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FOCUSSED RESEARCH REVIEW

Optimized dendritic cell-based immunotherapy for melanoma: the TriMix-formula

Sandra Van Lint · Sofie Wilgenhof · Carlo Heirman · Jurgen Corthals · Karine Breckpot · Aude Bonehill · Bart Neyns · Kris Thielemans

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Abstract Since decades, the main goal of tumor immunologists has been to increase the capacity of the immune system to mediate tumor regression. In this regard, one of the major focuses of cancer immunotherapy has been the design of vaccines promoting strong tumor-specific cytotoxic T lymphocyte responses in cancer patients. Here, dendritic cells (DCs) play a pivotal role as they are regarded as nature's adjuvant and as such have become the natural agents for antigen delivery in order to finally elicit strong T cell responses (Villadangos and Schnorrer in Nat Rev Immunol 7:543–555, 2007; Melief in Immunity 29:372–383, 2008; Palucka and Banchereau in Nat Rev Cancer 12:265–277, 2012; Vacchelli et al. in Oncoimmunology 2:e25771, 2013; Galluzzi et al. in Oncoimmunology 1:1111–1134, 2012). Therefore, many investigators are actively pursuing the use of DCs as an efficient way of inducing anticancer immune responses. Nowadays, DCs can be generated at a large scale in closed systems, yielding sufficient numbers of cells for clinical application. In addition, with the identification of tumor-associated antigens, which are either selectively or preferentially expressed by tumors, a whole range of strategies using DCs for immunotherapy have been designed and tested in clinical studies. Despite the evidence that DCs loaded with tumor-associated antigens can elicit immune responses in vivo, clinical responses remained disappointingly low. Therefore, optimization of the cellular product and route of administration was urgently needed. Here, we review the path we have followed in the development of TriMixDC-MEL, a potent DC-based cellular therapy, discussing its development as well as further modifications and applications.

Keywords 19th Danish Cancer Society Symposium 2013 · Dendritic cell · mRNA · Immunotherapy · Cancer

Abbreviations

API	Active pharmaceutical ingredient
caTLR4	Constitutive active form of Toll-like receptor 4
CD40L	CD40 ligand
CTLA-4	Cytotoxic T-Lymphocyte antigen 4
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
DTH	Delayed-type hypersensitivity
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GMP	Good manufacturing practice
HBSS	Hanks balanced salt solution
HLA	Human leukocyte antigen
i.d.	Intradermal

Sandra Van Lint and Sofie Wilgenhof contributed equally to this work.

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S. Van Lint · S. Wilgenhof · C. Heirman · J. Corthals · K. Breckpot · A. Bonehill · K. Thielemans (✉)
Laboratory of Molecular and Cellular Therapy & Dendritic Cell-bank, Vrije Universiteit Brussel, Laarbeeklaan 103E, 1090 Brussels, Belgium
e-mail: Kris.Thielemans@vub.ac.be

S. Van Lint
e-mail: sandra.van.lint@vub.ac.be

S. Wilgenhof · B. Neyns · K. Thielemans
Department of Medical Oncology, University Hospital Brussel (UZ Brussel), Brussels, Belgium

i.n.	Intranodal
i.v.	Intravenous
mAbs	Monoclonal antibodies
MP	Medicinal product
NGS	Next-generation sequencing
SKILs	Skin-infiltrating lymphocytes
T_H1	T helper 1
TLR	Toll-like receptor
Treg	Regulatory T cell
TriMixDC-MEL	TriMixDCs co-electroporated with mRNA encoding a fusion of DC-LAMP and one of four melanoma-associated antigens: gp100, tyrosinase, MAGE-A3 and MAGE-C2

Optimization of an ex vivo DC-based immunotherapy

Many of today's DC therapies rely on the use of ex vivo-modified autologous dendritic cells (DCs) that are re-administered into cancer patients. However, the development of strategies to increase the potency of DC therapy is needed. To further optimize this approach, several parameters can be scrutinized including the exploited DC subtype, as well as route, dose and frequency of DC-therapy administration. Moreover, a critical factor determining the efficacy of DC-based immunotherapy and its ability to reverse established T cell tolerance to tumor antigens and the induction of potent antitumor T cells is the activation or maturation status of DCs [6–11]. Consequently, much effort was put in the identification of strong activation stimuli that generate fully mature DCs not only by addition of inflammatory cytokines [12] or Toll-like receptors (TLRs) [9, 13] to the in vitro cultures but also by maturing DCs in vivo [14–17]. However, nowadays much effort is put into approaches which have the advantage of inducing maturation 'from within' [18, 19]. In this regard, Calderhead et al. [18] compared four different protocols for the maturation of DCs electroporated with antigen mRNA, including maturation using co-electroporation with mRNA encoding CD40L. Although they did not observe significant differences in the expression of co-stimulatory molecules nor in the expression of major histocompatibility complexes (MHC) comparing the four different maturation protocols, there was an induction of a potent pro-inflammatory cytotoxic T lymphocyte (CTL) response when DCs were matured by addition of cytokines followed by CD40L mRNA electroporation. We demonstrated maturation and increased T cell stimulatory capacity of DCs when DCs are electroporated with mRNA encoding CD40 ligand (CD40L), CD70 and a constitutive active form of TLR4 (caTLR4), referred to as TriMix [20]. The combination of CD40L and caTLR4 electroporation mimics CD40 ligation [21] and TLR4 triggering [22], respectively, resulting in the generation of phenotypically and functionally mature DCs. Moreover, introduction of CD70 into DCs provides a co-stimulatory signal to $CD27^+$ naive T cells by inhibiting activated T cell apoptosis and by supporting T cell proliferation [23, 24] (Fig. 1). Consequently, there is no need for incubation of the DCs for up to 48 h with soluble maturation signals such as pro-inflammatory cytokines or TLR ligands, actions that can render the DCs exhausted, resulting in DC-based vaccines of inferior capacity [25]. Indeed, electroporation of DCs with TriMix resulted in phenotypically mature, cytokine- and chemokine-secreting DCs that were able to potently activate $CD4^+$ T helper 1 (T_H1) cells. Besides enhanced secretion of the T_H1 cytokine IL-12, several pro-inflammatory cytokines, hematopoietic growth factors, IFN-γ and several chemokines, also an increased secretion of the immunosuppressive cytokine IL-10 by TriMixDCs was observed. However, this enhanced IL-10 secretion did not hamper the induction of T_H1 IFN-γ-secreting T cells. Moreover, the quantities of IL-10 produced by TriMixDCs did not lead to IL-4 or IL-10 secretion by $CD4^+$ T cells in vitro [20].

