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Physical transfection technologies for macrophages and dendritic cells in immunotherapy

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Abstract

Introduction: Dendritic cells (DCs) and macrophages, two important antigen presenting cells (APCs) of the innate immune system, are being explored for the use in cell-based cancer immunotherapy. For this application, the therapeutic potential of patient-derived APCs is increased by delivering different types of functional macromolecules, such as mRNA and pDNA, into their cytosol. Compared to the use of viral and non-viral delivery vectors, physical intracellular delivery techniques are known to be more straightforward, more controllable, faster and generate high delivery efficiencies. The most conventional physical delivery technique is electroporation, which is able to achieve efficient intracellular delivery of large nucleic acids such as mRNA and pDNA. However, it is associated with several drawbacks such as a low viability, a decreased migratory capacity and an altered cytokine secretion profile.

Areas covered: This review starts with electroporation as the most traditional physical transfection method, before continuing with the more recent technologies such as sonoporation, nanowires and microfluidic cell squeezing. A description is provided of each of those intracellular delivery technologies with their strengths and weaknesses, especially paying attention to delivery efficiency and safety profile.

Expert opinion: Given the common use of electroporation for the production of therapeutic APCs, it is recommended that more detailed studies are performed on the effect of electroporation on APC fitness, even down to the genetic level. Newer intracellular delivery technologies seem to have less impact on APC functionality but further work is needed to fully uncover their suitability to transfect APCs with different types of macromolecules.

Keywords: Cancer immunotherapy, Innate immunity, Dendritic cells, Macrophages, Cell engineering, Transfection, Electroporation, Sonoporation, NanoWires, Microfluidic cell squeezing, pDNA, mRNA, antigens

Article highlights

- Electroporation is able to achieve high delivery efficiency in patient-derived antigen presenting cells for different kinds of macromolecules, but recent reports point to unwanted effects on cell homeostasis and cellular functionality, including altered surface receptor expression, cytokine secretion and reduced migration capacity.
- Sonoporation is able to deliver macromolecules in APCs but less efficient compared to as electroporation. However, impairment of the expression of co-stimulatory molecules is seen and the technique can be associated with a high toxicity.
- NanoWires achieve a high delivery efficiency and viability, even though there is still doubt on the underlying mechanism. Successful intracellular delivery of large nucleic acids remains to be confirmed. Nevertheless, NanoWires do not influence the immune response or the expression of several immune response genes.
- Cell squeezing is an upcoming microfluidics based technique that is promising due to its efficacy, speed and simplicity. Novel technical variations on this concept seem to resolve shortcomings of the early designs and seems very promising for in the high-throughput production of therapeutic APCs.

1. Introduction on current transfection technologies to engineer dendritic cells and macrophages *ex vivo*

For the successful use of dendritic cells (DC) as cellular vaccines in the context of cancer immunotherapy, it is of importance to deliver antigens or antigen encoding genetic constructs (such as pDNA or mRNA) directly into the cytosol of DCs. Indeed, direct delivery of exogenous antigens into the cytoplasm favors major histocompatibility complex I (MHC I) presentation, rather than relying on cross-presentation after phagocytic uptake [1–5]. In addition, the cytoplasmic delivery of genetic constructs (such as pDNA or mRNA) can lead to higher transfection rates and as such further promote antigen expression delivered directly into the cytosol. Complementary to DCs, macrophages (Mφs) with a chimeric antigen receptor (CAR) have been proposed recently as well [6]. Therefore, the *ex vivo* cytosolic delivery of genetic constructs (such as pDNA or mRNA) into the cytosol of macrophages can be of importance to express and maintain the expression of CARs on the surface of macrophages.

One of the methods that could be utilized to transfect both DCs and macrophages, is the use of viral vectors such as a lentivirus or an adenovirus [6–14]. Viral vectors have advantages such as the possibility to incorporate large genes and their capacity to induce high transfection efficiencies in antigen presenting cells (APCs) [15–18]. However, they come with major drawbacks as well, including a high cost, being labor intensive and posing safety issues [19–22]. Moreover, viral vectors may trigger an immunogenic response themselves [23,24]. Indeed, several viruses have the potential to initiate the activation of an inflammasome response which could lead to increased cell death via pyroptosis [25–27].

Alternatively, transfection technologies making use of non-viral nanocarriers have been used for the cytosolic delivery of various compounds into macrophages and DCs. Several types

of nanocarriers have been shown to protect antigens against degradation and increase intracellular delivery of the antigens compared to spontaneous uptake of the antigens by itself [28]. The primary intended use of nanocarriers is for treatment of APCs *in vivo*, which is quite attractive as well as it would reduce the labor intensive isolation and *ex vivo* modification of APCs and the associated costs. However, at present this remains a great challenge and many steps still need to be taken, including specific in vivo targeting of APCs. Cationic nanocarriers such as liposomal formulations have been frequently explored to deliver pDNA, mRNA and siRNA into APCs [28–30]. However, lipofection experiments have shown variable results with quite low transfection efficiencies between 0 - 10% for pDNA [31–34] and 0 - 30% for mRNA [32,34–37]. For other cationic nanocarriers it was shown that delivery of model antigens (e.g., ovalbumin) into APCs an antigen specific immune response could be obtained which outperformed the response induced by soluble antigens alone [38,39]. A particular reported disadvantage of nanocarriers, especially for ex vivo modification of cells, is the potential induction of cellular toxicity [34,35,40–42]. Several nanocarriers were reported to influence some important functions of APCs like maturation, migration and antigen presentation [43]. Moreover, since nanocarriers are often charged, they can induce the activation of the NLRP3 response resulting in increased immunogenicity [44].

Physical transfection technologies, such as electroporation, on the other hand could have some promising features for the *ex vivo* engineering of macrophages and DCs. Physical delivery methods typically offer more precise control in an *ex vivo* setting, combined with high throughput treatment as well as freedom of the cargo that is to be delivered [45]. Physical transfection techniques have in common that they use a physical force to enhance the permeability of the cell's plasma membrane to allow influx of external molecules into the cell's cytoplasm. They will be explained in more detail throughout this review.

Below, we will first give an introduction of the antigen presenting cells and their application in cancer immunotherapy. Next, we will discuss the physical transfection technologies which have been successfully applied on both primary DCs and Mφs, including electroporation, sonoporation, nanowire poration and cell squeezing (**Figure 1**). We will briefly explain the mechanism of each method and give an overview of the studies in which they have been used, with special attention to their performance in terms of delivery efficiency and cytotoxicity. In particular we will focus on studies on primary human or animal (i.e. mice) derived DCs and Mφs as they are of most relevant compared to immortalized cell lines [46–48]. Finally, the most important findings, the current status of the technologies and potential improvements will be discussed in the expert opinion section.

2. Introduction to antigen presenting cells

The main focus of this review is to outline the current technologies which have been used to deliver macromolecules into APCs (i.e., Mφs and DCs, two of the most important innate immune cells). It is of utmost importance that only the delivered macromolecules (e.g., antigens) carry out their function and that the technologies do not lead to any unintended functional changes themselves. Therefore, in this section we will first discuss the main functions of the APCs and how these functions can change due to extracellular alterations (e.g., danger signals,…etc.).

In the presence of an infection, APCs like Mφs and DCs, play a crucial role in connecting innate and adaptive immune responses by engulfing antigens and presenting them to T cells [49–52]. Extracellular proteins are taken up via phagocytosis and end up in the phagosomes where they become degraded into peptides by proteases and are loaded onto the major histocompatibility complex II (MHC II) receptor which is then translocated to the surface of the cells [53–56]. They will interact mainly with CD4+ T helper cells, resulting in cytokine

release which regulates the immune response. In contrast, MHC-I presentation occurs when antigens are located in the cytosol. The cytosolic antigens are degraded into peptides by the proteasome and are translocated into the lumen of the endoplasmic reticulum (ER). There, the resulting peptides interact with the MHC I receptor which is then translocated to the surface of the cell. Through MHC I presentation of peptides to cytotoxic CD8+ T-cells a cellular response is triggered of the immune system against the antigen. Non-infected APCs may also obtain exogenous antigens by phagocytosis but still present them via the MHC I receptor through a mechanism known as cross-presentation, so as to ensure a more complete immune response [57–59]. For further details about the mechanism of cross-presentation we refer the readers to another excellent review [58]. The activation of the cytotoxic $CD8^+$ T cells via the MHC I complex is especially important to initiate a cytotoxic $CD8^+$ T cell mediated response against infections or tumors.

