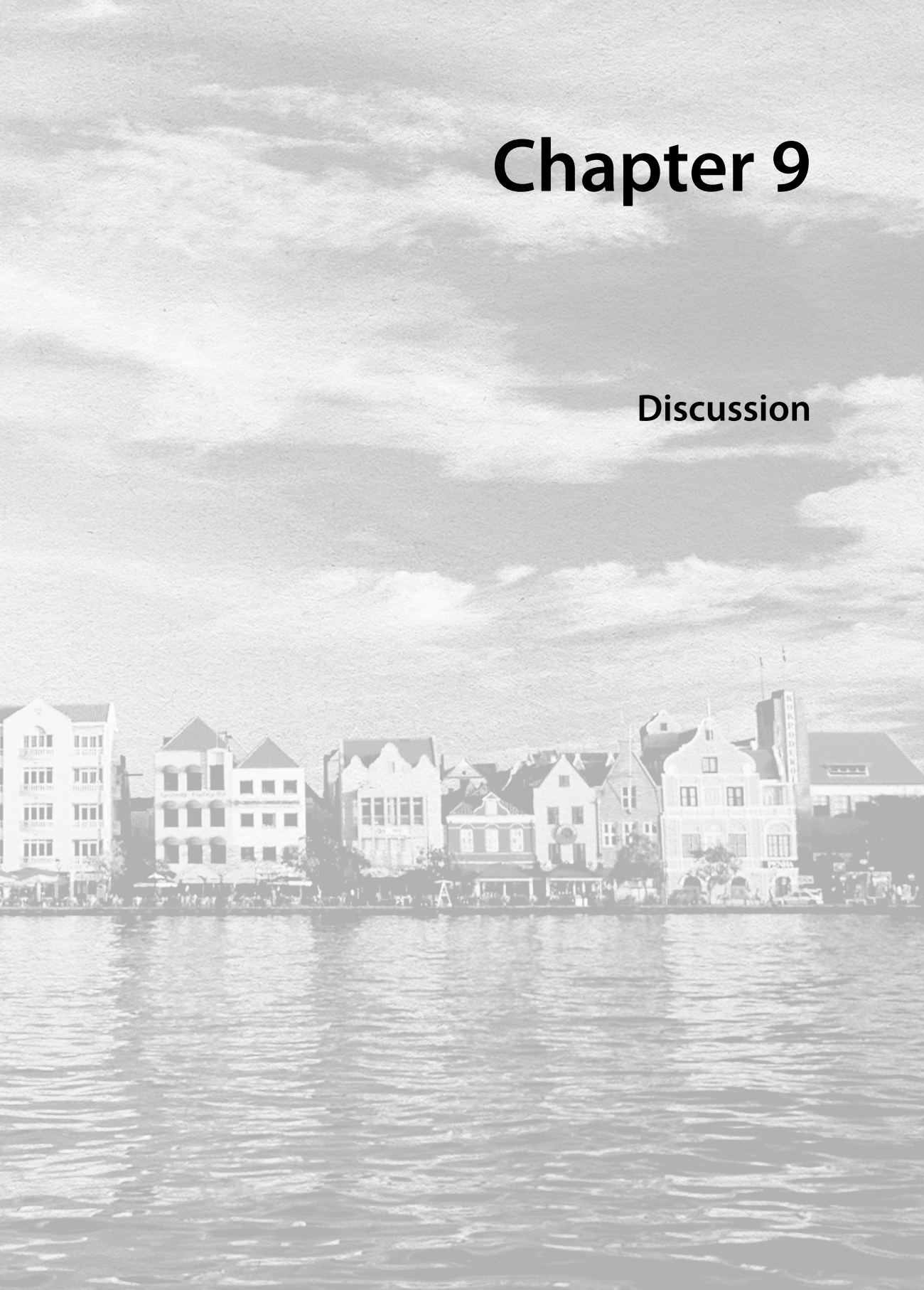
Chapter 9

Discussion



In this thesis we described our studies aimed at optimizing the efficacy of synthetic long peptide (SLP) vaccines via the encapsulation in Poly-(lactic-co-glycolic acid) (PLGA) particles. Encapsulation in biodegradable PLGA particles could serve to improve the delivery of the SLP to professional antigen presenting cells (APC), allow better control of the pharmacokinetics and biodistribution of the vaccine and finally, reduce the therapy related side effects associated with the current (pre-)clinical procedure of administering SLP vaccines delivery emulsified in water-in-oil preparations.