In a next step, tumor antigen mRNA was co-delivered as a replacement for peptide pulsing [26]. As such, our current DC therapy relies on the ex vivo generation of monocyte-derived DCs and their loading with tumor antigen encoding mRNA, while simultaneously potentiating them with TriMix mRNA (Fig. 2). This 'one-step' procedure has the advantage of minimizing the manipulations needed to generate the cellular product. As a result, this TriMixDC-cellular therapy can be injected directly into the patients, resulting in maturation and secretion of their immunostimulatory cytokines and chemokines in situ. More importantly, in situ maturation of DCs might lead to enhanced T cell immunity as it more closely resembles the physiological processes involved in response to pathogen infection [16].

Moreover, the use of this approach makes the treatment available to more patients as it avoids human leukocyte antigen (HLA) restriction as an inclusion criterion for clinical trials [27]. This can be explained by the fact that mRNA encoding the full-length tumor antigen allows presentation of all possible epitopes by the patient's HLA repertoire. We could show both stimulation of antigen-specific T cells in vitro and in vivo and the induction of antigen-specific T cell responses in melanoma patients through administration of TriMixDCs co-electroporated with mRNA encoding a fusion of DC-LAMP and one of four melanoma-associated antigens: gp100, tyrosinase, MAGE-A3 and MAGE-C2, referred to as TriMixDC-MEL. Furthermore, these T cells have an activated phenotype, show cytolytic capacity and produce inflammatory cytokines in response to specific stimulation [26]. More recently, we showed that CTLs activated by TriMixDCs are less prone to regulatory T (Treg)

