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SHORT COMMUNICATION

Polyinosinic polycytidylic acid prevents efficient antigen expression after mRNA electroporation of clinical grade dendritic cells

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Abstract Tumor-derived peptides are used frequently as antigen (Ag) source in dendritic cell (DC) therapy in cancer patients. An alternative is to load DC with tumor-associated Ag (TAA)-encoding RNA. RNA-loading obviates prior knowledge of CTL and Th epitopes in the Ag. Multiple epitopes for many HLA alleles (both MHC class I and class II) are encoded by the RNA and loading is independent of the patient's HLA make-up. Herein, we determined the optimal conditions for mRNA-electroporation of monocyte-derived DC for clinical application in relation to different maturation cocktails. The data demonstrate that TAA carcinoembryonic antigen, gp100 and tyrosinase are expressed already 30 min after electroporation with the encoding mRNA. Moreover, gp100-specific CTL are activated by gp100 mRNA-electroporated DC. Importantly, we show here that the presence of polyinosinic–polycytidylic acid [poly(I:C)] in the maturation cocktail prevents effective protein expression of the electroporated mRNA as well as subsequent CTL recognition. This effect of poly(I:C) correlates with the induction of IFN-induced genes and innate anti-viral effector molecules in DC. Together these data show that electroporation of mature DC with TAA-encoding mRNA is attractive for use in DC vaccination protocols in cancer patients, but protein expression should be tested for each maturation cocktail.

Keywords Dendritic cells · Maturation · Vaccination · Antigen presentation · Human · Cancer immunotherapy

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Abbreviations

CEA	Carcinoembryonic antigen
(c)DC	(Conventional cytokine cocktail-matured) dendritic cell(s)
MCM	Monocyte-conditioned medium
MDA-5	Melanoma differentiation-associated gene 5
2,5-OAS	2′-5′-Oligoadenylate synthetase
PKR	Double stranded RNA-activated serine/threonine protein kinase
poly(I:C)	Polyinosinic–polycytidylic acid
pIC-DC	Poly(I:C)-matured DC
R848-DC	R848-matured DC
R + P-DC	R848 + poly(I:C)-matured DC
RIG-I	Retinoic acid-inducible gene I
TAA	Tumor-associated Ag
TLR(-L)	Toll-like receptor (ligand)

Introduction

Dendritic cell (DC) vaccination studies showed the feasibility and safety of DC vaccinations (reviewed in [1, 2]). Clinical and immunological responses have been reported in some patients. One important aspect in DC-based vaccines is the maturation of DC. The cocktails used commonly for DC maturation in clinical studies [3] fail to induce IL-12p70 production in the DC. For cancer vaccine development DC producing IL-12p70 are desired because of the Th type 1 (Th1) and CTL inducing capacity [4]. It has been shown that ligation of particular Toll-like receptors (TLR) is a prerequisite to induce full maturation, enabling DC to produce IL-12p70 [5]. We and others have recently developed clinically applicable maturation cocktails, containing TLR ligands (TLR-L), to generate mature DC with high migratory and IL-12 producing capacity [6, 7].

Another crucial aspect in DC-based vaccines concerns the efficacy of Ag delivery to DC. To date, in most clinical studies DC loaded with tumor lysates or defined tumor peptides have been used for the induction of anti-tumor immunity [8–11]. Various methods have been designed to introduce whole tumor Ag into DC, including tumor mRNA or synthetic mRNA [12–14]. The advantage of endogenous expression of whole tumor Ag by the DC is that T cell epitopes do not need to be specified, HLA type is not a limiting factor and multiple epitopes (both CTL and Th epitopes) can be presented. DC transfected with mRNA encoding tumor-associated Ag (TAA) or with whole tumor mRNA can induce potent Ag- and tumor-specific T cell responses (reviewed in [15, 16]). Electroporation of mRNA is an efficient way of transfection [15, 16], leading to clear expression of the introduced gene. This strategy can be optimized for the treatment of cancer patients using synthetic RNA encoding tumor Ag, by analyzing protein expression levels in the electroporated cells and by testing efficiency of protein expression after mRNA electroporation in combination with different, clinical grade, maturation cocktails.

We optimized the electroporation protocol for transfection of synthetic mRNA to obtain a clinical grade mature monocyte-derived DC vaccine. This protocol was used for the electroporation of mRNA encoding TAA. We found that transfection of TAA carcinoembryonic antigen (CEA), gp100 and tyrosinase leads to expression and presentation of these Ag by clinical grade mature monocyte-derived DC. Moreover, we show that the presence of polyinosinic-polycytidylic acid [poly(I:C)], a TLR3 and melanoma differentiation-associated gene 5 (MDA-5) ligand, in the maturation cocktail interferes with effective transgene expression after mRNA electroporation of DC and that this correlates with the upregulation of genes involved in an anti-viral response by poly(I:C). These results may improve the generation of efficient DC-based vaccines for use in cancer patients.

Materials and methods

See supplementary material.

