Combinatorial prospects of nanoparticle mediated immunotherapy of cancer

Silva, C.G. da

8

GENERAL DISCUSSION

CHAPTER 8

General discussion

Immunotherapy is a rapidly growing class of cancer therapies that attempts to harness the power of the immune system to eradicate cancer cells. Despite great progress in recent years, a significant proportion of cancer patients remain unresponsive to (any) treatment and are in a dire need for new or improved therapies. The integration of nanotechnology in medicine has been remarkable in the last years, specifically concerning the improvement of the therapeutic index of existing drugs. Namely, nanotechnology can resolve specific problems faced by existing drugs, such as drug efflux mediated drug resistance, but also by enabling local slow and controlled drug release, as well as to enable the uptake of insoluble drugs by tumor cells. The integration of nanotechnology and immunotherapy, and specifically immunomodulatory drugs, brings the best of these two disciplines together to establish a new level of therapeutic benefit to eradicate cancer.

In this thesis, a novel combination of immunomodulatory drugs was tested that consisted of poly (I:C), resiquimod (also known as R848), and CCL20 (also known as MIP3α) for the treatment of cancer. The rationally combined drugs aim was to modulate the tumor microenvironment by reducing the immunosuppressive state, thereby introducing a less favorable milieu for cancer cells to survive immune attack. The combination of poly (I:C), an agonist of the endosomal Toll-Like Receptor (TLR)3, with R848, an agonist of the endosomal TLR7/8, was chosen based on the work of Tan et al. that described that the combination of poly (I:C) and R848, from several other combinations of TLR-agonists, induced the highest synergy in cytokine production in macrophages [1]. This was also later observed in human DCs and CD4 T cells [2–4] of which the mechanism was further studied

by Kreutz et al [5]. The effects on the immune system of poly (I:C) and R848 separately are quite distinct and it may therefore be a powerful combination that enhances different aspects of immune responses. Another advantage of utilizing agonists that target endosomal (viral sensing) TLRs, and not cell surface (bacterial sensing) TLRs (e.g. TLR2/4), is of relevance for human translational potential. Since humans are up to hundred fold more lethally sensitive to systemic introduction of endotoxins of bacterial original (e.g. LPS) compared to rodents such as mice and rats, it may declassify the usage of TLR2/4 agonists for direct cancer treatment [6]. Moreover, an increasing number of studies are revealing that the activation of surface TLR2/4, but much less so of endosomal TLR3/7/8/9, in tumors can have unwarranted pro-tumor effects [7]. On the other hand, humans can tolerate, up to a degree, the systemic introduction of TLR-agonists for the activation of endosomal TLRs (i.e. TLR3/7/8/9) without acute morbidity [8]. In part this is due to poor uptake of these TLR-agonists in somatic tissue since their respective receptors are located intracellularly but this also indicates that it is difficult to achieve an effective dose in the tumor. Alternatively, these endosomal TLR-agonists could be injected at high doses in the tumor directly to force a higher uptake but a swift diffusion of these drugs from the tumor microenvironment into the blood would greatly reduce the efficacy and likely induce unwarranted systemic side-effects [9]. In addition to Poly (I:C) and R848, MIP3α was added to the drug combination to enhance the recruitment of dendritic cells and lymphocytes to the tumor area [10–12]. The use of MIP3α in cancer treatment has been tested before and was found to be controversial. Although MIP3α can initiate the recruitment of these cells, the sole treatment of tumors with MIP3α (without any other treatment or drugs) in cancer patients has led to pleiotropic outcomes [13]. However, this reported phenomenon could indicate that the mere recruitment of immune cells to the tumor is insufficient because to the negative effects of the immune suppressed tumor microenvironment on these cells, and that aspect remained unaddressed. Indeed, Fushimi et al. has shown that MIP3α can induce anti-cancer effects directly but this observation was dependent on the tumor immune (suppressed) state and of the cancer model (i.e. more immunogenic, more responsive) [14]. In this sense, the combination of MIP3α with Poly (I:C) and/or R848 to ameliorate the suppressed environment, is rationally justifiable and we hypothesized less pleiotropic outcomes and instead more additive or synergism in therapeutic outcomes.