Since non-activated APCs have an inhibitory effect on the T cell response, APCs first have to become activated to provide T cells with a second co-stimulatory signal during the process of antigen presentation [60,61] and to provide APCs with the ability to migrate to the lymph nodes [55,56]. The activation of APCs is initiated upon encountering and recognizing pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) through endosomal or cytosolic pattern recognition receptors (PRRs) [62,63]. Several conserved PAMPs and DAMPs are specifically recognized by both cells via PRRs such as the toll-like receptors (TLRs). For instance, in case of endosomal PRRs, dsRNA is recognized by TLR3, lipopolysaccharide (LPS) is recognized by the TLR4, flagellin is recognized by TLR5 and unmethylated CpG DNA is recognized by TLR9 [50,64–66]. In case that DAMPs or PAMPs are present in the cytosol, recognition occurs via other kinds of cytosolic PRRs which can trigger different inflammatory responses. For example, dsRNA is recognized by RIG-I or MDA5, flagellin is recognized by NAIP5/NLRC4 and dsDNA can be recognized by AIM2,

IFI16, or DDX41 [67–69]. This then leads to the activation of a specific signaling pathway which in turn leads to the expression of several inflammatory cytokines [63]. Interestingly, several cytosolic PRRs have the ability to trigger the activation of an inflammasome response which eventually can lead to a certain kind of cell-death known as 'pyroptosis' [70–72]. For a more detailed overview of how inflammasome activation can shape the adaptive immune system, we refer the reader to another review [70]. Finally, upon migration to the lymph nodes, the processed foreign antigens can be presented as peptides by either the MHC II or MHC I complex to respectively 'naïve' $CD4^+$ T cells or 'naïve' $CD8^+$ T cells [51,53,54]. Despite the similar function of antigen presentation for both DCs and Mφs, there are still some differences which makes the DC a more specialized form of APC which will be discussed below.

DCs were first discovered in 1973 by Ralph Steinmann who laid the foundation of the overlap between the innate and adaptive immune system [73]. In their immature state (iDCs) they act as sentinels throughout the body who are able to recognize and capture antigens via phagocytosis. In the activated mature state (mDCs) a shift in both functional and phenotypic properties is observed as they start to migrate to the lymph node and interact with the T-cells from the adaptive immune system [74]. Mφs, on the other hand, were already discovered in 1883 by Elie Methnikoff who uncovered the basic principles of the host defense mechanism against microbial invaders [75]. Over the years, it has become clear that Mφs act as sentinels as well and are the first line defense mechanism of the human host. Based on their activation state, Mφs can be further divided into two subtypes of respectively the M1 Mφ and the M2 Mφ [76–78,79,p.2]. M1 Mφs are characterized by pro-inflammatory properties by responding to pathogen or danger signals. In contrast, M2 Mφs are more immunosuppressive and participate in remodeling of the tissue environment after inflammation, thus controlling tissue homeostasis [76,77]. In response to a foreign signal, IFN-γ is one the early initiators of Mφ polarization to one of both phenotypes and is responsible for the antimicrobial and antitumor response.

Moreover, IFN-γ also functions as an activator for several genes which are responsible for antigen processing and presentation [80,81]. One of the properties that M1 Mφs gain is the ability to directly eliminate pathogens or cancer cells via phagocytosis [80,82,83]. Polarization to the M1 Mφ phenotype can be further enhanced through the production of IFN-γ by activated T-cells [84]. Furthermore, M1 Mφs have the ability to produce a large amount of nitric oxide (NO) which is important in the early battle against infections or tumors as it has cytotoxic properties [56,76,79,84].

Interestingly, compared to Mφs, mDCs migrate to the lymph nodes to a much higher extent where they can provide 'naïve' non-primed T-cells with a second co-stimulatory signal (first signal is the interaction between MHC-antigen complex and T cell receptor). Moreover, DC presentation is much more robust and more efficient compared to Mφ presentation [51,55,56]. For instance, it has been shown that a 10-fold higher amount of antigens is required for macrophages to prime naïve T cells at the same extent as DCs [51]. Additionally, DCs have a much slower degradation of internalized antigen into peptides than Mφs, ensuring that the antigen presentation can last over a longer period of time [85,86]. A slower degradation also increases the efficiency of cross-presentation as there is for instance more time left for the antigen to transfer to the cytosol [85,87]. For these and several other reasons, DCs could be considered as the most professional APCs to elicit a robust primary immune response [52,76,88,89].

Next, the use of both cell types in the application of cancer immunotherapy will briefly be discussed.

3. Cell-based cancer immunotherapy

3.1. Dendritic cell based cancer immunotherapy

Cancer immunotherapy aims to boost the patient's own immune system in order to specifically recognize and kill tumor cells. This is possible because cancer cells develop both autologous and non-autologous antigens through several mutations. The shared antigens are also known as tumor associated antigens (TAAs) and the non-shared antigens are termed as neoantigens. In contrast to TAAs, which are expressed at a higher extent in tumor tissue compared to normal tissue, neoantigens have a much stronger immunogenicity and stronger affinity to MHC receptors [90]. In DC based immunotherapy, DCs are first modified and loaded *ex vivo* with a tumor antigen and then injected into the patients as such that an immune response against the tumor is initiated. Traditionally, TAAs were used as epitopes for the presentation to 'naïve' T cells [91]. However, recent reports have shown that neo-antigen based vaccination approaches can increase clinical efficacy as they can while they also provide a more personalized and precise immunotherapeutic DC vaccine. In addition, this approach should result in a much lower risk of autoimmunity and an enhancement of the anti-tumorigenicity [90,92].

In cancer immunotherapy, DCs, considering their property to elicit a robust immune response, have been the target of choice for the development of a cancer vaccine [93]. Both *in vivo* and *ex vivo* engineering of DCs has been used in the development of a cancer vaccine [94,95]. However, in this review, only *ex vivo* engineering will be considered as this has the advantage that modifications can be precisely and carefully controlled and several clinical studies have demonstrated the efficacy and safety of *ex vivo* engineered DC-based immunotherapy [96–99]. Basically, for human trials, human iDCs are first differentiated *ex vivo* from autologous monocytes by an appropriate cytokine cocktail mostly consisting of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4

[100,101]. In contrast, for pre-clinical experiments, mice derived iDCs can be differentiated from bone-marrow derived progenitor cells by an appropriate cytokine cocktail [102,103]. DCs are subsequently loaded with tumor antigens or tumor antigen encoding constructs either by simple incubation and subsequent phagocytic uptake, or by the use of different cytosolic delivery technologies. The tumor antigen can be administered as respectively a peptide, a tumor lysate, as well as mRNA or DNA encoding for the antigen [104]. The choice of TAA mainly depends on the available information of tumor antigen expression by the tumor. Indeed, in case the antigen is defined, one could opt to use antigens such as gp100, tyrosinase or CEA [104,105]. However, the use of mRNA or pDNA has several advantages over the use of peptides and proteins [99,106]. Gene constructs can be produced more easily and ensure a sustained antigen presentation. Nowadays, mRNA is generally preferred over pDNA as it does not pose the risk of the integration into the genome and does not require translocation across the nuclear envelope. An interesting feature of genetic constructs is also that multiple tumor antigenic epitopes can be included at once [30,99,107]. Whole tumor lysates and total RNA from autologous tumors also have been used to target multiple undefined antigens [104,105,108]. However, the latter also depends on the availability of sufficient quantities of the tumor tissue for further processing.

Pro-inflammatory cytokines or TLR ligands can be used as adjuvants to achieve maturation of iDCs [109–111]. During the maturation process, several receptors such as the chemokine receptor 7 (CCR7), which is involved in DC migration towards the lymph nodes, and several costimulatory receptors such as CD40, CD80 and CD86 become upregulated. Finally, the therapeutic vaccine with engineered mDCs can be reinjected into the patients via various administration routes [93,95,112–115].

