SUMMARY AND CONCLUSIONS

Adoptive cell therapies have provided great advancements in the field of cancer immunotherapies. Since the approval of the first adoptive cell therapy, Provenge, in 2010, eight more products have been approved by the Food and Drug Adminsitration (FDA). Noteably, all but one of these approved therapies rely on the engineering of autologous T cells. Their *ex vivo* engineering enables T cells to target specific tumor-associated antigens and achieve remarkable therapeutic results. However, the use of autologous T cells imposes particular challenges on the manufacturing of the therapies and is associated with severe side-effects. As an alternative, NK cells are being explored for adoptive cell therapies.

NK cells offer a specific set of advantages compared to T cells including a lower risk for severe side effects (e.g. CRS and neurotoxicity) and the possibility to generate off-the-shelf products. Whilst they also provide the advantage of intrinsic cytotoxicity, engineering of NK cells is still desired to improve their overall antitumor responses and provide prolonged persistence *in vivo*. In **Chapter 1** we discuss the different approaches that are being explored to enhance the efficacy of NK cells. The current approved T cell therapies rely on the use of viral transduction which is associated with several drawbacks such as safety concerns, limited cargo capacity, regulatory hurdles and high product cost. Additionally, viral transduction of NK cells lacks the high efficiences obtained for T cells. This is attributed to reliance of NK cells on pattern recognition signaling for the identification of aberrant cells, which enhances their sensitivity to transduction. As a result, in **Chapter 1**, we summarized the different non-viral transfection technologies that are being evaluated for efficient engineering of NK cells. In this chapter we focussed not only on the operating mechanism and performance of the techniques but also on the ability to maintain a healthy cell state and optimal cell functionality.

One particular non-viral transfection technology that has gathered significant interest over the last years is nanoparticle-sensitized photoporation. This technique relies on the combination of photothermal nanoparticles and their irradiation with pulse laser light to induce transient permeabilization of the plasma membrane, enabling the entry of external molecules into the cytosol. In **Chapter 1** we review the transition of nanoparticle-sensitized photoporation using gold nanoparticles (AuNPs) towards biocompatible and biodegradable polydopamine nanoparticles (PDNPs), to improve the clinical translation of the technique and avoid the possible detrimental effects of fragmented AuNPs after laser irradiation. In this PhD thesis we assessed the potential of

nanoparticle-sensitized photoporation using PDNPs for the development of engineered NK cell therapies.

In **Chapter 2** we provided an initial proof-of-concept for the delivery of macromolecules (e.g. FD500 and eGFP mRNA) in NK-92MI cells. PDNPs of 250 nm and 500 nm were employed and after the optimalization of different photoporation parameters (e.g. nanoparticle concentration and laser fluence) and we demonstrated efficient transfection of the cells while maintaining high cell viability. In addition, we compared these results to state-of-the-art electroporation. This provided us with a relevant comparison since electroporation is currently the most commenly used non-viral transfection technology for NK cell engineering. Here we found that photoporation was able to outperform electroporation in terms of transfection yield, thanks to being much gentler to the cells. However, electroporated cells outperformed photoporated cells in in terms of higher expression levels per cell within the viable population. The significant differences in viability after photoporation and electroporation encouraged us to further investigate the quality of the cells after the treatments. Here we found no differences in the proliferation capacity of the cells and their expression of various surface markers. We did observe alterations in the release of cytokines by the cells after electroporation, which was not observed for the photoporated cells. And lastly, we showed that the cytotoxic potential of photoporated NK-92MI cells did not differ from non-treated cells. Altogether these results demonstrate that PDNP-mediated photoporation enables efficient and gentle transfection of NK-92MI cells, while preserving cell phenotype.