Results

Expression and presentation of TAA after electroporation of mRNA differs for various tumor Ag

Electroporation is an efficient way to transfect DC with mRNA [15, 16]. Because studies comparing mRNA transfection of immature and mature DC are contradictory [14, 17, 18], we compared the efficiency of electroporation of immature and mature DC with our settings. As shown in Fig. 1a the level of transgene expression was higher in mature DC than in immature DC.

We decided to electroporate mature DC with gp100, tyrosinase and CEA as TAA, because in our ongoing clinical trials peptides derived from these Ag are used to load DC [19–21]. First we studied the expression of TAA after electroporation of the encoding mRNA. As shown in Fig. 1b, 4 h after electroporation, gp100, tyrosinase and CEA proteins were clearly detectable in the electroporated DC both by intracellular FACS analysis and by staining of cytospins. All three TAA were already highly expressed 30 min after electroporation (Fig. 1c), but tyrosinase protein levels decreased to background levels within 24 h after electroporation (Fig. 1c), whereas both gp100 and CEA expression could still be measured 96 h after electroporation (data not shown). The percentage of cells expressing

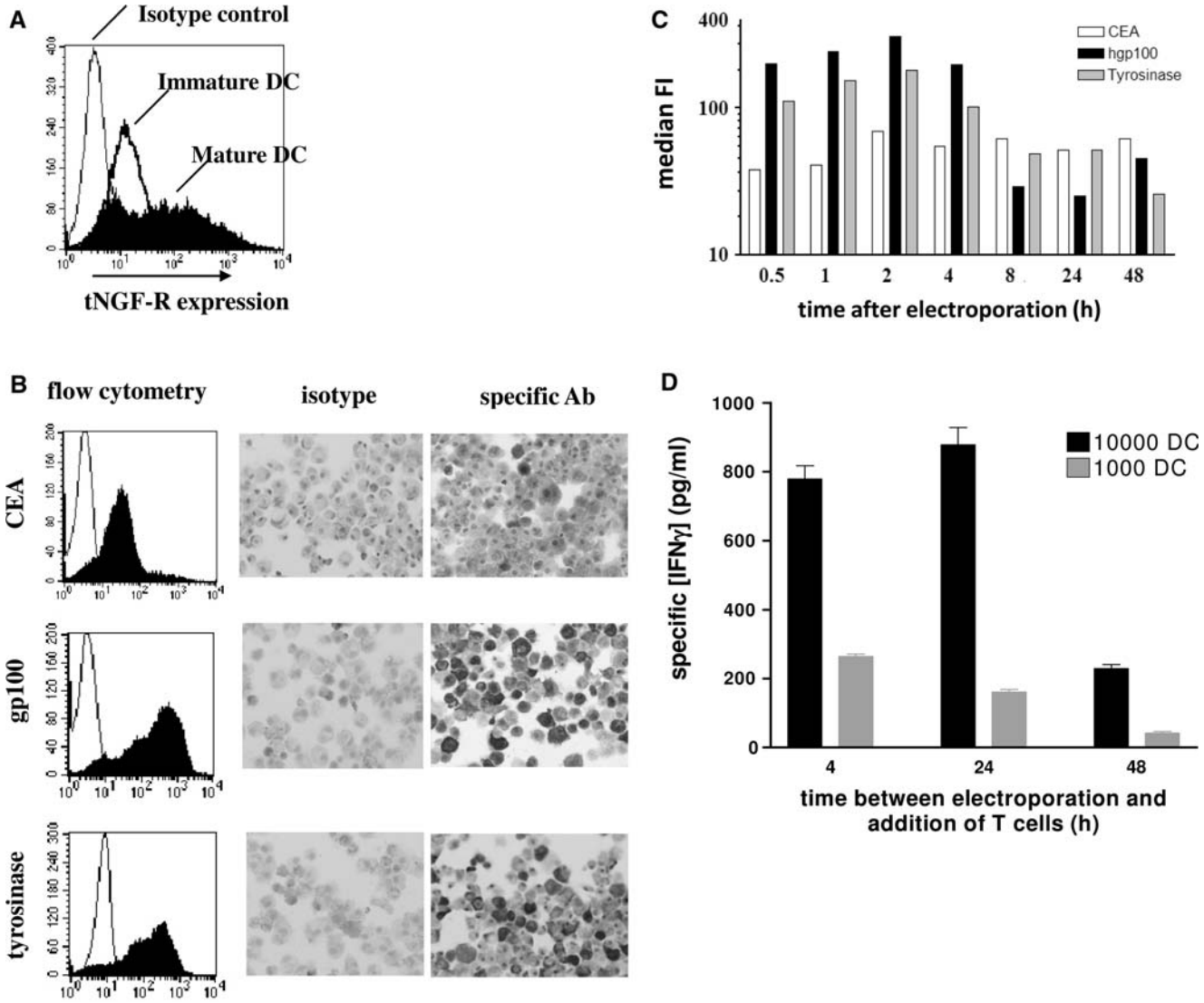

Fig. 1 Electroporation of DC with mRNA encoding TAA results in expression of the respective proteins and presentation of gp100-derived epitope to specific CTL. **a** DC were incubated with cytokine cocktail (mature DC) or not (immature DC). After 48 h, DC were electroporated with mRNA encoding the tNGF-R. tNGF-R expression at the cell surface was determined by FACS analysis 24 h after electroporation. Histograms show staining with specific antibody [*filled curve* (mature DC) and *thick-lined curve* (immature DC)] or isotype control antibody (*thin-lined curve*) after electroporation by exponential decay pulse. **b** Cytokine cocktail-matured DC (cDC) were electroporated with mRNA encoding CEA, gp100 or tyrosinase by exponential decay pulse. Intracellular FACS analysis was performed 4 h after electroporation. Histograms showing isotype control antibody (*thin-lined curves*) and specific antibody (*filled curves*) are presented in the *left panels*. Cytospins were prepared 4 h after electroporation and stained with isotype control antibody (*middle panels*) or specific antibody (*right panels*). **c** cDC were electroporated with mRNA encoding CEA, gp100 or tyrosinase by exponential decay pulse. At different time points after electroporation intracellular FACS analysis was performed. Median fluorescence intensity of the staining of positive cells is shown for one representative experiment of three performed. **d** cDC were electroporated with mRNA encoding for CEA (control) or gp100 by exponential decay pulse. At different time points after electroporation 2.5×10^4 TIL1200 cells (gp100-specific CTL line) were incubated for 24 h with titrated amounts of electroporated DC. [IFNγ] was measured in the supernatants by specific ELISA. Data shown are IFNγ production induced by gp100 mRNA-electroporated DC, corrected for IFNγ production induced by CEA mRNA-electroporated DC (control) and are mean $\pm$ SEM of triplicates from one representative experiment of four performed. IFNγ production induced by CEA mRNA-electroporated DC (control) was 52, 174 and 57 pg/ml for 10,000 DC and 59, 168 and 23 pg/ml for 1,000 DC; when added 4, 24 and 48 h after electroporation. Stimulation indices were 16, 6 and 5 for 10,000 DC and 6, 2 and 3 for 1,000 DC; when added 4, 24 and 48 h after electroporation. Stimulation indices for the three experiments that were not shown were 9, 2 and 35 for 1,000 DC added 24 h after electroporation