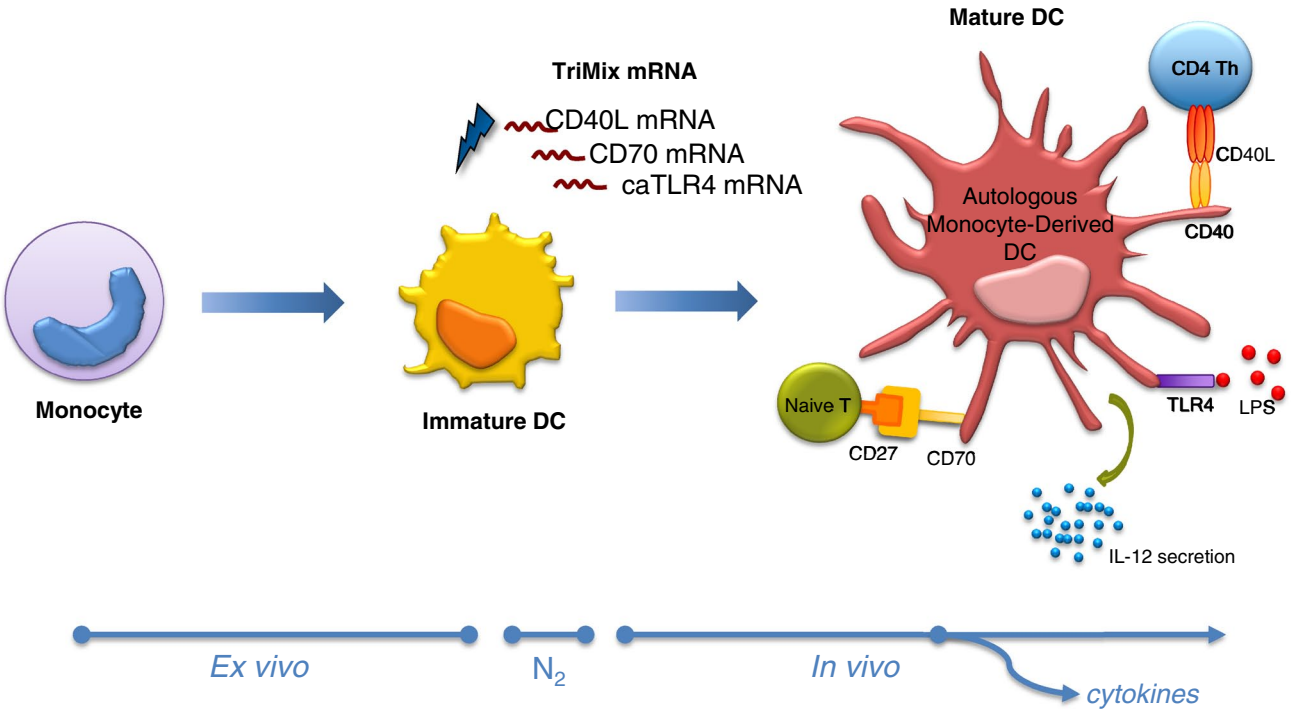

Fig. 1 Maturation induction 'from within'. Today, monocytes derived from the blood of patients can easily been differentiated ex vivo into immature DCs. To obtain fully mature DCs, these autologous monocyte-derived DCs are electroporated with mRNA encoding CD40L, a constitutive active form of TLR4 and CD70. Delivery of CD40L mimics ligation of CD40L normally expressed by $CD4^+$ T_H1 cells with CD40 expressed by DCs. Electroporation with caTLR4 will result in constitutive triggering of TLR4 pathway and mimics addition of LPS. Both result in the maturation of DCs, characterized by the enhanced secretion of cytokines such as IL-12 and chemokines, and enhanced T helper 1 (T_H1) cell skewing. Introduction of CD70 into the DCs provides a co-stimulatory signal to $CD27^+$ naive T cells by inhibiting activated T cell apoptosis and supporting T cell proliferation. These activated autologous monocyte-derived DCs can be stored in nitrogen or used directly

cell-mediated suppression [28]. In addition, TriMixDCs were shown to suppress the function of Treg cells and even reprogram them to T_H1 cells in vitro [28].

From bench to bedside: clinical studies with TriMixDC-MEL

In 2007, we initiated a pilot clinical trial and investigated the intradermal (i.d.) administration of TriMixDC-MEL in 35 melanoma patients [27]. We demonstrated that this therapy was safe and feasible. TriMixDC-MEL-related adverse events comprised grade 2 local injection site reactions in all patients and grade 2 fever and lethargy in two patients. Immunomonitoring was performed by analyzing skin-infiltrating T lymphocytes (SKILs) from a biopsy of a delayed-type hypersensitivity (DTH) test. Vaccinal antigen-specific SKILs were not found in patients tested at treatment initiation, whereas these were detectable in 12 out of 21 patients (57.1 %) assessed after the fourth TriMixDC-MEL injection. Although we could demonstrate that TriMixDC-MEL treatment was immunogenic in this cohort of melanoma patients, no objective tumor responses [according to Response Evaluation Criteria in Solid Tumors (RECIST)] were observed.

Preclinical insights into the organ-specific trafficking of vaccine-induced T cell populations indicated that combination of different routes of administration may be beneficial to target different tumor locations [29, 30]. Also, the intravenously (i.v.) administered DC-like product Sipuleucel-T demonstrated an improved overall survival for castration-resistant prostate cancer patients in a randomized phase III trial [31]. Based on these insights, we investigated TriMixDC-MEL when administered by the combined i.d. and i.v. route in a follow-up phase IB study [32]. In this study, the ratio of i.d./i.v. administered TriMixDC-MEL was decreased in sequential cohorts: Cohort-1: 20.106/4.106 [2 pts], Cohort-2: 12.106/12.106 [3 pts], Cohort-3: 4.106/20.106 [6 pts] and Cohort-4: 0/24.106 [4 pts]. TriMixDC-MEL were administered every 2 weeks (four times), and a 5th administration on week 16. Adverse events related to TriMixDC-MEL were mild. Local skin reactions (grade 1–2) were observed in all patients receiving i.d. administrations. Grade 2 flu-like symptoms and