Immunotherapy based on SLP-vaccines has resulted in strong tumor specific immune response [1-4] and importantly, improved clinical benefit in patients with pre-malignant lesions [5].

SLPs used as vaccines are overlapping synthetic peptides of 15–35 amino acids; thus considerably longer than the minimal CTL-epitopes of 8–9 amino acids. SLP vaccines will 1) cover the entire sequence of the native TAA-encoding protein to which an immune response is targeted. SLPs 2) require internalization and processing by DCs for optimal presentation in MHC class I and class II molecules [3,6,7]. 3) SLP vaccines makes the necessity for HLA-typing of cancer patients obsolete because internalization of overlapping peptides by APCs allows natural epitope processing and selection in vivo based on the patient's own HLA-profile. APC-specific processing of SLPs will 4) facilitate simultaneous priming of T cells against multiple epitopes, both dominant and sub-dominant. Thus vaccinations will result in a broad T-cell response which decreases the chance of tumor escape related to loss of expression of immuno-dominant TAA-epitope [8-11]. With each novel TAA discovered/ described, SLP-vaccines targeting this TAA can be manufactured in advance, in large quantities and under the required strict GMP-quality standards making SLP-vaccines a clinically and pharmaceutically attractive therapeutic compound.

One important drawback associated with SLP-vaccines is their current form of administration in montanide, a clinical grade water-in-oil (w/o) emulsion. Montanide and Incomplete Freunds Adjuvant (IFA), the emulsion used for pre-clinical studies, consist of mineral oils with mannide monooleate as a surfactant emulsified with saline.

Aside from its slow release characteristics and protection of the antigen for degradation, w/o emulsions induce local inflammation and attraction of immune cells to the site of injection [12]. Montanide however causes significant side-effects at the site of injection [5,13]. Overwijk et al. showed that short peptide vaccines administered in w/o emulsions lead to poor tumor bed infiltration by activated $CD8^+$ T cells which was a consequence of

the primed $CD8^+$ T cells being "trapped" in the high inflammatory environment at the injection site containing the water-in-oil emulsion Ag depot [14]. Interestingly, substituting short peptide vaccines with SLP-vaccines abrogated this negative effect and resulted in superior tumor control.

The aim of this Ph.D project was to device an alternative method of delivery which overcomes the drawbacks associated with the use of Montanide. For this purpose we explored the use of PLGA (nano)particles (NP) as a delivery vehicle for SLP. Several important aspects of SLP encapsulated in PLGA NP (PLGA-SLP) were assessed in this thesis; from the pharmaceutical formulation to the immunological characterization of different PLGA-SLP preparations.

Together, the data presented in this thesis show that PLGA-NP mediated delivery of SLP is a very efficient method to target, load and mature Dendritic cells (DCs) as immune stimulatory compounds can be co-encapsulated with the vaccine Ag.

Although the results of these studies have been discussed in detail in the separate chapters preceding this general discussion, some remaining points will be discussed below including the applicability of PLGA particles vaccines to improve SLP-based immunotherapy and the tasks which need to be overcome to successfully translate this promising therapeutic application to the clinic.

Efficient internalization and processing of vaccine Ag by DCs is an important aspect to consider when developing peptide vaccines for therapy against cancers. The use of synthetic long peptide (SLP) vaccines based on the elongation of the N- and/or C-terminus [15,16] of the minimal cytotoxic $CD8^+$ T cell or $CD4^+$ T cell peptide epitopes were shown by our group [5,17-20] and others [2,21-23] to elicit, robust and sustained *in vivo* T cell responses with the capacity to control the growth of experimental tumors and eradicate pre-malignant lesions of the vulva. In patients with late-stage cancers, SLP-vaccines in the majority of cases failed to induce durable clinical responses even though the administration of the vaccines strongly enhanced specific immunity to tumor associated antigens (TAA) [24]. SLP-vaccines have also been studied in combinatorial approaches with DNA-vaccines as a therapy against AIDS [25,26]. These studies have led to encouraging observations; the vaccines elicited high frequencies of simian immunodeficiency virus-specific IFN-γ-positive T cells which could afford partial protection against a subsequent viral challenge.