3.2. Redirecting the phagocytic function of macrophages and stimulating the adaptive immune system with a CAR

Another approach that has been considered for cell based cancer immunotherapy is the use of M1 Mφs for the direct killing of cancer cells considering their phagocytic capacity and cytotoxic properties due to the production of the cytotoxic NO. First, monocytes are collected and further differentiated into Mφs. Next, the Mφs are polarized into the cytotoxic M1 Mφs via an IFN-γ activation protocol and intravenously re-injected into the patient. After homing to the tumor region, the M1 Mφs should have the ability to destroy the cancer cells. Despite the fact that several clinical trials have shown a good safety profile, clinical efficacy has been lacking [82,116–118]. In a very recent approach, a chimeric antigen receptor (CAR) was introduced into Mφs with the purpose to redirect their phagocytic capacity towards tumor cells [14,119]. Furthermore, Klichinsky et al. introduced a CAR into macrophages to enable specific antigen recognition of (Her-2) tumor cells and stimulate adaptive responses [6]. These CAR-macrophages were able to selectively phagocytose the antigen expressing tumor cells that they were designed to target. Moreover, *in vitro* experiments showed that CAR M1 Mφs had the ability to polarize the bystander M2 Mφs into pro-inflammatory M1 Mφs. CAR M1 Mφs were able to activate and mature DCs as well as recruit and activate T-cells which resulted in CAR-Mφs and T-cell synergy *in vivo*.

3.3. Electroporation

Basically all cells have a resting electric potential over the plasma membrane which is also called the resting transmembrane voltage (TMV). In electroporation, an electric field is applied

which changes the TMV. Above a certain cell-type dependent threshold, the plasma membrane undergoes both structural and chemical changes which result in the permeabilization of the membrane [120,121]. It is believed that first water molecules start to penetrate through the lipid bilayer (**Figure 2A**) which eventually leads to the formation of water channels (**Figure 2B**). The hydrophilic head groups of the lipid bilayer then start to point towards the water channels (**Figure 2C**), finally leading to the formation of transient hydrophilic pores (**Figure 2D**). While smaller molecules can enter by diffusion, entry of large charged molecules mostly rely on directed electrophoretic mobility. This is why very large multicharged molecules such as pDNA require mostly a long pulse duration in the order of milliseconds [121]. At the end of the exposure of the electric field, the created hydrophilic pores start to reseal again in the order of seconds to minutes. Under moderate conditions electroporation can thus be a reversible process [120–123].

3.3.1. Electropration of Dendritic cells

3.3.1.1. Transfection efficiency and viability

About 25 years ago, several reports on electroporation of human primary DCs for the delivery of pDNA started to emerge. pDNA encoding for the enhanced green fluorescent protein (eGFP) was used to quantify the transfection efficiency (TE), defined as the percentage of cells with a detectable eGFP signal. Van Tendeloo et al. initially optimized the conditions to perform electroporation of $CD34^+$ progenitor cell-derived DCs (PC-DCs), Langerhans cells (PC-LCs) and monocyte derived DCs (Mo-DCs) [31]. The first results were rather disappointing as only TEs of 12%, 16% and 1% were measured 24h after treatment for respectively PC-DCs, PC-LCs and Mo-DCs. Worryingly, the TE's further dropped to 1% after

96h, likely due to electroporation being a fairly harsh process since cell viabilities were respectively 55%, 60% and 40% as measured via flow cytometry with ethidium bromide staining. Another study was conducted by Strobe et al [32] where pDNA was delivered into immature Mo-DCs (iMo-DCs). After 48 hours again a rather low TE of 6.2% was achieved. Sæbøe-Larssen et al [124] transfected human DCs with pDNA, reaching an efficiency of 15% and a viability of 50%, even though the type of viability assay was not mentioned. Later on several other studies [125–127] made use of nucleofection (i.e. a proprietary form of electroporation marketed by Lonza) for the delivery of pDNA in iDCs. Compared to previous studies remarkably high efficiencies of around 50% could be achieved after 24 and 48 hours [125,126]. However, this was accompanied by a significant drop in viability [125–127] which event went down to around 10% or even less [126,127] as measured by flow cytometry [125–127].

Apart from pDNA, electroporation of mRNA has been tried as well because it offers a better safety profile, as already mentioned above. However, unmodified mRNA is prone to enzymatic degradation and can elicit a strong immune response through recognition by PRRs [30,128,129]. This is why chemical modifications are applied to mRNA nowadays, such as the inclusion of a long poly(A) tail and the use of chemically modified nucleosides [30,130]. Strobe et al [32] was one of the first to deliver mRNA into iMo-DCs via electroporation. While the first experiments resulted in a quite low TE of 7.6%, the authors showed that mRNA transfected DCs were way more efficient in inducing the proliferation of antigen specific T-cells compared to pDNA transfected DCs. Later on, Grünebach F et al [131] delivered mRNA into iMo-DCs with slight modifications in the electroporation protocol. The TE could be increased up to 29% with a viability of more than 80%, although the exact viability assay that was used, was not reported by the authors.

Van Tendeloo et al. [35] also performed mRNA delivery into three subtypes of human DCs (i.e. 34-DCs, 34-LCs and iMo-DCs). In contrast to their first results with pDNA, eGFP-mRNA could be delivered into 34-DCs, 34-LCs and iMo-DCs with a TE of respectively 73%, 50% and 63% with a cell viability above 80% as measured by flow cytometry. They observed that eGFP expression could last up to at least 5 days (31% positive cells, Mo-DCs). Surprisingly, the delivery of mRNA in matured Mo-DCs (mMo-DCs) was substantially lower (TE of 33%) compared to iMo-DCs (63%).

The electroporation protocol for the delivery of mRNA into both iMo-DCs and mMo-DCs was further studied by several other groups as well (**Table 1**) [40,125,132–134]. For iMo-DCs, this resulted in TEs of 70% or more with viabilities of more than 80% as measured with flow cytometry using propidium iodide (PI) or ethidium bromide (EB) staining. Several other studies observed even better efficiencies between 80% to 95% with viabilities ranging from 50% to 90% measured via flow cytometry in the majority of the studies (**Table 1**) [124,135–139]. Interestingly, in contrast to the above mentioned study of Van Tendeloo et al. [35], the TE for mMo-DCs did not differ much from the iMo-DCs [132,135–137,139,140]. Other studies paid particular attention to the duration of eGFP-mRNA expression considering that DC migration takes place between 24 and 48 hours after injection [38,112,115]. Michiels et al. [135] showed that eGFP expression for the transfection with mRNA could be followed up to 5 days after transfection at which point still 76% and 65% of cells had a detectable eGFP signal for respectively iDCs and mDCs. Landi et al. in turn showed that electroporated iDCs which were transfected with mRNA still had a TE of more than 60% after 3 days with a viability of more than 80% [125]. Met et al. [139] achieved even a mRNA TE of around 90% which remained stable up to 3 days after electroporation. However, after 4 and 5 days, they noted that the percentage of positive cells dropped to respectively around 50% and 30%. Interestingly,

contrary to previous studies they found that electroporation of mDCs resulted in a much higher viability and eGFP expression level compared to iDCs.

It is important to note that in the majority of electroporation studies cell viability is quantified by flow cytometry using live-dead stainings such as EB, 7-amino-actinomycin D (7-AAD) or PI. The apparent viability percentages quantified this way are relative to the number of intact cells, since cells that are potentially lysed during the electroporation process are no longer detectable. Indeed, Javorovic M et al [136] found that at least 50% of the cells were completely lost after electroporation (viability assay not reported) using similar electroporation protocols, a fact which is neglected when reporting flow cytometry based viability results. Similar studies made use of a microscopic analysis of trypan blue staining, which indeed showed a rather existential reduction in cell viability up to 40-80% cell death after electroporation [134,136–138,140–142].

3.3.1.2. Loading DCs with mRNA and siRNA

Electroporation was also used to transfect DCs with mRNA encoding for tumor associated antigens. Both iDCs and mDCs were transfected with mRNA encoding for tumor antigens such as MAGE-3, Melan-A/MART-1, and survivin antigen [35,137]. In addition, several other studies [131,138] showed successful delivery of whole tumor RNA, mRNA or amplified mRNA which resulted in a tumor specific cytotoxic T lymphocyte (CTL) response even at very low gene expression levels. However, in spite of the long lasting eGFP expression reported in previous studies, mDCs transfected with mRNA encoding for an antigen showed a fast decay in the potential to stimulate T cells during the first 48 hours [137,139]. It was found, compared to the more stable eGFP, that the MAGE-3 and MelanA proteins almost completely disappeared

during the first 24 hours, while the survivin antigen could only be detected during the first 6 hours.