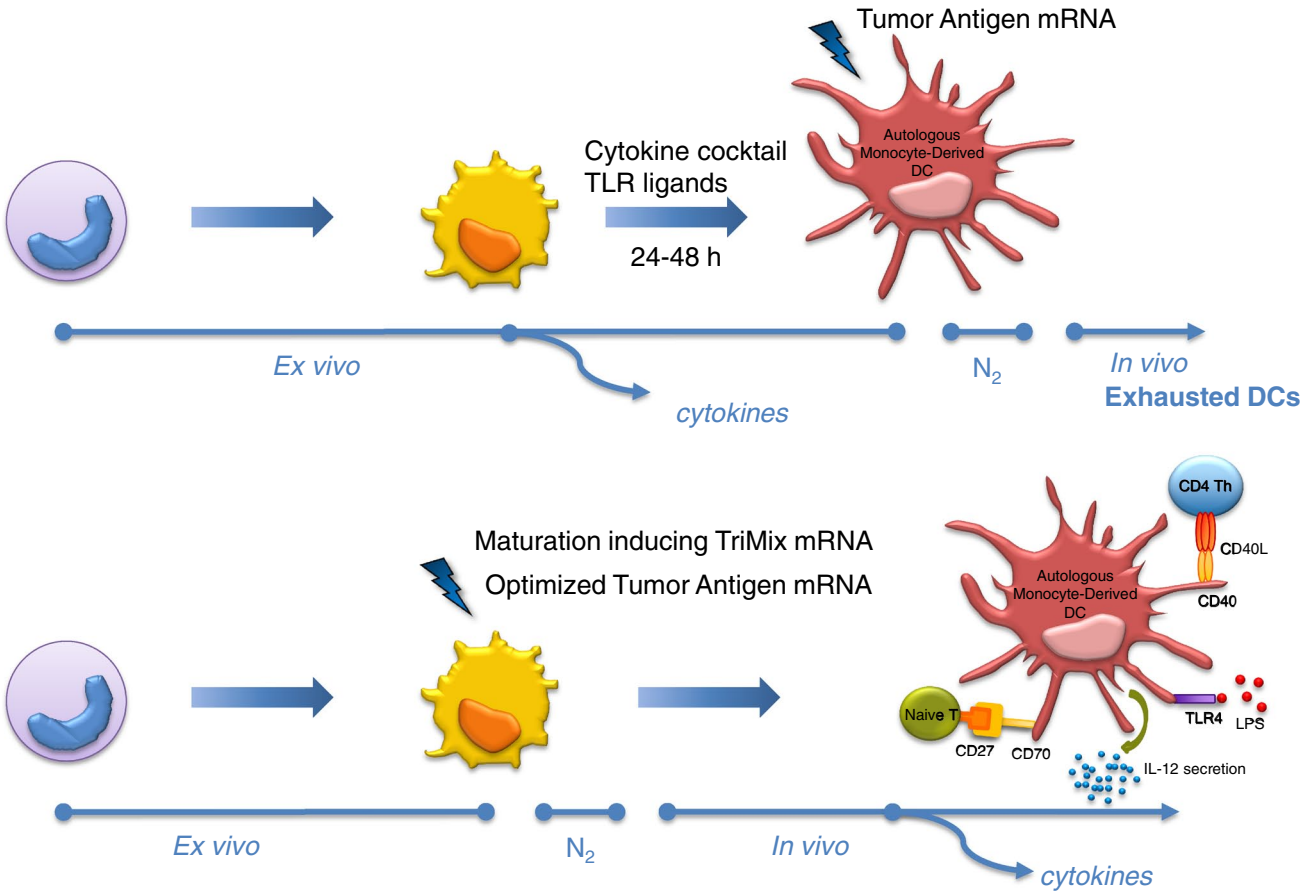

Fig. 2 Maturation induction and antigen loading in a one-step procedure. In the upper panel, autologous monocyte-derived DCs are matured by addition of soluble maturation signals such as pro-inflammatory cytokines or TLR ligands during a 24–48-h culture. These matured DCs, that already secreted their cytokines and chemokines in vitro, need to be further loaded with information regarded tumor antigens, in an additional step. Long in vitro maturation might possibly exhaust the DCs, resulting in insufficient cytokine secretion at the time of injection. Consequently, these exhausted DCs might induce poor T cell stimulation in vivo. In contrast, in the lower panel, DCs can be co-electroporated with mRNA encoding TriMix and optimized tumor antigen in a one-step procedure. As maturation induction starts 'from within', this will result in maturation and secretion of immunostimulatory cytokines and chemokines in situ

fever (38–39 °C) that persisted for 2–3 days after the TriMixDC-MEL injection were reported by, respectively, 8 and 3 out of 15 patients. Post i.v. infusion chills (grade 2) occurred in 3 out of 15 patients. Chills were associated with a post-infusion cytokine release (including pro-inflammatory cytokines IL-1β, IL-6, IFN-γ, TNF-α, IL-8 and IL-17, and chemokines MIP-1α and MIP-1β). Tumor response assessments were performed by 2-Fluoro-2-deoxy-D-glucose positron emission tomography/computed tomography (^{18}F-PET/CT) at baseline and on week 8, 16 and 24. Objective tumor responses (two complete responses and two partial responses according to RECIST) were documented in 4 out of 15 patients (27 %), comparing favorably with our prior experience with i.d. administrations only ($P = 0.026$). All objective tumor responders and one patient with a disease stabilization are progression-free after a follow-up of, respectively, 18+, 22+, 23+, 27+ and 28+ months. Post-therapy antigen-specific SKILs were documented in 6 out of 12 patients, and antigen-specific $CD8^+$ T cells were detected in the blood of 4 out of 5 patients studied. In conclusion, this study demonstrated that intravenous administration of TriMixDC-MEL is safe, feasible and immunogenic, and results in encouraging durable clinical responses.

In June 2010, ipilimumab, an anti-cytotoxic T-Lymphocyte antigen 4 (CTLA-4) monoclonal antibody, was the first treatment to demonstrate an improved survival for advanced melanoma patients, hereby underlining its potential and promise for cancer immunotherapy. Antigen-specific immunotherapy (TriMixDC-MEL) could potentially be complemented by other antigen unspecific immunostimulating therapies such as ipilimumab that are dependent on the presence of immune effector cells for their anti-tumor activity. The group of James Allison pioneered work in

which in vivo blockade of CTLA-4 with monoclonal antibodies (mAbs) resulted in rejection of experimentally transplanted tumors in mice, and this effect was clearly enhanced when combined with a granulocyte/macrophage colony-stimulating factor (GM-CSF) expressing tumor cell vaccine [33]. Administration of ipilimumab together with TriMixDC-MEL therapy may therefore be a more effective treatment as compared to either modality alone. Based on this hypothesis, we initiated in February 2011 a single-arm, two-stage phase II trial with TriMixDC-MEL therapy in combination with ipilimumab in patients with previously treated unresectable stage III or IV melanoma. The primary objective of this study is disease-control rate (according to the immune-related response criteria). Secondary objectives include safety, tumor response, progression-free and overall survival, and immunomonitoring (including DTH analysis, serum cytokine analysis and phenotypic T cell monitoring in peripheral blood before and after treatment). We recently reported that encouraging anti-tumor activity was observed in the first patients enrolled in this study (three partial responses out of the first 6 patients treated with TriMixDC-MEL and ipilimumab (presented at the 2011 American Society of Clinical Oncology (ASCO) meeting and published in the conference abstractbook) [34].

In vivo TriMix-based mRNA vaccines

Recently, the use of mRNA was introduced as a novel vaccine platform combining the induction of strong immune responses together with simplicity and a good safety profile. Together with its versatility and the other advantages it offers, mRNA can now be seen as an active pharmaceutical ingredient (API) in cancer vaccinology [35]. In this regard, mRNA has been mainly used for ex vivo modifications of DCs as described above. However, nowadays much effort is taken to investigate the effect of mRNA as a medicinal product (MP) and its application as an off-the-shelf immunotherapeutic.

