by CeGaT GmbH, and BN by Roche Diagnostics. H-GR has ownership interest (including patents) in Immatics, CureVac, and Synimmune.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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