To summarize, the use of SLP-vaccines leads to the potent $CD4^+$ and $CD8^+$ T cell responses with broad a cytokine profile and anti-tumor effector functions. Importantly, administering

cocktails of overlapping SLP covering the whole sequence of the native protein more effectively boosts the immune system when compared to vaccination with the native protein [27]. Nevertheless, owing to the low clinical benefit in cancer patients, it is clear that therapies based on SLP-vaccines require optimization. To proceed in this process, it is vital to understand how DCs "handle" long peptide Ag.

In **chapter 2** we showed the superior capacity of both mouse and human DCs to cross-present long peptides in MHC class I molecules in contrast to whole protein Ag. Interestingly, we were able to detect SLP at early time-points outside of endo-lysosomes implying that SLP internalized from the extracellular environment are translocated to the cytosol very fast once inside the DC. We were puzzled by this observation and considered the possibility that the long peptides directly access the cytosol via passive diffusion through the cell membrane. Others have shown that facilitated Ag-delivery into the cytosol endows even non-professional APC with MHC class I cross-presentation capacity [28] which would suggest that all immune cells can potentially cross-present SLP. However, internalization via passive diffusion of SLP into the cytosol through the cell membrane is unlikely; *in vitro* and *ex vivo* MHC class I Ag cross-presentation was specifically detected only when DCs were used as APC (**chapter 2**, Figure 2.5) and not by other immune cells, such as B cells. The transport of SLP into the cytosol was recently described by Menager et al. who using human DCs established a role for the ER-associated degradation machinery (ERAD)-related protein p97/VCP in the transport of SLP from early endosomes to the cytosol [29]. We also studied the method of SLP internalization using murine and human DCs. But the uptake and MHC class I cross-presentation of SLP(-OVA_{24aa}) by DC cultured in the presence of Filipin (caveolae-dependent endocytosis), dimethyl amiloride (macropinocytosis), monodansylcadavanine (inhibitor of clathrin-mediated endocytosis) or cytochalasin D (inhibitor of actin polymerization dependent phagocytosis) were inconclusive. Another group, using cytochalasin B, another inhibitor of receptor-mediated endocytosis, showed, that treatment of human DCs during SLP-Ag loading decreased, but not fully blocked, $CD8^+$ T cell activation [30] demonstrating that DC internalize long peptides at least partially via a yet unknown endocytic receptor.

Taken the data described in **chapter 4** into consideration one can conclude that MHC class I Ag cross-presentation by DC of long peptides or protein Ag is influenced by the capacity to rapidly internalize large amounts of Ag and the localization of the Ag inside the cell post-internalization. Soluble SLP (sSLP) are taken up much faster by DC than whole protein resulting in better MHC class I and II Ag presentation. Soluble proteins in contrast are

internalized slower and collect inside endolysosomal compartments where it poorly gains entry in the cytosol resulting in poor MHC class I and II presentation. The poor translocation of proteins into the cytosol is likely related to the much larger size compared to SLP (43 kD vs 2.4 kD). Size of an Ag critically determines the extent of transport to the cytosol [31].

Despite of the high amounts of sSLP internalized in the first hours, peptides were barely detectable inside the cytosol after 24 hr suggesting near complete degradation by ubiquitin proteasome system (UPS) [32,33]. Interestingly, encapsulation of SLP in PLGA-NP seems to re-route or, alternatively, store the SLP inside the endo-lysosomal compartments where it can be detected up to the 72 hr post Ag loading. This observation suggests that the Ag present inside endo-lysosomal compartments is protected from the rapid degradation by the UPS as occurs with sSLP. This mechanism of sustained antigen presence in storage compartments has also been observed using immunecomplexed protein and TLRL-SLP conjugates [34].

The prolonged $CD8^+$ T cell activation observed using PLGA-SLP points to the possibility that the internalized NP gradually releases the encapsulated SLP inside the endo-lysosomal compartments, via hydrolysis of the polymer. The released SLP is then transported into the cytosol which results in the continuous proteasome processing and sustained MHC class I Ag presentation. The enhanced MHC class I presentation observed using PLGA-SLP compared to sSLP is most probably related to the sheer higher amounts of Ag taken up through phagocytosis of NP compared to sSLP. DCs loaded with PLGA-SLP showed modest capacity to sustain *in vivo* $CD8^+$ T cell responses. Co-encapsulation of Pam3CSK4, a TLR2 agonist, significantly enhances the capacity for DCs to prolong MHC class I Ag presentation and $CD8^+$ T cell activation *in vivo*. TLR stimulation promotes Ag processing and increases the half-life of MHC class I molecules on the cell surface [35-37] which together with the prolonged presence of PLGA-SLP/TLR2L inside endolysosomal compartments can account for the sustained $CD8^+$ T cell activation we described.