Having the tools, we explored the use of mRNA as investigational MP for in vivo modification of DCs by its direct administration in a mouse model. However, the success of mRNA vaccination strongly depends on the engulfment by DCs and its potential to mature these DCs. Consequently, the route of mRNA delivery and the modus of DC maturation are parameters that will critically affect the efficacy of the mRNA vaccine. Since lymph nodes are at the center of our immune system harboring a relatively high number of DCs that are in close contact with T cells, we evaluated the intranodal (i.n.) delivery of TriMix and tumor antigen mRNA [36].

Before focusing on the induction of immunotherapy responses, we first identified the optimal buffer for the in vivo delivery of mRNA, since immunobioavailability of antigen mRNA is a success limiting factor in view of cancer therapy [37]. High antigen expression was observed when mRNA was delivered in $Ca2^{+}$ containing HBSS or in 0.8 volumes of the clinically applied Hartmann's solution, confirming the dependency of $Ca2^{+}$ for efficient uptake of mRNA [38]. Furthermore, selective uptake and translation of mRNA by lymph node resident DCs were shown after i.n. delivery of the mRNA, resulting in the direct modification of these DCs.

As with ex vivo-modified DC therapy, it is of utmost importance that in situ-modified DCs are ideally equipped to stimulate effector T cells. Although it has been described that mRNA itself can trigger several pattern recognition receptors [35], as such providing immune-stimulating capacities, much effort is put into the identification of an applicable additional adjuvant. However, the type of adjuvant should be chosen carefully as several adjuvants could hamper mRNA uptake. This phenomenon can be explained by the fact than mRNA uptake is critically dependent on macropinocytosis, a process that is rapidly down-regulated upon DC activation [39]. Through i.n. delivery of TriMix mRNA, we provide a strong activation stimulus for DCs, resulting in the induction of a T cell attracting and stimulatory environment. Moreover, when antigen mRNA was co-delivered together with TriMix, this resulted in the recruitment of antigen-specific $CD4^{+}$ and $CD8^{+}$ T cells as well as CTLs against various tumor antigens. This might be explained by the timing of DC activation as i.n. delivery of TriMix initiates maturation of DCs after the uptake and translation of the mRNA as such still allowing strong antigen expression by these DCs. Simultaneous delivery of TriMix and antigen mRNA significantly enhances the induction of antigen-specific T cells compared to i.n. delivery of antigen mRNA alone. These observations point toward the value of co-delivering TriMix mRNA for the induction of strong antigen-specific immune responses against cancer.

Since immunotherapy with human DCs electroporated with tumor antigen and TriMix mRNA has shown promise in clinical evaluation [27], we further compared the efficacy of ex vivo TriMixDC-cellular therapy to the direct application of TriMix and antigen mRNA. Our results indicate that i.n. immunization with TriMix and antigen mRNA is at least as efficient in both stimulation of CTLs and therapy as immunization with ex vivo-modified TriMixDCs in a mouse model [36].

These preclinical data together with the GMP compliant mRNA production have led to the preparation of clinical trial protocols for i.n. treatment with TriMix and tumor antigen mRNA of patients suffering from melanoma or hepatocellular carcinoma. Health Authorities recently approved these clinical trial protocols, resulting in the