Van Meirvenne S et al [40] studied the capacity of electroporated DCs to present antigens via both MHC I and MHC II receptors. To this end, mRNA encoding for the antigen ovalbumin (OVA) was additionally modified with an endosomal targeting sequence, derived from the invariant chain (Ii80), such that both MHC I and MHC II presentation could take place. After electroporation, mDCs, had a much higher potency to induce a CTL response compared to iDCs [132,139]. In addition, DCs transfected with Ii80.tOVA encoding mRNA were administered as a vaccine into mice initiating an excellent *in vivo* tumor-eradicating T cell response [132]. Similar to the study above, Van Nuffel et al [143] performed the delivery of mRNA encoding tumor associated antigens linked to an MHC II targeting sequence into DCs via electroporation. The transfected DCs were clearly able to perform a stimulation of both antigen specific $CD4^+$ and $CD8^+$ T cells in several melanoma patients.

Upon maturation of iDCs with pro-inflammatory cytokines or TLR ligands, an upregulation of PD-L1 and PD-L2 co-inhibitory molecules is observed which can provide T-cells with an inhibitory signal [113,140,144]. Therefore, it could be of interest to additionally combine DC modification with the delivery of siRNA against PD-L1 and PD-L2 [113,144]. Several groups have performed the delivery of siRNA molecules into DCs via electroporation. To be able to quantify the amount of transfected cells, siRNA was labeled with fluorescent dyes. siRNA could be delivered into iMo-DCs, mMo-DCs and iBM-DC with an efficiency of more than 70% (**Table 1**) [145,146], resulting in efficient gene silencing. These studies additionally reported that the delivery of siRNA via electroporation did not induce any changes in morphology and functionality of the cells [145,146]. Also Hobo et al [113] successfully delivered siRNA via electroporation and showed that both PD-L1 and PD-L2 could be silenced for up to 5 days.

3.3.1.3. Functional changes after electroporation

DCs transfected with tumor antigens should be able to migrate to the lymph nodes where priming of naïve T cells can occur. Therefore, it is of utmost importance that the physical delivery method does not induce any functional changes to the treated cells. Several studies showed that electroporation did not induce any change in cellular morphology, the expression of surface molecules, the maturation capacity or the ability of mDCs to stimulate a T cell response [32,40,116,132,138,139]. In contrast, other studies did find slight changes in maturation of iDCs after electroporation [126,135,141]. Lenz et al [126] showed that nucleofection of iDCs caused a downregulation of CD80 expression and the nucleofected iDCs failed to undergo maturation upon the treatment with LPS. Michiels et al [135] observed that iDCs which were first electroporated and then matured, showed a lower maturation status compared to mDCs which were electroporated as mDCs. Chung DJ et al [141] showed that electroporated DCs and LCs have an altered expression level of maturation markers such as CD80, CD83 and CD86 receptors. Moreover, the migration capacity of post-electroporated mDCs seemed to be significantly affected [135,147]. Based on these contradictory results, a more thorough study on the functional effects of DCs, with standardized electroporation settings should be performed. Indeed, as the transfection technique shouldn't change the functionality of your cells, it would be an interesting option to first transfect iDCs prior to the maturation step. In this way, deviations on the maturation potential would probably allow to foresee functional changes due to the treatment.

As the secretion of cytokines drives the polarization of the T-cell response (e.g. sufficient amounts of IL-12 drives a type 1-polarized response), it is very important to evaluate cytokine release of electroporated DCs as well [140,148]. Lenz et al [126] observed that, after LPS treatment of nucleofected iDCs, the secretion of several cytokines (i.e. IL-1β, TNFα and IL-6)

was decreased while IL-12 was augmented. Several other studies [15,125,134] showed that electroporated DCs had a much lower secretion of IL-12 compared to non-electroporated DCs. Dullaers et al [15] observed a 10-fold decrease in the secretion of IL-12p70 by electroporated mDCs compared to non-electroporated mDCs which also resulted in a reduced *in vivo* immune response. This negative effect on the secretion of cytokines was also observed by Van Meirvenne et al [132] for both electroporated iMo-DCs and mMo-DCs. Indeed, electroporated DCs showed a significant decrease in the secretion of several important cytokines such as IL-12p70, IL-6, IFN-α, IFN-ϒ and tumor necrosis factor (TNF)-α.

The latter observations indicate that electroporation potentially has a big effect on the gene expression. This is in line with other recent studies on the impact of electroporation on the morphology, functionality and transcriptional activity of other kinds of primary human cells (i.e. T cells and hematopoietic stem cells). The studies clearly demonstrated several functional changes on respectively the morphology, expression of surface receptors and cytokine release [149,150]. Interestingly, despite that the viability of primary cells was not significantly affected, it could be seen that electroporation was associated with a dramatic dysregulation of functional pathways and, more importantly, the gene expression. Moreover, they observed that electroporated T cells did show a reduced *in vivo* functional activity [150]. These effects could somehow be expected as the cell's DNA and other components are also exposed to the applied electric fields. Taken together, it is our opinion that a more thorough analysis at the genomic level should be performed for DCs as well to have a better understanding of which important genes could be affected by the electroporation procedure.

3.3.2. Electroporation of macrophages

One of the first reports on electroporation of macrophages was published about 30 years ago. Stacey et al. [48] performed electroporation of primary bone marrow derived macrophages (BMDMs) for the delivery of pDNA. Although the TE was not reported, it did provide a first indication of cell viability. Whereas electroporation without pDNA yielded a viability of around 80% according to an MTT assay, electroporation in the presence of different constructs of pDNA showed a remarkable dose dependent decrease of cell viability. In addition, this effect was also observed when electroporation was used to deliver poly (I):poly (C) dsRNA into the cytosol. As already mentioned in the general introduction of this review, it has become clear that the cytosolic presence of dsDNA or dsRNA can result in PRRs triggering and activation of the inflammasome complex, eventually leading to pyroptotic cell death [25,151,152,153,p.2]. This mechanism could be a potential explanation of the high cell death rates that were reported after cytosolic pDNA delivery in both macrophages and DCs. Even though RNA molecules can also trigger an immune response by themselves, this can however be reduced by making use of chemically modified siRNA or mRNA [30,154,155]. Therefore, both siRNA and mRNA can be used for the delivery into respectively macrophages and DCs for cell based therapies. However, it is not known whether the immune response could be triggered by a dose dependent effect.

The delivery of mRNA into primary macrophages has not been studied to the same extent as for primary DCs. Indeed, the majority of macrophage studies focused on the delivery of siRNA in order to study their immunobiology. Wiese M et al [156] showed that electroporation of fluorescently labeled siRNA was feasible in BMDMs with an efficiency of more than 95%. The resulting viability was around 90 to 100% as measured with both MTT and trypan blue exclusion assays. Neither mock electroporation nor siRNA electroporated BMDMs showed any change in respectively the morphology, functionality, production of inducible nitric oxide synthase (iNOS) and antimicrobial activity. In another study Jensen et al. [29] performed the

delivery of fluorescently labeled siRNA into primary bovine monocyte-derived macrophages (Mo-DMs) via electroporation and obtained a markedly lower efficiency of around 55%. This was accompanied by a viability of around 80% as measured with flow cytometry by staining the dead cells with a Sytox dye. Another study [157] made use of the lactate dehydrogenase (LDH) cytotoxicity assay to quantify the viability of mock electroporated BMDMs, arriving at a cell viability of about 70% after electroporation.

Wiese et al. [156] showed that electroporation of macrophages did not impair the production of NO or the iNOS expression. Moreover, no changes were observed on the secretion of TNF or IL-6 when the electroporated cells were exposed to LPS. Most importantly, the antimicrobial capacity of electroporated macrophages was similar to the untreated macrophages. However, based on the sometimes conflicting results for DCs, more thorough studies should be performed for macrophages as well.

3.4. Mechanically induced poration

An interesting alternative to more locally induce the formation of pores could be the use of mechanical forces that act specifically at the level of the cell membrane. This in contrast with electroporation, where the whole cell is exposed to the electric field, which may contribute to the adverse effects seen on the functionality of DCs and other immune cells, as discussed above. Below we will discuss physical delivery technologies that mechanically increase cell membrane permeability and their results on macrophages or DCs.