As described above, the poor uptake of Poly (I:C) and R848 by tumor cells and the swift diffusion of these drugs from the tumor microenvironment could hamper therapy responses. Specifically to address these issues, nanotechnology in the

form of nanoparticles can provide solutions by enhancing the uptake of Poly (I:C) and R848 by tumor cells, via pinocytosis, while simultaneously enhancing the slow and controlled release both intra and extracellularly [15, 16]. Both properties can be utilized when the nanoparticles are administered directly in the tumor. There, a significant portion of the intact nanoparticles is taken-up by cells, where they first start to slowly release their cargo in early and late endosomes activating TLR3 and 7/8, and then in lysosomes after which the remaining cargo is released in the cytoplasm [16]. The MIP3**α** that was also released intracellularly, would be lost in the lysosomes. However, the fraction of nanoparticles that was not taken-up by any cells also start to slowly release their cargo in the extracellular space, which includes the building of a gradient concentration of MIP3**α**.

In **chapter 3,** it was determined whether the PLGA nanoparticle backbone technology could indeed reduce drug diffusion from the tumor area when injected directly in the tumor, subcutaneously elsewhere, or intravenously. In addition, the biodistribution into vital organs and blood concentration of a drug surrogate (i.e. ICG) was studied to determine the drug release kinetics. Here it is shown that the intratumoral injection of the nanoparticles is the most effective administration method to achieve the highest concentration of nanoparticles in the tumor. Generally, the method of intratumoral administration of cancer drugs is rapidly increasing for several tumor types, including less accessible tumors in the thorax and abdominal area [17, 18]. The same trend is also applicable for the administration of nanomedicine. For instance, Hensify® received recent approval by the EMA as a nanoparticle formulation for the combinatorial treatment of sarcoma to be administered by intratumoral injection [19]. Although the treatment is performed locally and the nanoparticles are unlikely to reach the metastases themselves, systemic protection for metastases control can be attainable via the abscopal effect, by which locally activated tumor-specific immune cells will migrate and eradicate distant lesions.[20]. Hence, the intratumoral administration method was chosen to establish a proof of principle for the nanoparticle technology developed specifically for the work presented in this thesis.

In **chapter 4,** the efficacy of the nanoparticle mediated therapy with poly (I:C), R848, and MIP3**α** was tested in vivo on the TC-1 cancer model. The TC-1 model, compared to MC-38 or CT-26, is considerably less responsive to conventional (immune)therapies, including chemotherapy, except for specific immunotherapy in the form of a therapeutic cancer vaccine. Hence, the TC-1 model is one of the most relevant cancer models for human cancer patients, because it very difficult to treat

and cure, similar to human cancers, and as such by achieving TC-1 cures, it would increase the translation relevance to human cancers. When TC-1 tumor-bearing mice were treated with the nanoparticle mediated treatment with poly (I:C), R848, and MIP3**α**, they were found to be irresponsive. This could indicate that, despite the presence of highly immunogenic antigens in TC-1 cells, no effective immunity (i.e. cognate T cells) is present that can be enhanced by the nanoparticle treatment. To overcome this problem, doxorubicin was added to the drug combination to induce TC-1 cancer cell death and to release antigens to which immune cells could target. This resulted in a nanoparticle mediated chemoimmunotherapy modality consisted of poly (I:C), R848, MIP3**α**, and of doxorubicin. The co-loading of doxorubicin not only did improve the overall survival (cures) but also improved the progression-free survival time, which was nearly doubled. Moreover, it was established that the nanoparticle mediated delivery of these drugs for the therapeutic efficacy is pivotal, as established by the observation that TC-1 tumor-bearing mice treated with equal concentrations of the drugs injected intratumorally (but not loaded in nanoparticles) induced no cures and the gain of the progression-free survival time was not nearly as significant. The MC-38 model was found to be much more responsive to the chemoimmunotherapy modality than TC-1, but also in this model the nanoparticle mediated delivery was found pivotal to achieve higher percentages of cures. These results warranted a more in-depth literature study of the current development stages of chemoimmunotherapy and most specifically when the treatment is mediated by nanotechnology. The results of the literature study and the discussion thereof is presented in **chapter 2**.