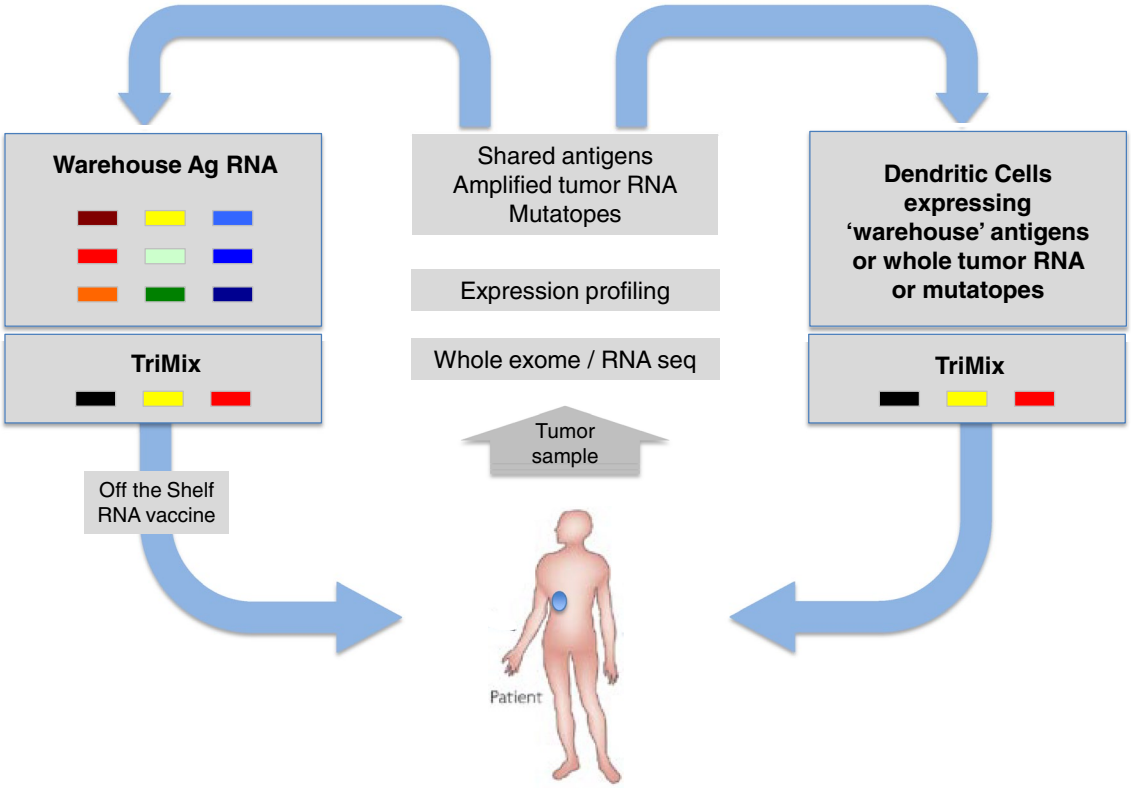

Fig. 3 The future concept of RNA-based immunotherapy. Future therapy will most likely be based on the sequencing of the entire tumor exome by which the expression profile of shared tumor antigens, total tumor RNA or mutatopes can be defined. Based on these expression profiles of the tumor, two different vaccination strategies can be offered to the patient. On the one hand, an off-the-shelf mRNA vaccine can be generated by which TriMix mRNA, as a potent adjuvant, can be combined with tumor antigen mRNA selected from a warehouse created by sequencing of the tumor. On the other hand, an ex vivo-modified DC-based vaccine can be generated by co-electroporation of the patients autologous DCs with mRNA encoding warehouse antigens, total tumor RNA or mutatopes in combination with TriMix

recruitment of patients with hepatocellular carcinoma and the initiation of a first clinical trial.

The future of mRNA-based immunotherapy

Since mRNA became the focus of research in molecular medicine, it has mainly been described as a vehicle to deliver tumor antigens as well as activation stimuli for the induction of immune responses against cancer [24, 35, 40–45]. In this regard, most research groups describe vaccination strategies based on the use of mRNA encoding multiple tumor antigens or the use of total tumor mRNA both for the ex vivo modification of antigen-presenting cells such as for DCs as well as for the direct in vivo application [35]. However, a key challenge in cancer immunotherapy is tolerance against self-proteins. Of note, most tumor antigens applied today are considered 'self' by the immune system. In contrast, tumor-specific mutations are not subjected to central tolerance mechanisms. Therefore, much effort is put in targeting of the tumor mutanome [46, 47]. Indeed, as cancer is a disease caused by DNA aberrations [48], mutations provide a unique difference between tumor and normal cells. This strategy might be a promising future direction for personalized vaccination therapy (Fig. 3). Profiling of cancer and normal cells to identify somatic mutations is possible via a technology platform called next-generation sequencing (NGS) [46, 47, 49]. In this regard, the capability of NGS to provide a comprehensive map of somatic mutations in individual tumors, also called 'the mutanome,' provides a powerful tool to better understand cancer and intervene. Moreover, NGS can be applicable for both whole exome sequencing or for gene expression profiling (RNA-sequencing). In this regard, Castle et al. [47] completed the first mouse tumor exome by using NGS and described the identification of non-synonymous somatic mutations, which were shown to be highly immunogenic and moreover confer anti-tumoral vaccine activity. Whole exome sequencing in combination with transcriptome profiling enables the discovery of the tumor-specific mutated

epitopes (mutatopes) and can provide an insight into the molecular nature of potential driver mutations in cancer.

So far, neither the concepts nor the processes for their implementation for the sake of cancer patients have been realized. Moreover, shared mutations are rare and the great majority of mutations are patient-specific, which has hindered exploitation of the mutanome for the development of broadly applicable drugs. However, personalized therapy concepts that integrate personal disease genetics to create customized therapies are promising alternatives to existing approaches. Next-generation immunotherapy will therefore probably pioneer a new drug platform by targeting the individual tumor antigen signature, thus creating a biomarker-guided personalized treatment of cancer. This pioneering research will take cancer immunotherapy to the next level of individualized medicine. The proposed approach will exploit the large unique antigenic target repertoire of each single individual patient, instead of searching for the small common denominator shared by patients.

So far, challenges tackling clinical grade processes and technologies for the rapid determination of individual tumor antigen signatures and mutations by sequencing of whole exomes are addressed. Together with a GMP compliant, cutting edge RNA vaccine platform that enables the design, the manufacture and the release of fully tailored pharmacologically optimized RNA vaccines within a short time frame, a warehouse of antigen mRNA for off-the-shelf RNA vaccination or personalized therapy can be generated. This personalized next-generation vaccination strategy opens new opportunities to solve critical problems in current cancer development and will address both broad inter-individual variability and intratumoral clonal heterogeneity.

The use of mRNA in immune checkpoint blockade

It is believed that the limited effect of antitumor immune responses and as a consequence limited clinical benefit for cancer patients is due to several reactions driven by the tumor and its suppressive microenvironment [50–52]. This is highlighted by the fact that the ability of cancer cells to evade antitumor T cell activity in the microenvironment was pinpointed as one of the hallmarks of cancer progression. Immune checkpoint blockade that aims to inhibit molecular pathways that are crucial for cancer cell growth and maintenance and have gained attention as targets for novel immunotherapeutic strategies. Since there are multiple inhibitory ligands and receptors expressed by tumor cells, tumor-associated myeloid cells and tumor-infiltrating lymphocytes, respectively, there are many opportunities to enhance antitumor immunity through blockade of immune checkpoints. In this regard, blockade of CTLA-4, a regulator of early-stage T cell activation in response to antigen and blockade of the PD-1/PD-L1 pathway, known to inhibit T cell function of activated T cells has been studied. Clinical trials using fully humanized neutralizing monoclonal antibody therapy against these immune modulators already showed durable responses and activation of long-term immunological memory, resulting in promising clinical outcome [53–55]. Moreover, preliminary data of our own clinical trial using ipilimumab, an anti-CTLA-4 monoclonal antibody together with antigen-specific immunotherapy (TriMixDC-MEL), showed promising results [34]. To avoid systemic autoimmune toxicity, which have been described with anti-CTLA-4 treatment, mRNA encoding factors that have the ability to block CTLA-4 or PD-1 can be used [56], either for ex vivo transfection of DCs or via direct local administration of the mRNA. Therefore, to increase safety and to avoid autoimmune toxicity by systemic administration of immune modulators, the use of mRNA encoding soluble immune modulators is a promising strategy.