For successful implementation of PLGA-NP as a delivery vehicle for SLP-vaccines in the clinic, the pharmaceutical formulation should be straightforward, reproducible and meet GMP quality requirements and regulations. Of importance, PLGA-NP should be applicable to encapsulate cocktails of various SLP with different physicochemical properties, for example the overlapping long peptides encoding the HPV-16 oncoproteins. Our initial attempts to successfully encapsulate the model Ag, SLP-OVA_{24aa} using the well-known "double emulsion method with solvent evaporation" technique commonly used to encapsulate

proteins or short peptides [38-42] in PLGA-particles required considerable modifications, as we reported in **chapter 3**. Several formulation parameters were modified, especially the inner and outer emulsion compositions which led to successful encapsulation of SLP-OVA_{24aa}. One important advantage of PLGA-particles is the capacity to control the release kinetics of the encapsulated Ag from the particles upon s.c. administration. Ag dosing over time *in vivo* is crucial as it determines the breadth of an immune response [43]. With our novel PLGA-NP formulation method we were able to encapsulate SLP-OVA_{24aa} with up to 40% encapsulation efficiency, exhibiting minimal burst release, and a total peptide release of circa 30% over the first 24 hr. We applied the novel formulation method to encapsulate SLP-OVA_{17aa} and two versions of gp100-SLP encoding for an immunodominant CTL-epitope present in this melanoma associated differentiation Ag [44]. Thus different SLP can be successfully encapsulated inside PLGA-NP using our novel "double emulsion and evaporation" formulation technique. This PLGA-NP formulation wil allow us to encapsulate the overlapping SLP encoding the HPV-E6 or HPV-E7 proteins inside PLGA-NPs and test these vaccines in pre-clinical models or on patient peripheral blood samples.

The use of particulate vaccine delivery systems is considered a promising method to achieve the desired robust and potent T cell responses that is sometimes lacking when administering vaccines in soluble form emulsified in Montanide or mixed in PBS.

The unexpected challenges at the start of the Ph.D project associated with the poor encapsulation of SLP-OVA_{24aa} and SLP-OVA_{8aa} initiated a parallel project pursuing the use of liposome based vaccine delivery strategies, which were argued to offer facilitated incorporation of SLPs compared to PLGA-NP delivery systems. Liposomes encapsulating the SLP-$MUC\text{-}1_{25aa}$ and MPLA-4, L-BLP25, have led to significant survival benefits in stage III non-small cell lung cancer patients compared to the patients receiving the standard treatment alone [45]. Despite these promising results, the authors describe that liposomal formulation includes several disadvantages that possibly limit its application in large clinical settings. Most of these disadvantages are related to the pharmaceutical formulation of the liposomes; such as laborious and expensive procedures, difficulties to scale-up the formulation process and production of sterile products. In contrast to liposomes, the formulation process of PLGA-NPs and scaling-up are easier and cost-effective [46]. Of importance, the use of PLGA co-polymers provides extra advantages over liposomes related to the ability to manipulate several physicochemical properties of the particle carrier and control the degradation rate and Ag release kinetics as discussed before in more detail in **chapter 3**.

Regarding the encapsulation of protein, it was convincingly shown that PLGA-particles are superior carriers to induce T cell responses with the capacity to control a bacterial challenge. In this study, PLGA-NP and Liposome-NP induced similar antibody titers, however liposomes failed to trigger a robust T cell response, whereas PLGA-particles showed potent capacity to induce IFN-γ producing, memory phenotype, $CD8^+$ T cells [35-37,47]. However, results obtained with protein Ag do not necessarily predicts results using long peptides as vaccine-Ag. Therefore, the ongoing studies with liposomes in our group, will address which particulate carrier is the best suited for the *in vivo* delivery of SLP-vaccines.