In **chapter 5**, the individual therapeutic potential of poly (I:C), R848, and MIP3**α** was studied. Since the TC-1 model is responsive to a therapeutic cancer vaccine, but generally little to no cures are attained long-term with only vaccination, it provided an ideal basis to establish possible improvements with other therapeutic combinations. When mice bearing TC-1 tumors were vaccinated and the tumors treated with nanoparticles containing either one or more combination of drugs, it was observed that both poly (I:C) and R848, but not MIP3**α**, separately increased the percentages of overall survival. However, the triple combination of poly (I:C), R848, and of MIP3**α** induced significantly better overall survival outcomes and the progression-free survival time was nearly doubled. The same combination of drugs was also tested on the RMA cancer model. Although a significant therapy response was attained, the RMA model was found to be much less responsive than the TC-1 model. This observation underlines the potential of the modality to improve distinct therapeutic cancer vaccines, but the actual enhancing potential of the modality

is dependent on the model and on the initial potency of the therapeutic cancer vaccine itself. Nonetheless, this study established a proof of principle that the immune modulation of tumors with the nanoparticle delivery of poly (I:C), R848, and MIP3α can improve response of therapeutic cancer vaccines.

In **chapter 6**, the nanoparticle modality was combined with photodynamic therapy and tested on the TC-1, MC-38, and CT-26 cancer models. The co-treatment induced high overall survival percentages on both MC-38 and CT-26 models. Also, the nanoparticle treatment alone without photodynamic therapy enhanced the overall survival percentages of MC-38 and of CT-26. However, the co-treatment with photodynamic therapy did not improve the overall survival percentages on the TC-1 model, however, the progression-free survival time was observed to increase significantly. Kleinovink et al. has reported that the local treatment of tumors with photodynamic therapy affects the growth of distant tumors, a process that is likely mediated by the abscopal effect via CD8 cytotoxic effector T cells [21]. In chapter 6, this effect was reproduced and further enhanced with the co-treatment of the nanoparticle modality. This effect that was most pronounced in the CT-26 cancer model. This suggests that the combination of photodynamic therapy with immunomodulatory nanoparticles are an ideal combination for the treatment of tumors and of metastases.

FUTURE PERSPECTIVES

To date, fifty-one nanomedicine formulations of existing drugs are FDA/EMA approved and are used to treat cancer in humans, and many more are in clinical trials or pending approval [22, 23]. In general, nanomedicine formulations tend to face additional problems during research & development phases and the production under GMP conditions before attaining clinical approval compared to 'regular' drug development [24–26]. For instance, common variations between production batches during smaller scale production and pre-clinical phase, issues during large-scale manufacturing, and overall cost-effectiveness compared to 'regular' drugs are additional obstacles faced during the development of nanoparticle formulations [27]. Despite these caveats, the advantages of nanoparticle formulations can outweigh the disadvantages, but only in specific cases. One case is Doxil®, a nanomedicine formulation of doxorubicin [28]. In this example, Doxil® resolves cardiotoxicity by reducing biodistribution (i.e. to the heart; as it is a major limiting adverse effect of doxorubicin), which Doxil® reduces significantly without loss of therapeutic efficacy. Although the production costs are higher