Conclusion

Over the years, much effort has been invested in the design of DC-based immunotherapies promoting strong tumor-specific CTL responses and more importantly in the identification of a strong activation stimulus that provides DCs with all required features. Recently, tremendous progress has been made which has led to the successful application of immunotherapy in cancer treatment. However, continuing progress in development should be taken not only to boost cancer-specific T cells to fight against cancer but also to induce immunological memory to control metastatic and recurring cancer cells. We have shown that by combined intradermal and intravenous administration of our optimized DC formula (TriMixDC-MEL) durable anti-tumor activity can be achieved in a subset of melanoma patients. The search for the optimal treatment will depend on many factors, and RNA vaccines might be prime candidates to become the next vaccine technology platform in the near future and result in a personalized vaccination approach. To finally further improve survival of cancer patients, antigen-specific immunotherapies (TriMixDC-MEL, future RNA vaccines) could be combined with therapeutic strategies such as immune checkpoint blockade to outsmart tumor escape mechanisms and induce an equilibrium state of cancer immunoediting. As such, future treatment strategies may shift cancer from the devastating disease it is now, toward a disease that can be durably controlled by the patient's own immune system.

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Conflict of interest The use of dendritic cells electroporated with tumor antigen mRNA and TriMix is the topic of a patent (W2009/034172) on which Dr. A. Bonehill and Prof. Dr. K. Thielemans are filed as inventors. None of the authors receive any support or remuneration related to this platform. No potential conflict of interests were disclosed.

References

1. Villadangos JA, Schnorrer P (2007) Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol 7:543–555
2. Melief CJM (2008) Cancer immunotherapy by dendritic cells. Immunity 29:372–383
3. Palucka K, Banchereau J (2012) Cancer immunotherapy via dendritic cells. Nat Rev Cancer 12:265–277
4. Vacchelli E, Vitale I, Eggermont A et al (2013) Trial watch: dendritic cell-based interventions for cancer therapy. Oncoimmunology 2:e25771
5. Galluzzi L, Senovilla L, Vacchelli E et al (2012) Trial watch: dendritic cell-based interventions for cancer therapy. Oncoimmunology 1:1111–1134
6. Jonuleit H, Giesecke-Tuettenberg A, Tüting T et al (2001) A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. Int J Cancer 93:243–251
7. De Vries IJM, Lesterhuis WJ, Scharenborg NM et al (2003) Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. Clin Cancer Res 9:5091–5100
8. Steinman RM, Nussenzweig MC (2002) Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. Proc Natl Acad Sci U S A 99:351–358
9. Lutz MB, Schuler G (2002) Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol 23:445–449
10. Cools N, Van Tendeloo VFI, Smits ELJM et al (2008) Immunosuppression induced by immature dendritic cells is mediated by TGF-beta/IL-10 double-positive CD4+ regulatory T cells. J Cell Mol Med 12:690–700
11. Enk AH (2005) Dendritic cells in tolerance induction. Immunol Lett 99:8–11
12. Jonuleit H, Kühn U, Müller G et al (1997) Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur J Immunol 27:3135–3142
13. Mailliard RB, Wankowicz-Kalinska A, Cai Q et al (2004) Alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. Cancer Res 64:5934–5937
14. Vonderheide RH, Flaherty KT, Khalil M et al (2007) Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. J Clin Oncol 25:876–883
15. Turner JG, Rakhmilevich AL, Burdelya L et al (2001) Anti-CD40 antibody induces antitumor and antimetastatic effects: the role of NK cells. J Immunol 166:89–94
16. Nair S, McLaughlin C, Weizer A et al (2003) Injection of immature dendritic cells into adjuvant-treated skin obviates the need for ex vivo maturation. J Immunol 171:6275–6282
17. Adema GJ, de Vries IJM, Punt CJA, Figdor CG (2005) Migration of dendritic cell based cancer vaccines: in vivo veritas? Curr Opin Immunol 17:170–174
18. Calderhead DM, DeBenedette MA, Ketteringham H et al (2008) Cytokine maturation followed by CD40L mRNA electroporation results in a clinically relevant dendritic cell product capable of inducing a potent proinflammatory CTL response. J Immunother 31:731–741
19. DeBenedette MA, Calderhead DM, Tcherepanova IY et al (2011) Potency of mature CD40L RNA electroporated dendritic cells correlates with IL-12 secretion by tracking multifunctional CD8(+)/CD28(+) cytotoxic T-cell responses in vitro. J Immunother 34:45–57
20. Bonehill A, Tuyaerts S, Van Nuffel AMT et al (2008) Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. Mol Ther 16:1170–1180
21. Kikuchi T, Moore MA, Crystal RG (2000) Dendritic cells modified to express CD40 ligand elicit therapeutic immunity against preexisting murine tumors. Blood 96:91–99
22. Cisco RM, Abdel-Wahab Z, Dannull J et al (2004) Induction of human dendritic cell maturation using transfection with RNA encoding a dominant positive toll-like receptor 4. J Immunol 172:7162–7168
23. Borst J, Hendriks J, Xiao Y (2005) CD27 and CD70 in T cell and B cell activation. Curr Opin Immunol 17:275–281
24. Van Lint S, Van Nuffel AM, Wilgenhof S, et al. (2013) Priming of cytotoxic T lymphocyte responses by dendritic cells: induction of potent anti-tumor immune responses. Cytotoxic T lymphocytes Mech Dev Dis, Horizons i. Nova Science Publishers, p volume 51
25. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F (2000) Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol 1:311–316
26. Bonehill A, Van Nuffel AMT, Corthals J et al (2009) Single-step antigen loading and activation of dendritic cells by mRNA electroporation for the purpose of therapeutic vaccination in melanoma patients. Clin Cancer Res 15:3366–3375
27. Wilgenhof S, Van Nuffel AMT, Corthals J et al (2011) Therapeutic vaccination with an autologous mRNA electroporated dendritic cell vaccine in patients with advanced melanoma. J Immunother 34:448–456
28. Pen JJ, De Keersmaecker B, Maenhout SK et al (2013) Modulation of regulatory T cell function by monocyte-derived dendritic cells matured through electroporation with mRNA encoding CD40 ligand, constitutively active TLR4, and CD70. J Immunol 191:1976–1983
29. Fong L, Brockstedt D, Benike C et al (2001) Dendritic cells injected via different routes induce immunity in cancer patients. J Immunol 166:4254–4259
30. Mullins DW, Sheasley SL, Ream RM et al (2003) Route of immunization with peptide-pulsed dendritic cells controls the distribution of memory and effector T cells in lymphoid tissues and determines the pattern of regional tumor control. J Exp Med 198:1023–1034
31. Kantoff PW, Higano CS, Shore ND et al (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363:411–422
32. Wilgenhof S, Van Nuffel AMT, Benteyn D et al (2013) A phase IB study on intravenous synthetic mRNA electroporated dendritic cell immunotherapy in pretreated advanced melanoma patients. Ann Oncol 24:2686–2693
33. Van Elsas A, Hurwitz AA, Allison JP (1999) Combination immunotherapy of B16 melanoma using anti-cytotoxic T

lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied. J Exp Med 190:355–366
34. Neyns B, Wilgenhof S, Van Nuffel AMT et al (2011) A phase I clinical trial on the combined intravenous (IV) and intradermal (ID) administration of autologous TriMix-DC cellular therapy in patients with pretreated melanoma (TriMixIDIV). ASCO Meet Abstr 29:2519
35. Van Lint S, Heirman C, Thielemans K, Breckpot K (2013) mRNA: from a chemical blueprint for protein production to an off-the-shelf therapeutic. Hum Vaccin Immunother 9(2):265–274
36. Van Lint S, Goyvaerts C, Maenhout S et al (2012) Preclinical evaluation of TriMix and antigen mRNA-based antitumor therapy. Cancer Res 72:1661–1671
37. Kuhn AN, Diken M, Kreiter S et al (2011) Determinants of intracellular RNA pharmacokinetics: implications for RNA-based immunotherapeutics. RNA Biol 8:35–43
38. Probst J, Brechtel S, Scheel B et al (2006) Characterization of the ribonuclease activity on the skin surface. Genet Vaccines Ther 4:4
39. Diken M, Kreiter S, Selmi A et al (2011) Selective uptake of naked vaccine RNA by dendritic cells is driven by macropinocytosis and abrogated upon DC maturation. Gene Ther 18:702–708
40. Kreiter S, Diken M, Selmi A et al (2011) Tumor vaccination using messenger RNA: prospects of a future therapy. Curr Opin Immunol 23:399–406
41. Pascolo S (2006) Vaccination with messenger RNA. Methods Mol Med 127:23–40
42. Pascolo S (2004) Messenger RNA-based vaccines. Expert Opin Biol Ther 4:1285–1294
43. Bringmann A, Held SAE, Heine A, Brossart P (2010) RNA vaccines in cancer treatment. J Biomed Biotechnol 2010:623687
44. Kuhn AN, Beißert T, Simon P et al (2012) mRNA as a versatile tool for exogenous protein expression. Curr Gene Ther 12:347–361
45. Ulmer JB, Mason PW, Geall A, Mandl CW (2012) RNA-based vaccines. Vaccine 30:4414–4418
46. Kreiter S, Castle JC, Türeci O, Sahin U (2012) Targeting the tumor mutanome for personalized vaccination therapy. Oncoimmunology 1:768–769
47. Castle JC, Kreiter S, Diekmann J et al (2012) Exploiting the mutanome for tumor vaccination. Cancer Res 72:1081–1091
48. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674
49. Britten CM, Singh-Jasuja H, Flamion B et al (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31:880–882
50. Le Dieu R, Taussig DC, Ramsay AG et al (2009) Peripheral blood T cells in acute myeloid leukemia (AML) patients at diagnosis have abnormal phenotype and genotype and form defective immune synapses with AML blasts. Blood 114:3909–3916
51. Ramsay AG, Clear AJ, Kelly G et al (2009) Follicular lymphoma cells induce T-cell immunologic synapse dysfunction that can be repaired with lenalidomide: implications for the tumor microenvironment and immunotherapy. Blood 114:4713–4720
52. Ramsay AG, Johnson AJ, Lee AM et al (2008) Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. J Clin Invest 118:2427–2437
53. Hodi FS, O'Day SJ, McDermott DF et al (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363:711–723
54. Topalian SL, Hodi FS, Brahmer JR et al (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366:2443–2454
55. Brahmer JR, Tykodi SS, Chow LQM et al (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366:2455–2465
56. Pruitt SK, Boczkowski D, de Rosa N et al (2011) Enhancement of anti-tumor immunity through local modulation of CTLA-4 and GITR by dendritic cells. Eur J Immunol 41:3553–3563