In **chapter 6**, we studied the role of particle size to induce an immune response. Our results point towards the use of NPs instead of microparticles (MPs) for the purpose of cancer immunotherapy as the first induced much better $CD8^+$ T cell responses. Other studies have led to similar conclusions [48,49]. Taking into consideration particle size, Ag release kinetics, adjuvanticity and in vivo uptake we can conclude that a robust $CD8^+$ T cell response is obtained by using nanoparticles, but not microparticles, co-encapsulating potent immunostimulatory agents (**chapter 4, 5 and 8**). The formulated particles should have a low burst release of the encapsulated Ag for optimal MHC class I presentation (**chapter 3**), to allow uptake of the particle co-encapsulating Ag and TLRL [50] by APC. Succeeding the initial phase when the PLGA-particles comes in contact with an aqueous environment, the release of the encapsulated Ag should be gradual, in other words "slow" rather than "fast", to induce an humoral response and most likely also efficient $CD8^+$ T cell responses [47]. Interestingly, we observed better $CD8^+$ T cells responses using PLGA-NP in comparison to IFA as a vaccine delivery system. Thus, our data and other studies [47,51] support the application of PLGA-NP delivery systems for vaccination purposes. Biodegradable NPs are a suitable replacement for w/o emulsions or Aluminium based adjuvants for the delivery of protein vaccines for cancer immunotherapy.

In **chapter 7** and **chapter 8** we explored novel targeting strategies to enhance the immune activating potency of PLGA-NP based vaccines. CD40, DEC-205 and CD11c molecules, which are highly expressed on the cell surface of DCs were the receptors of choice for these studies. Given the essential role that CD40 molecules have in DC activation and subsequent T cell priming, we hypothesized that delivering PLGA-NP encapsulated Ag via CD40 would convey two complementary effects; 1) enhance DC activation and 2) improve Ag uptake, MHC class I and II presentation and subsequent T cell priming.

Ag delivery via the targeting of the C-type lectin DEC-205 has been successfully used by many others and is therefore an established method to enhance T cell responses with tumor controlling properties [52-54]. Interestingly, DEC-205 has also been used a targeting molecule with the aim to facilitate the induction of immune tolerance, through the priming of Ag-specific Tregs [36,37]. DEC-205 targeting promotes vaccine delivery to $CD8\alpha^+$ secondary lymphoid organ resident DCs and $CD103^+$ migratory skin-resident DC. Especially the latter has been associated with strong induction of Ag-specific immune tolerization [55-57]. CD11c, an integrin, likewise has been studied in the murine system as a method to increase delivery of several types of vaccines to DC to potentiate tumor immunity. Indeed, targeting DC via CD11c significantly enhances anti-tumor immune responses [58,59] or anti-viral immunity [60]. We compared the efficacy to prime $CD8^+$ T cells injecting particles containing TLR7 and TLR8 and protein Ag targeted to these three receptors vs the control formulation without a targeting mAb. Our results point to the importance of targeting to achieve efficient priming of T cell responses as the non-targeted NPs poorly triggered $CD8^+$ T cell activation. Interestingly, we did not detect significant differences to stimulate OT-I $CD8^+$ T cells or induce *in vivo* cytotoxicity by endogenous T cells using the three targeting strategies.

As we discussed in **chapter 8**, it is possible that the efficacy of cancer immunotherapy based on PLGA-NPs is not critically dependent on the DC subtype targeted as has been shown for soluble Ag [61] as long as the vaccine is efficiently transferred to the early endosomes [62,63]. Indeed, the necessity to target DC has been questioned recently by Figdor and colleagues [64]. One study analysing the requirement for DC to prime cytotoxic T cells showed conclusively that $CD8^+$ T cells could be primed after vaccination of PLGA-particles in the absence of $CD8\alpha^+$ and $CD103^+$ DCs implying the in vivo other APC, considered to be inferior cross-presenting cells are playing a role in the priming of vaccine Ag-specific $CD8^+$ T cells. In fact, the same study pointed to the priming of $CD8^+$ T cells by MΦ and $CD8\alpha^-$ DCs. However our data in **chapter 7** show that upon injection, CD40-targeted PLGA-NP preferably bound $CD11c^+CD8\alpha^+$ DCs but poorly bound MΦ or $CD8\alpha^-$ DCs.