and technically more difficult to produce than doxorubicin, Doxil® is a common chemotherapy administered to cancer patients for the treatment of several cancer types. However, in many other cases the nano formulation products were discontinued in early clinical phases due to a discrepancy between preclinical and clinical outcomes. The safe delivery of drugs to cancer cells, while sparing healthy cells, is commonly claimed but this effect is often later not observed in cancer patients [29, 30]. Since many applications of nanomedicine are currently designed as therapeutics for the treatment of cancer via systemic administration (i.e. intravenous administration) and did show significant improvement and therapeutic effect in preclinical (murine) models, they also often fail to show improvement in clinical trials. Arguably, one of the several reasons for this failure is related to the enhanced permeability and retention (EPR) effect. Many publications regarding nanomedicine formulations of oncological drugs lean greatly on the premises of the EPR effect to explain the observed therapeutic effects observed in mice. However, the existence of EPR effect in human cancers is of a strong debate and even if it exists, whether the EPR effect may be pronounced enough and as such, whether EPR-dependent nanomedicine formulations present any value for large scale application for the treatment of human cancers if they are truly dependent on the EPR effect for the therapeutic efficacy. Another reason for nanomedicine discontinuation is the considerable pathological and physiological variations between cancer patients compared to the uniformity of tumor specific preclinical (murine) models, albeit this is also applicable for non-nanomedicine formulations. However, this suggests that the putative absence of the EPR effect is not the only reason for the unsuccessful application of nanomedicine drugs in clinical trials. A potential solution for this problem may be the intratumoral administration of the nanomedicine, which would be less dependent on the EPR effect, and is in fact already becoming a more common method of administration indeed. Another potential solution that would not depend on the EPR effect to access cancer cells directly is the active extravasation of nanoparticles into tumors, but progression in this area is slow [31]. On the other hand, the application of nanomedicine to improve immunotherapeutics, that largely target immune cells rather than cancer cells, would also not depend on the EPR effect. As such, the outlook on the future of nanomedicine is still looking promising and likely to improve the therapeutic index of many drugs in the future but researchers should consider the known disadvantages of nanomedicine application during the nanomedicine design phase to reduce early clinical trial failures.

Nonetheless, new proof of principle research exploring bright new ideas in the field of nanomedicine research are still accomplished and are pushing the field forward. Another aspect to be considered are the varying therapy responses between the different cancer models to the same therapy. For instance, it would be interesting to determine which populations of immune cells are pivotal for tumor regressions after treatment in these distinct models. This could be studied in the TC-1 model by combining the therapeutic cancer vaccine with the immune modulation nanoparticles and then perform cell type clonal deletions of NK cells, macrophages, etc. The contribution of both adaptive and of the innate immune system could be further established by depleting CD8 T cells while applying therapeutic pressure with immune modulatory nanoparticles (targeting innate immune cells) in the tumor. The dose-response of the immunomodulatory nanoparticles was not determined in this work, but it is probably a relevant aspect for therapeutic efficacy. For instance, it has been described that the dose concentration of STING-agonist in the tumor determine the type of immune response, and it is currently not know whether this is similar for TLR-agonists [32]. Moreover, the effectiveness of the immunomodulatory nanoparticles combined with different immune checkpoint inhibitors, such as those targeted against PD-1, CTLA-4, LAG-3, or other small molecules, was not studied here and the combination is likely be of great therapeutic benefit.

Another important fact to be considered is that successes of pre-clinical mice studies are not always reproducible in clinical studies in humans. Should in the future the immunomodulatory nanoparticles presented in this thesis be considered for a future clinical trial, then the combination of with other ablative modalities would be suggested because of the induction of abscopal effects as indicated with chemotherapy (chapter 4) or photodynamic therapy (chapter 6). Similarly, the potential to improve the efficacy of therapeutic cancer vaccines with the immunomodulatory nanoparticles could also be further studied for cancer patients eligible for such specific immunotherapy.

Besides the challenges of production under GMP conditions of the immunomodulatory nanoparticles, neither poly (I:C), resiquimod, or CCL20 are currently FDA/EMA approved for the direct therapy of cancer. Only resiquimod is approved for topical application in the treatment of cutaneous T cell lymphoma. Also, the patents of these immune adjuvants have expired decades ago and unfortunately, drugs that are not protected by non-expired patents are often

considered economically non-viable, specially to larger pharmaceutical companies. However, there are subsidies and procedures available within the European Union and the EMA to conduct clinical trials and the economic exploitation of (orphan) drugs. Alternately, novel and perhaps more powerful immune adjuvants currently in development should consider the usage of nanomedicine technology as well as the intratumoral administration for possible improved therapeutic outcomes.