The engineering of NK-92(MI) cells is relevant as they are currently being evaluated in various clinical trials for cancer immunotherapy. However, as an immortalized cell line, NK-92(MI) cells require γ-irradiation prior to administration, to prevent uncontrolled proliferation *in vivo*. Alternatively, primary human NK cells do not require require γ-irradiation which allows for a longer persistence of the cells *in vivo*. Moreover, primary human NK cells are more heterogeneous and provide a better representation of patient-derived NK cells. For these reasons, primary human NK cells are often preferred over NK-92MI cells for therapeutic applications. This led us to investigate the engineering of the cells with PDNP-mediated photoporation in **Chapter 3**. Similar as in **Chapter 2**, we optimized the different photoporation parameters (e.g. particle size, particle concentration and laser fluence) and compared the obtained results to electroporation. Photoporation demonstrated efficient transfection of the cells while preserving high cell viability, which prompted a 3-fold higher transfection yield compared to electroproration. Transfection of the NK cells through electroporation, on the other hand, introduced higher expression levels per cell but was accompanied by high cell

toxicity. Next, we examined if photoporation using PDNPs could be used to deliver Cas9 ribonucleoproteins (RNPs) in NK cells. The Cas9 RNPs targeted the inhibitory NKG2A receptor, as this is a commonly used strategy to enhance NK cell toxicity (see **Chapter 1**). In this chapter we demonstrated the successful knock-out of the receptor with photoporation, which outperformed electroporation in terms of knock-out yield. Due to the significant differences in cell viability observed between electroporation and photoporation, we decided, very similar to our approach in **Chapter 2**, to investigate the conditions of the cells following the application of these technologies. Neither photoporation nor electroporation had any significant impact on the phenotype of the cells, their proliferation capacity or their cytolytic activity. Based on these results, we could conclude that photoporation serves as a gentle transfection technology for the engineering of primary human NK cells.

Nanoparticle-sensitized photoporation has demonstrated successful delivery of various macromolecules in different cell types, including NK cells, as discussed above. However, the technique requires further optimalization to deliver large constructs such as large mRNA strands and pDNA. This limitation was addressed recently in the work of Fraire *et al.* where they synthesized self-assembled nanostructures called 'nanobombs' (NBs). These NBs were composed of a photothermal iron oxide core particle (IONP) to which smaller entities or 'nanoprojectiles' were attached. Similar as before, the IONP would generate VNBs upon irradiation with pulsed laser light. However, in this case, the nanoprojectiles would be propelled into the surrounding medium and cell membranes enabling the delivery of pDNA. In **Chapter 4**, we set out to synthesize NBs for the transfection of NK cells with mRNA and especially pDNA. Unfortunately, the IONPs that were previously used by Fraire *et al.* were discontinued, which led us to evaluate new IONPs but also new strategies to attach the nanoprojectiles. In total we synthesized six different NBs, using four different IONPs and two distinct coupling strategies, and evaluated their morphology. As time was limited, we could only evaluate one NB for intracellular delivery of FD500 in HeLa cells, which was unfortunately unsuccessful. If possible, this work should be continued by also evaluating the other NBs for the delivery of macromolecules in cells. In addition, more systematic experiments should be conducted such as a more depth-analysis of the attachment of the nanoprojectiles to the core, their release upon laser irradiation and evaluation of their presence in cells.

Lastly, in **Chapter 5** we provide the broader international context, relevance and future perspectives of this work. We start by describing the evolution of cell-based cancer immunotherapies over the last years and provide an overview of the currently approved therapies. While greatly

successful, these therapies are limited by their use of autologous cells and risk for severe side-effects (as discussed above). We explain how this has driven the field to explore a variety of cell types, such as NK cells, for the development of innovative cell therapies. Furthermore, the currently approved therapies face challenges related to lengthy and costly manufacturing processes, resulting in extended vein-to-vein times. We discuss how this has increased interest in non-viral engineering approaches (e.g. electroporation, microfluidic devices), which offer solutions to these challenges. Additionally, we highlight the potential of nanoparticle-sensitized photoporation by reviewing the promising restuls presented in this PhD thesis. Finally, we examine the benefits of decentralized and automated manufacturing systems to deliver faster, more affordable therapies, alongside the ability of NK cells to provide off-the-shelf cell therapies.