In conclusion, targeting PLGA-NP to DC is essential to stimulate a robust $CD8^+$ T cell mediated response. But the critical importance of targeting only one DC subtype based on the specific expression of a specific cell-surface receptor, for optimal immune activation, remains to be studied and clarified.

For the clinical application, the results described in this thesis present sufficient evidence for the use of PLGA-NP as a vaccine delivery system to target DC and being an alternative

for montanide as clinical vaccine carrier. However, the formulation, reproducibility and GMP-production of large amounts of different PLGA-NP vaccines might be a hurdle as multi-compound vaccines will probably require fine-tuning and modification of the original double-emulsion-method-with-solvent-evaporation technique to successfully encapsulate the desired SLP and/or adjuvant which each having unique physicochemical properties. Of importance, approval of multi-compound vaccines for clinical use will require that each individual compound encapsulated in the NP is tested first for toxicity and potentially also for therapeutic benefit.

From immunological perspective, however, we have strong evidence that encapsulation of SLP together with a potent immunostimulatory agent in PLGA-NP is a suitable strategy to optimize SLP-vaccines and enhance the clinical efficacy in cancer patients.

Future perspectives on the use of SLP-vaccines and particulate-based delivery methods for their delivery

The potency of SLP-vaccines to boost TAA-specific $CD4^+$ and $CD8^+$ T cell responses is clear as shown by our group and others. But despite the significant induction of tumor-specific immunity post-vaccination with SLP, objective and durable clinical responses have been rare.

Tumor immunity is complex phenomena consisting of pro-inflammatory anti-tumor effector mechanisms and, arguably of even more significance, anti-inflammatory, suppressive mechanisms that unfortunately turns out very hard to overcome in cancer patients. In this thesis we have described our studies how to enhance the pro-inflammatory milieu anti-tumor effector mechanisms by co-encapsulating long peptide Ag, or alternatively protein Ag, in PLGA-NP. Our results show the potency of PLGA-NP-vaccines to prime $CD8^+$ T cells which could control tumor growth.

Bearing in mind the several promising cancer vaccines formulated in our research groups but also the multitude of other vaccines undergoing (pre-)clinical testing or already successfully tested in phase I and/or II trials, I believe that the main obstacle for the use of therapeutic (SLP) cancer vaccines will not lie with the incapacity to prime strong T cell responses. Instead, immunologists and clinicians should implement combinatorial treatment approaches to maintain the potency of these vaccine-induced T cell responses. It is well established that T cell exhaustion and the induction of tumor-induced immune-suppression have a detrimental effect on the clinical efficacy of cancer vaccines.

Anti-CTLA-4, anti-PD1 and anti-PDL1 mAbs are immune checkpoint blockade inhibitors which are emerging as very potent immunotherapies. Their administration has led to several cases of durable clinical benefits in end-stage cancer patients. One can envision a treatment strategy starting with a cocktail of TAA-encoding SLP-vaccines to stimulate a broad T cell response followed by the administration these modulators of anti-CTLA-4, anti-PD1 or both to sustain the potency of the ongoing anti-tumor immune response.

Another attractive strategy and the topic of my current post-doctoral research project is the combination of SLP vaccines with cytokine immunotherapy. Combining IL-2/anti-IL-2 mAb complexes (IL-2-Cx) with SLP-vaccines leads to synergistic anti-tumor responses resulting improved tumor control compared to single therapies of SLP-vaccines or IL-2-Cx.

Addition of IL-2-Cx not only boosted the effector functions also seemed to rescue tumor infiltrating lymphocytes from exhaustion, evident by decreased PD-1 expression. Therefore, the provision of IL-2-Cx post SLP-vaccines will 1) supply vaccine-activated T cells with essential growth-factors and enhance their numbers and 2) maintain their pro-inflammatory phenotype by promoting their effector functions such as IFN-y and granzyme B production. Finally, IL-2-Cx will 3) counter T cell exhaustion.

Based on these arguments I propose combining IL-2-Cx with the "best SLP-vaccine on the market" to boost tumor specific T cell responses. I strongly support the use of DC-targeting vaccines. Following vaccination, treatment cycles with IL-2-Cx will sustain the ongoing anti-tumor responses.

This combinatorial immunotherapy approach will likely result in significantly better therapeutic benefits over the individual monotherapies, consisting of only SLP-vaccines or IL-2-Cx, and lead to improved patient treatment with manageable adverse effects.

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