3.4.1. Sonoporation

Sonoporation makes use of ultrasound as a physical trigger, often in combination with microbubbles. Microbubbles are gas-filled micron sized structures which are usually stabilized by a polymeric or lipid shell. Under ultrasound stimulation, microbubbles will start to compress and expand as a result of the high and low pressure phases of the ultrasound wave. Depending on the amplitude of the ultrasound wave, the bubbles can undergo stable cavitation (low amplitude) or inertial cavitation (high amplitude). These two types of cavitation can have different biophysical effects at the level of the plasma membrane. Indeed, stable cavitation can lead to the transient formation of small pores through (1) expansion or compression of the microbubble near the cell membrane (**Figure 3i)**, (2) pressure gradients displacing and pushing the bubble through the membrane (**Figure 3ii)** or (3) microstreaming that results in a mechanical shear stress on the membrane (**Figure 3iii**). Inertial cavitation instead can form pores through the collapse of the microbubble, resulting in the creation of an acoustic shock wave (**Figure 3iv)** or a liquid jet towards the membrane (**Figure 3v)**. In contrast to stable cavitation, inertial cavitation has the ability to generate much larger pores and can do so from a larger distance away from the cell membrane [158]. For a more detailed description of these biophysical effects the interested reader is referred to this review [159].

3.4.1.1. Dendritic cells

One of the first reports on *ex vivo* DC sonoporation came from the group of Suzuki et al. [160]. In their study, ovalbumin was delivered into DCs through the use of lipid stabilized bubbles, and ultrasound. The delivery efficiency was assessed by measuring the uptake of fluorescently labelled ovalbumin with confocal microscopy. Successful cytosolic delivery could be observed as a diffuse fluorescent pattern while endocytic uptake resulted in the appearance of dots due to the labeled protein being trapped in endosomes. The sonoporation

treatment appeared to be relatively gentle to the cells as more than 80% of the cells were still viable according to an MTT assay. In addition, they showed that ovalbumin delivered into the cytosol resulted in MHC class I presentation to $CD8^+$ T cells, leading to a strong *in vivo* CTL response.

The transfection of iBM-DCs with mRNA via sonoporation was performed for the first time almost 10 years ago [161]. In this study mRNA was loaded onto the microbubbles to minimize degradation and to lower the required concentration of mRNA. This was done by first complexing mRNA with cationic liposomes which were then bound to the surface of microbubbles through an avidin-biotin coupling (**Figure 3B)**. Following sonoporation, fluorescently labeled mRNA lipopexes could be delivered to 58% of the cells [161]. However, the eGFP expression of mRNA was rather low with a TE of 18-24%, probably due to incomplete mRNA release from the liposomes [161,162]. On the upside, the sonoporation treatment resulted in a viability of around 84% as measured with flow cytometry using a Sytox dye [161]. However, a slight increase in the expression of the CD40 and CD86 co-stimulatory surface receptors could be observed when mRNA was sonoporated into the iBM-DCs [161,162], which could be the result of the sonoporation process as such or could be induced by an innate immune response to the cationic lipids which were used to form the lipoplexes [162]. The main advantage of ultrasound and microbubbles as a physical delivery technique is their potential to allow in vivo transfections as both, ultrasound and microbubbles are widely used for contrast imaging in the clinic and several microbubble formulations are available for clinical use. However, further research is required to elucidate the different mechanisms underlying sonoporation in order to optimize the delivery efficiency. In particular the use of monodisperse microbubbles could therefore be essential as this would allow to match microbubbles size and ultrasound frequency which could lead to more controlled microbubble cavitation regimes.

3.4.1.2. Macrophages

Lemon JCM et al [163] were the only group who used sonoporation for the *ex vivo* transfection of primary macrophages. They made use of an approach referred to as microbubble-mediated intra-phagosomal sonoporation (MIPS) for the cytosolic delivery of pDNA. MIPS differs from the sonoporation methods described above in that microbubbles were first engulfed by the macrophages before applying ultrasound stimulation. To this end, microbubbles coated with a cationic lipid layer and pDNA, were opsonized with an immunoglobulin (IgG) antibody to increase internalization into the phagolysosomes of the macrophages. After phagocytic uptake of the pDNA loaded microbubbles, ultrasound was applied to induce poration of the phagosomal membrane which led to the cytosolic delivery of pDNA. However, this procedure only resulted in a rather poor TE of 2% to 8% and was accompanied with a strong drop in viability. Indeed, for a TE of 5 % the viability decreased to around 40%. Likely the biophysical effects of these microbubbles cause too much damage when generated from within the cells.

An advantage of sonoporation is that microbubbles have been approved for clinical use and that ultrasound doesn't induce any harmful biological effects. However, the inertial cavitation of such big microbubbles in the proximity of the cells could potentially cause an irreversible pore formation and hence lead to an increased cell death. Therefore, as mentioned above, the use of different viability assays will be necessary to determine the immediate and long-term viability effects.

3.4.2. NanoWire induced permeabilization

The use of NanoWires (NWs) has been recently introduced as a form of mechanical poration of a variety of cell types [164–167]. NWs are vertical structures composed, for instance, of silicon, gallium phosphide or Al_2O_3 and which are engrafted on a substrate (**Figure 4A**). The process starts with the sedimentation of the cells on top of the substrate. Next, the cells adhere on the substrate which is thought to result in plasma membrane penetration at the tips of the nanowires [168,169]. For cytosolic delivery, macromolecules such as proteins, siRNA and pDNA can be bound non-covalently to those tips (via an electrostatic or van der Waals interaction) so that it can slowly be released into the cytosol (**Figure 4B**). Indeed, through this method a variety of macromolecules such as peptides, proteins, siRNA and pDNA have been introduced into the cytosol [166,167,170]. The efficiency and viability can be carefully controlled by varying the length, thickness and the density of the NWs.

3.4.2.1. NanoWire induced permeabilization of dendritic cells and macrophages

The first use of NWs for the delivery of various compounds into primary DCs (**Figure 4C**) and macrophages (**Figure 4C**) has been performed by Shalek et al. [167]. In their study, silicon NWs (siNWs) were first functionalized with a layer of aminosilane which was then precoated with biomolecules of interest via a non-specific electrostatic or van der Waals interaction. DCs and macrophages required the use relative short siNWs of 1-2 µm at a density of 0.15-0.2 per µm². The biomolecules (pDNA, siRNA, proteins and peptides) were fluorescently labeled such that the cytosolic delivery could be assessed by confocal microscopy. The authors reported that basically all cells were positive for the different macromolecules without any influence on viability. The viability for the NWs alone or the NWs in combination with siRNA was measured with the CellTiter Glo metabolic assay. The viability for the NWs in combination with other

macromolecules such as pDNA, proteins and peptides was assessed by confocal microscopy after performing the live-dead staining. Upon delivery of siRNA into primary DCs and macrophages, a knock-down efficiency between 70-90% could be observed. Furthermore, the authors showed that the siNWs do not trigger an innate immune response nor do they influence the maturation profile or the secretion of cytokines. Moreover, an array of 300 immune response genes were screened showing no dysregulation by siNWs treatment.

These very positive results are at least to some extent surprising, however. As previously mentioned, the cytosolic presence of pDNA into macrophages as well as DCs can compromise cell viability due to the inflammasome response. Since in this study not any influence on the viability was observed nor on the innate immune response, it does raise the question if pDNA was really present in the cytosol. Indeed, other studies did not observe any rupture of the plasma membrane when culturing cells on NWs [171–173]. Instead, the cell membrane seems to wrap around the NWs with only a few long NWs having direct access to the cytosol [172]. Nair et al. [170] attributed the delivery of siRNA to the perturbation of lipids in the plasma membrane which could increase the passage of small molecules such as siRNA. Indeed, as shown in that study, small molecules such as siRNA could reach the cytosol, while in a previous study it was shown that this was not the case for much bigger molecules such as pDNA (<1%) [164]. Taken together it seems that further studies on NWs would be needed to confirm that NWs are suitable for transfecting APCs with large cargo molecules such as pDNA. For instance cytosolic delivery of large molecules such as pDNA and mRNA could be quantified by flow cytometry using eGFP-pDNA or eGFP-mRNA. Another aspect that is of interest to look into is the leaking of important intracellular proteins outside the cells during the time that they are cultured on the nanowires. This could for instance have important consequences on the metabolic activity of the cells. Finally, reactive oxygen species (ROS) induced inflammasome activation should be investigated as it has been shown that NWs have the ability to produce ROS which could result

in DNA damage [174]. Apart from that, NWs certainly could prove useful for intracellular delivery in APCs, especially since siRNA mediated down-regulation of PD-L1 and PD-L2 in DCs has already been shown, as mentioned above.