CONCLUSIONS

The work presented in this thesis forms the basis of a proof of principle treatment for the immunomodulation of tumors upon the intratumoral administration of poly (I:C), R848, and MIP3α in mice. The nanoparticle mediated delivery of these drugs was repeatedly shown to be pivotal for enhanced therapeutic outcomes. A discrepancy of responses to this treatment was observed between different cancer models, since the modality independently of ablative co-modalities was quite effective to treat the colon cancer models MC-38 and CT-26, but not the TC-1 or RMA models. When combined with ablative co-modalities, the immunomodulatory nanoparticles have shown remarkable good adjuvant potential when combined with chemotherapy and photodynamic therapy, but also with therapeutic cancer vaccines in the TC-1 and RMA models. This underlines the therapeutic benefit of the combinational treatment of these modalities with immunomodulatory nanoparticles due to their enhancing potential of the abscopal effect to control distant metastases. Furthermore, it was established that the combination of poly (I:C) and R848 is of therapeutic benefit and that the addition of MIP3α increases the therapeutic potential further. Mechanistically, a phenotype shift of tumor-associated macrophages towards inflammatory monocytes within tumors and tumor-draining lymph nodes was recurrently observed, which underlines the importance of the collaboration between the adaptive and innate immunity to achieve durable anti-cancer responses. Collectively, the immunomodulatory nanoparticles have great potential to mediate the local controlled delivery of synergistic drug combinations and can be further tailor-made as an ideal adjuvant therapy for exiting treatment modalities of several different cancer types.

REFERENCES

[1] Ting Tan, R. S.; Lin, B.; et al. The Synergy in Cytokine Production through MyD88-TRIF Pathways Is Co-Ordinated with ERK Phosphorylation in Macrophages. Immunol. Cell Biol. 2013; 91: 377–387.

[2] Pearson, F. E.; Chang, K.; et al. Activation of Human CD141 + and CD1c + Dendritic Cells in Vivo with Combined TLR3 and TLR7/8 Ligation. Immunol. Cell Biol. 2018; 96: 390–400.

[3] Surendran, N.; Simmons, A.; et al. TLR Agonist Combinations That Stimulate Th Type I Polarizing Responses from Human Neonates. Innate Immun. 2018; 24: 240–251.

[4] Madera, R. F.; Wang, J. P.; et al. The Combination of Early and Rapid Type I IFN, IL-1α, and IL-1β Production Are Essential Mediators of RNA-Like Adjuvant Driven CD4+ Th1 Responses. PLoS One 2011; 6: e29412.

[5] Kreutz, M.; Bakdash, G.; et al. Type I IFN-Mediated Synergistic Activation of Mouse and Human DC Subsets by TLR Agonists. Eur. J. Immunol. 2015; 45: 2798–2809.

[6] Munford, R. S. Murine Responses to Endotoxin: Another Dirty Little Secret? J. Infect. Dis. 2010; 201: 175–177.

[7] Kaczanowska, S.; Joseph, A. M.; et al. TLR Agonists: Our Best Frenemy in Cancer Immunotherapy. J. Leukoc. Biol. 2013; 93: 847–863.

[8] Shi, M.; Chen, X.; et al. Application Potential of Toll-like Receptors in Cancer Immunotherapy: Systematic Review. Medicine (Baltimore). 2016; 95: e3951.

[9] Engel, A. L.; Holt, G. E.; et al. The Pharmacokinetics of Toll-like Receptor Agonists and the Impact on the Immune System. Expert Review of Clinical Pharmacology, 2011, 4, 275–289.

[10] Dieu, M. C.; Vanbervliet, B.; et al. Selective Recruitment of Immature and Mature Dendritic Cells by Distinct Chemokines Expressed in Different Anatomic Sites. J. Exp. Med. 1998; 188: 373–386.

[11] Liao, F.; Rabin, R. L.; et al. CC-Chemokine Receptor 6 Is Expressed on Diverse Memory Subsets of T Cells and Determines Responsiveness to Macrophage Inflammatory Protein 3 Alpha. J. Immunol. 1999; 162: 186–194.

[12] Al-Aoukaty, A.; Rolstad, B.; et al. MIP-3alpha, MIP-3beta and Fractalkine Induce the Locomotion and the Mobilization of Intracellular Calcium, and Activate the Heterotrimeric G Proteins in Human Natural Killer Cells. Immunology 1998; 95: 618–624.

[13] Ranasinghe, R.; Eri, R. Modulation of the CCR6-CCL20 Axis: A Potential Therapeutic Target in Inflammation and Cancer. Medicina (B. Aires). 2018; 54: .