3.4.3. Microfluidic cell squeezing

Cell squeezing is another method which leads to the permeabilization of the plasma membrane through the use of mechanical energy. Sharei et al. [175] developed a microfluidic device consisting of parallel channels comprising several constrictions sites with a dimension smaller than the diameter of the cells. When cells in suspension flow through the microchannels and are squeezed through the constrictions, it leads to the deformation of their plasma membrane due to the resulting shear forces. This leads to transient permeabilization of the membrane so that external macromolecules can enter the cells (**Figure 5**). An optimal delivery efficiency for a certain cell type can be achieved by varying respectively the fluid speed, the constriction dimensions and geometry, and the number of subsequent constrictions [175–177].

3.4.3.1. Microfluidic cell squeezing of dendritic cells and macrophages

The microfluidic cell squeezing technique has been used for the intracellular delivery of macromolecules into both primary macrophages and DCs [175,176,178]. Fluorescently (FITC) labeled dextran molecules, with a molecular size of respectively 3 kDa (FD3) and 70 kDa (FD70), were used to quantify the delivery efficiency. It was shown that FD3 and FD70 could be delivered with an efficiency of respectively around 35% and 25% into DCs. Macrophages could be labeled with a similar efficiency of about 50% and 30% for respectively FD3 and

FD70 [175]. In a later study [176], the delivery of FD3, FD70 and a fluorescent IgG1 antibody (Ab) into DCs was further enhanced by varying the dimensions of the constrictions, achieving a delivery efficiency of around 60%, 40% and 35% for respectively FD3, FD70 and Ab. For macrophages this led to an optimized efficiency of around 80%, 40% and 35% for respectively FD3, FD70 and Ab. The viability was around 70%, although this was again only quantified by flow cytometry with PI staining. In addition, cell squeezing was successfully used to deliver siRNA into DCs. Interestingly, in comparison to electroporation, microfluidic cell squeezing of primary human cells (i.e. T cells) was shown to have minimal adverse effects on their functional properties, such as the secretion of cytokines [150]. Similarly it had much less effect on the gene expression level as compared to electroporation. Combined with an immense throughput (order of 10^6 cells per second), clearly cell squeezing is a very promising technology for the production of clinically relevant therapeutic batches of cells.

Yet, as any technology it also comes with some drawbacks. For instance, clogging of the microfluidics channels is an annoying practical problem apart from the fact that for every cell type a new device with optimized channel dimensions has to be developed [175]. Recently, technical variations of the cell squeeze concept such as the hydroporation method [179,180] and microfluidic vortex shedding (μVS) [181] have been developed to overcome clogging which is one the major drawbacks of cell squeezing. Interestingly, μVS has also proven to achieve efficient delivery of mRNA in primary human T cells without a huge loss in cell viability [181]. Considering that T cells are notoriously difficult to transfect, it suggests that it may be useful for engineering DCs and macrophages as well.

Table 2 gives a qualitative summary on how the different delivery technologies perform for transfecting APCs.

4. Conclusion

In summary, the delivery of functional molecules *ex vivo* in patient derived DCs and macrophages is an important step towards improved cell based cancer immunotherapy. Physical transfection technologies are gaining interest as they offer excellent control of the delivery process combined with high efficiency and throughput. Electroporation remains the most used technique to date with the ability to deliver a variety of macromolecules, including mRNA, into primary DCs and macrophages. However, recent literature points to adverse effects on the level of cell homeostasis and functionality, including alteration of cytokine secretion, disturbed migration capacity and misexpression of genes. To reduce these unwanted effects that may reduce the cell's therapeutic potential, novel delivery technologies are emerging, such as sonoporation, nanowires and cell squeezing. Contrary to electroporation they only act at the level of the outer cell membrane, which provides them with a better safety profile. Although research is still ongoing, they already have been successfully applied for the cytosolic delivery of macromolecules in DCs and macrophages. Combined with the rise of other new physical transfection technologies which already have shown promising results in other cell types [22], chances are high that modification of DCs and macrophages will be possible with steadily increasing efficiency while preserving cell viability and functionality.

5. Expert opinion

The production of therapeutic APCs for cancer immunotherapy requires the intracellular delivery of macromolecules. It is important that the delivery technology used enables a high delivery rate without impacting on cellular viability or functionality. Unfortunately, however, there is at present no consensus on a standardized manner to report acute cell toxicity. Indeed, the viability percentages which are quantified by flow cytometry are based on live-dead

stainings and are relative to the number of intact cells rather than the entire initial population. This leads to an overestimation of cell viability since lysed cells are no longer detected and are as such not taken into account. Instead, to obtain a complete picture on acute cytotoxicity it is recommended to quantify more precisely the remaining percentage of intact cells, e.g. by cell counting, and to measure the metabolic activity of the remaining cells (MTT, MTS, WST-1, CellTiter Glo). This could be further complemented with quantification of cell death metabolites (LDH assay). Preferably these cell viability assays should be performed as a function of time since the modified cells are expected to be effective for at least several days. Only when the field adopts a standardized set of toxicity assays, at least per cell type, it will remain difficult to compare different delivery technologies in terms of their safety profile.

More recently it has become apparent that measuring acute toxicity alone is not sufficient. In order to obtain highly potent cellular vaccines, it is of equal importance to look into more subtle effects of the treatment on cellular homeostasis and functionality. Indeed, even if acute toxicity can be kept to an acceptable level with state-of-the-art electroporation devices, recent studies have pointed out that the surviving 'viable' cells may suffer from alterations in gene expression, in the expression of surface receptors, in migration capacity and secretion of important cytokines. Therefore, independent of the transfection technology used, in our opinion future studies should not only report viability values but additionally focus on the effects on gene expression and cell functionality before moving a cell therapy product forward to clinical trials. This not only true for DC vaccines, but also applies to CAR macrophages which are relatively novel, but feature some interesting immunological anti-cancer effects. Expression of a CAR receptor requires transfection of macrophages with CAR-mRNA, for which electroporation is the typically used delivery technology. Also here it remains to be investigated to which extent viability and functionality of electroporated macrophages is affected over time.

Of the three alternative intracellular delivery technologies used for DCs or macrophages, being microfluidic cell squeezing, nanowires and sonoporation, microfluidic cell squeezing clearly has progressed the furthest so far. Indeed, it has been shown that this technique has the potential to deliver a variety of macromolecules into both DCs and macrophages without strongly affecting cell viability or functionality. In a collaboration with Roche, cell squeezing is now used in a clinical trial where antigen presenting cells are modified for cancer immunotherapy. But there are other upcoming new physical delivery techniques that may prove valuable as well in the near future.

One example is photoporation [182–184], which makes use of a pulsed laser light source to induce photothermal effects from cell-attached nanoparticles (e.g. AuNPs) leading to enhanced membrane permeability. For instance our group has recently demonstrated that primary T-cells as well as macrophages can efficiently be transfected via photoporation without adverse effects on cell homeostasis or functionality (*submitted*). Clearly technological improvements in delivery technologies are expected to continue to emerge in the coming years, so that we are optimistic that safe engineering of therapeutic cells will become a future reality.

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7. Declaration of interest

‘The authors declare no competing interests’

8. References

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Table legends

Table 1: Overview of electroporation results on DCs and macrophages for the delivery of respectively siRNA, mRNA and pDNA.

Table 2: Comparison between transfection technologies for APCs.

Figure legends

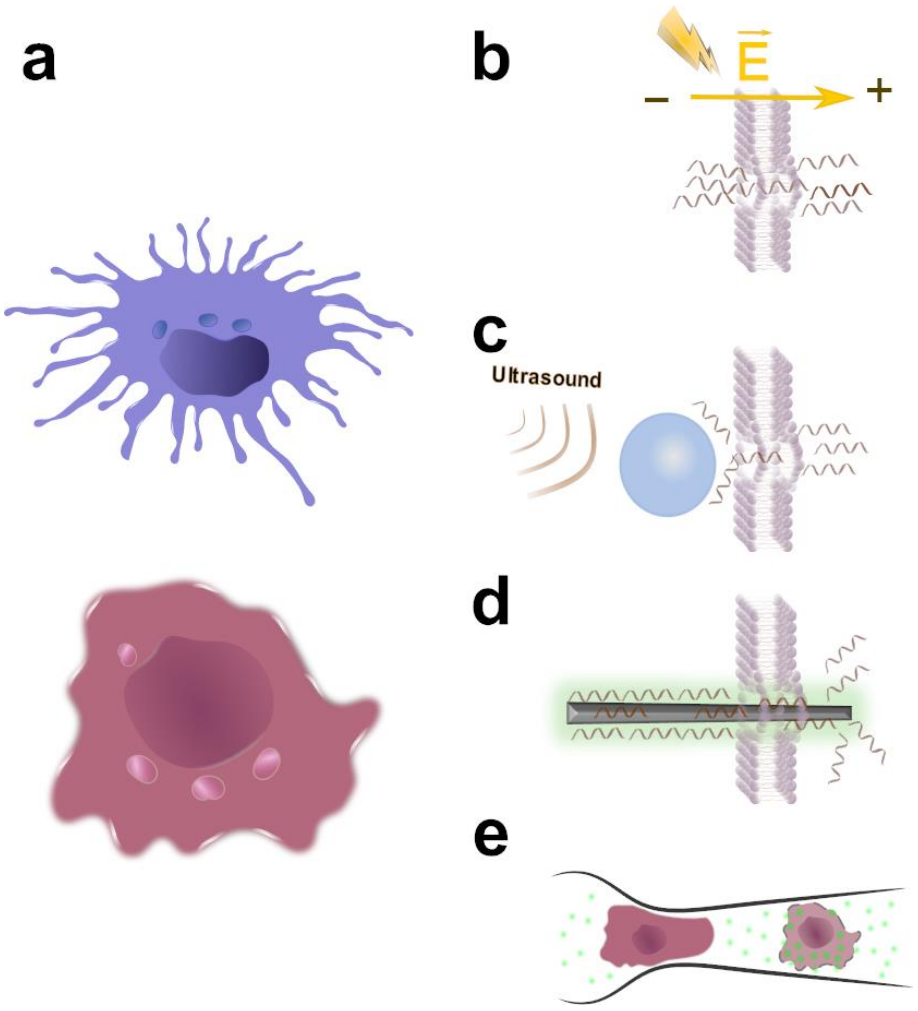

Figure 1: Schematic illustration of the different physical transfection technologies is shown here. (a) Illustration of dendritic cells and macrophages. (b) Electroporation induced

permeabilization of the plasma membrane which allows for the delivery of different macromolecules. (c) Ultrasound mediated permeabilization of the plasma membrane (= sonoporation) which allow for the passage of different macromolecules through the pores. (d) Nanowires, coated with the macromolecules of interest, spontaneously cause a rupture of the plasma membrane which allows for the cytosolic delivery of macromolecules. (e) Macrophages are squeezed through narrow constrictions which transiently permeabilizes the plasma membrane.

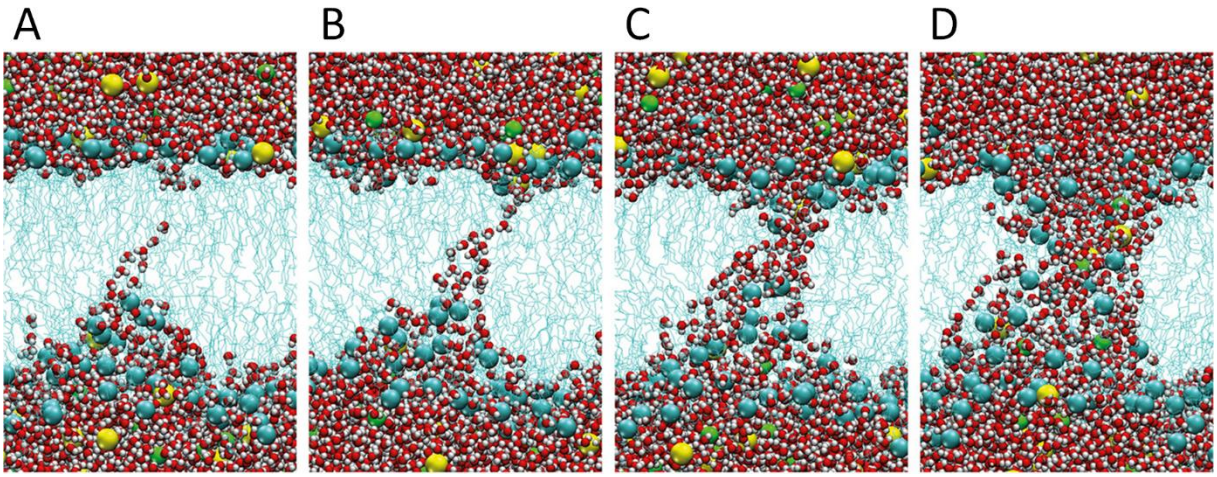

Figure 2. Illustration of electroporation induced pore formation in a simulated (palmitoyl-oleoyl-phosphatidylcholine) POPC bilayer system. A) The water molecules (red and white spheres) penetrate the lipid core (cyan lines). B) Formation of a water file through the bilayer. C) The zwitterionic phospholipids bilayer head groups (cyan spheres) start to point towards the file. D) Further expansion of the hydrophilic pore through which molecules can get through. (Hydrogen: white spheres; Oxygen: red spheres; sodium ions: yellow spheres; chloride ions: green spheres). Reproduced with permission [120]. Copyright 2017, Elsevier.

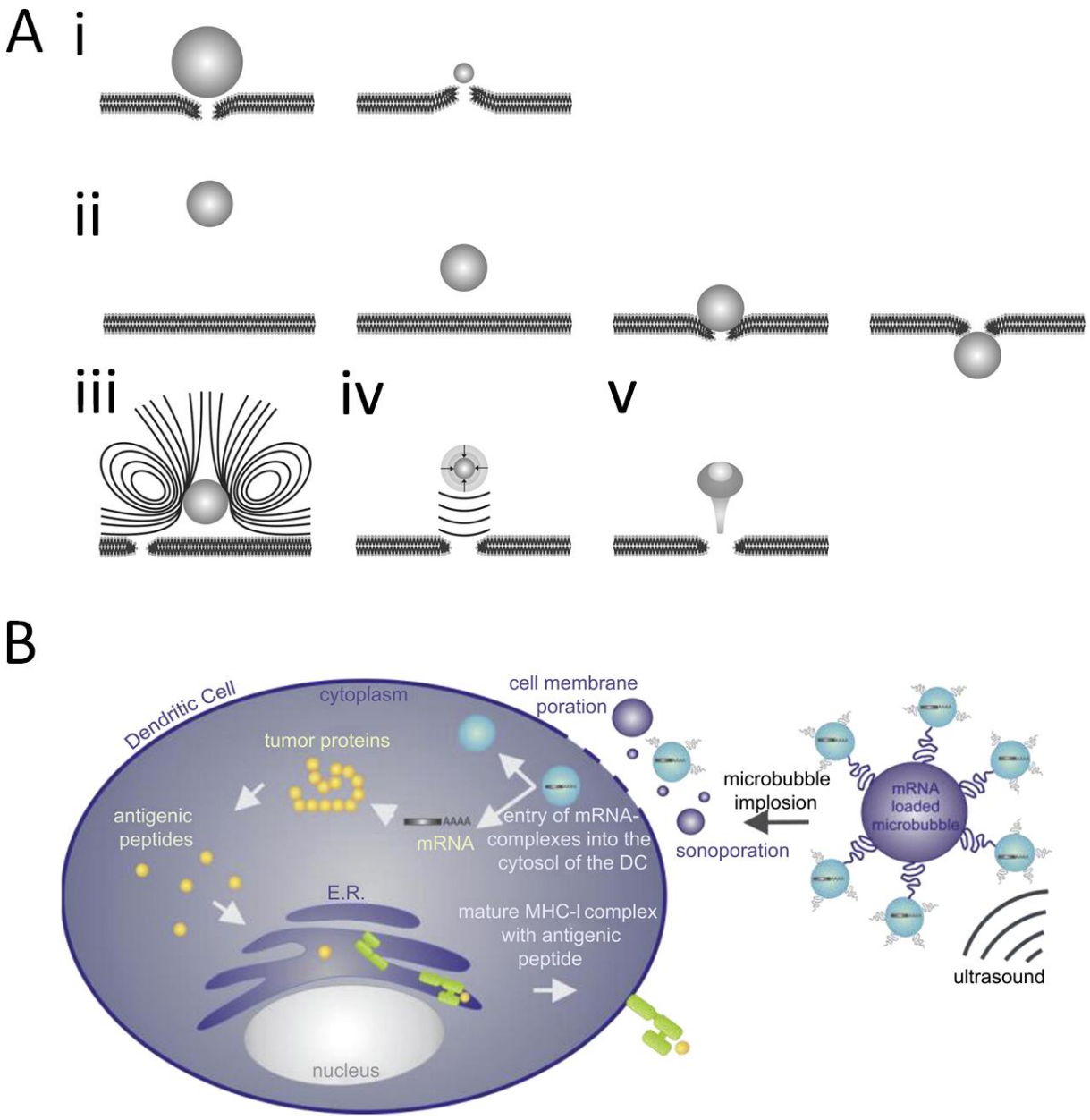

Figure 3. The mechanism of sonoporation and its application for the mRNA delivery into DCs. (A) Pore formation in the plasma membrane through different biophysical mechanisms. i-iii) Stable cavitation can respectively lead to i) expansion or compression of the microbubble, ii) pressure gradients displacing and pushing the bubble through the membrane, iii) microstreaming causing a mechanical shear stress on the membrane. iv-v) Inertial cavitation can lead to iv) the creation of an acoustic shock wave or v) a liquid jet towards the membrane. Reproduced with permission [159]. Copyright 2014 Elsevier Ltd. B) Schematic illustration of sonoporated iDCs for the delivery of mRNA. Upon ultrasound application, bubbles start to