[14] Fushimi, T.; Kojima, A.; et al. Macrophage Inflammatory Protein 3alpha Transgene Attracts Dendritic Cells to Established Murine Tumors and Suppresses Tumor Growth. J. Clin. Invest. 2000; 105: 1383–1393.

[15] Hines, D. J.; Kaplan, D. L. Poly(Lactic-Co-Glycolic) Acid-Controlled-Release Systems: Experimental and Modeling Insights. Crit. Rev. Ther. Drug Carrier Syst. 2013; 30: 257–276.

[16] Cartiera, M. S.; Johnson, K. M.; et al. The Uptake and Intracellular Fate of PLGA Nanoparticles in Epithelial Cells. Biomaterials 2009; 30: 2790–2798.

[17] Hamid, O.; Ismail, R.; et al. Intratumoral Immunotherapy—Update 2019. Oncologist 2020; 25: .

[18] Hong, W. X.; Haebe, S.; et al. Intratumoral Immunotherapy for Early-Stage Solid Tumors. Clinical cancer research : an official journal of the American Association for Cancer Research, 2020, 26, 3091–3099.

[19] Bonvalot, S.; Rutkowski, P. L.; et al. NBTXR3, a First-in-Class Radioenhancer Hafnium Oxide Nanoparticle, plus Radiotherapy versus Radiotherapy Alone in Patients with Locally Advanced Soft-Tissue Sarcoma (Act.In.Sarc): A Multicentre, Phase 2–3, Randomised, Controlled Trial. Lancet Oncol. 2019; 20: 1148–1159.

[20] Kaminski, J. M.; Shinohara, E.; et al. The Controversial Abscopal Effect. Cancer Treat. Rev. 2005; 31: 159–172.

[21] Kleinovink, J. W.; Fransen, M. F.; et al. Photodynamic-Immune Checkpoint Therapy Eradicates Local and Distant Tumors by CD8 + T Cells. Cancer Immunol. Res. 2017; 5: 832–838.

[22] Bobo, D.; Robinson, K. J.; et al. Nanoparticle-Based Medicines: A Review of FDA-Approved Materials and Clinical Trials to Date. Pharm. Res. 2016; 33: 2373–2387.

[23] Anselmo, A. C.; Mitragotri, S. Nanoparticles in the Clinic: An Update. Bioeng. Transl. Med. 2019; 4: .

[24] Shi, J.; Kantoff, P. W.; et al. Cancer Nanomedicine: Progress, Challenges and Opportunities. Nature Reviews Cancer, 2017, 17, 20–37.

[25] Heiligtag, F. J.; Niederberger, M. The Fascinating World of Nanoparticle Research. Materials Today, 2013, 16, 262–271.

[26] Santiago, I. Nanoscale Active Matter Matters: Challenges and Opportunities for Self-Propelled Nanomotors. Nano Today 2018; 19: 11–15.

[27] Hua, S.; de Matos, M. B. C.; et al. Current Trends and Challenges in the Clinical Translation of Nanoparticulate Nanomedicines: Pathways for Translational Development and Commercialization. Frontiers in Pharmacology, 2018, 9, 790.

[28] Gyöngyösi, M.; Lukovic, D.; et al. Liposomal Doxorubicin Attenuates Cardiotoxicity via Induction of Interferon-Related DNA Damage Resistance. Cardiovasc. Res. 2020; 116: 970–982.

[29] Pasut, G. Grand Challenges in Nano-Based Drug Delivery. Front. Med. Technol. 2019; 1: 1.

[30] Wilhelm, S.; Tavares, A. J.; et al. Analysis of Nanoparticle Delivery to Tumours. Nature Reviews Materials, 2016, 1, 1–12.

[31] Moghimi, S. M.; Simberg, D. Nanoparticle Transport Pathways into Tumors. J. Nanoparticle Res. 2018; 20: .

[32] Sivick, K. E.; Desbien, A. L.; et al. Magnitude of Therapeutic STING Activation Determines CD8+ T Cell-Mediated Anti-Tumor Immunity. Cell Rep. 2018; 25: 3074-3085.e5