cavitate and can as such of form pores into the membrane of DCs and deliver lipoplexes containing mRNA. Reproduced with permission [161]. Copyright 2011 Elsevier Ltd.

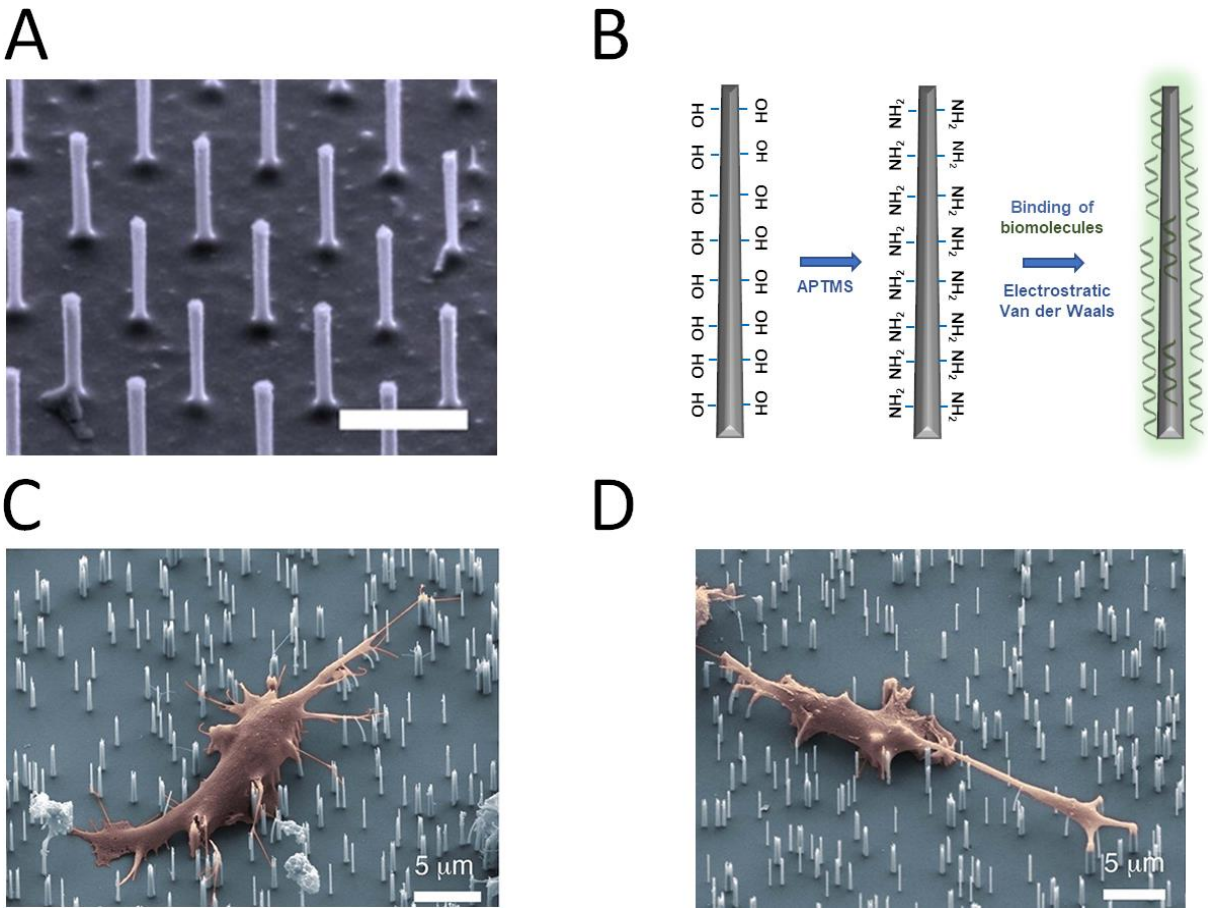

Figure 4. Illustration of siNWs for the delivery of different macromolecules into DCs and macrophages. (A) Scanning electron microscopy (SEM) image of vertical siNWs (light color) on a silicon substrate (dark color). Scale bar = 1 µm. Reproduced with permission [166]. Copyright 2010 PNAS (B) Schematic illustration of siNWs functionalized with 3-aminopropyltrimethoxysilane (APTMS) on which biomolecules are bound on the surface via an electrostatic or van der Waals interaction. (C-D) SEM images of (C) DCs and (D) macrophages seeded on top of the siNWs. Scale bars = 5 µm. Reproduced with permission [167]. Copyright 2012 American Chemical Society.

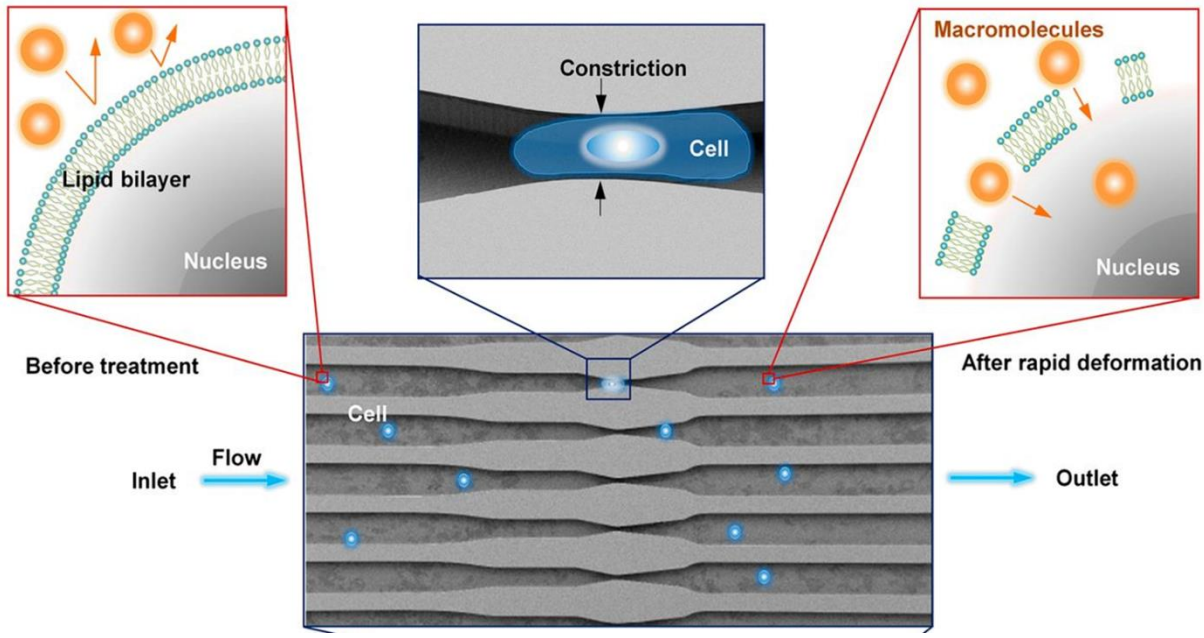

Figure 5. Schematic illustration of the microfluidic cell squeezing method for the delivery of macromolecules. Cells pass through the parallel channels with a certain flow speed. Upon the passage through the narrow constriction, the shear forces deform the cell membrane, leading to increased permeability of the cell membrane and allowing external macromolecules to reach the cytosol. Reproduced with permission [175]. Copyright 2013 PNAS.