gp100, tyrosinase and CEA was 81, 12 and 76%, respectively 24 h after electroporation and 59, 1 and 71%, respectively 48 h after electroporation.

To examine whether DC electroporated with gp100-encoding mRNA are capable of presenting gp100-derived epitopes to specific T cells we used the T cell line TIL1200

[22–24], specific for the $gp100_{154}$ and $gp100_{457}$ CTL epitopes presented in HLA-A2.1 [19]. DC were electroporated with gp100-encoding mRNA and incubated with TIL1200 cells at different time points after electroporation. As shown in Fig. 1d, high IFNγ production was induced in specific CTL by gp100 mRNA-electroporated DC as soon as 4 h after electroporation and this capacity to stimulate specific CTL was retained for at least 48 h. Taken together the data presented here show that electroporation of clinical grade, monocyte-derived DC in the mature state leads to expression of tumor Ag in the DC and efficient presentation of TAA-derived epitopes to specific CTL for a durable time period.

Protein expression after mRNA electroporation is dependent on DC maturation stimuli

To date the most widely applied DC vaccines are matured with a defined cocktail of pro-inflammatory cytokines: IL-1β, IL-6, TNFα and prostaglandin E_2 (PGE_2) [3] or with monocyte-conditioned medium (MCM) combined with TNFα and PGE_2 (conventional DC, cDC) [25]. We used this cytokine mixture to optimize the electroporation protocol. Monocyte-derived cDC produce very low to undetectable levels of IL-12p70 [6, 26]. Recently, maturation cocktails containing TLR-L have been developed that induce the production of IL12p70 by DC [6, 7, 26]. We tested the efficiency of transgene expression after electroporation of DC with gp100-encoding mRNA after maturation with TLR3 and MDA-5 ligand poly(I:C) (pIC-DC), TLR7/8L R848 (R848-DC) and the combination (R + P-DC). All maturation cocktails were supplemented with PGE_2, because this is required to enhance migration [6] and therefore will be added to the maturation cocktail for clinical application. All DC (cDC, pIC-DC, R848-DC and R + P-DC) were phenotypically mature as determined by FACS analysis (data not shown and [6]). However, poly(I:C) and/or R848 induced much higher levels of IL-12 than cytokine mixture (data not shown and [6]). As shown in Fig. 2a, gp100 expression levels after gp100 mRNA electroporation were equal for R848-DC and cDC. In contrast, the presence of poly(I:C) in the maturation cocktail decreased the gp100 protein expression both at 4 and 24 h after electroporation. As shown in Fig. 2b, the capacity to activate gp100-specific T cells was also decreased for DC matured with a cocktail containing poly(I:C). Poly(I:C) is a synthetic viral dsRNA analog and we have shown recently that treatment of DC with poly(I:C) induces a strong upregulation of mRNA and protein for viral sensors, including the RNA helicases retinoic acid-inducible gene I (RIG-I) and MDA-5, and effector molecules like the double stranded RNA-activated serine/threonine protein kinase (PKR) and 2′-5′-oligoadenylate synthetase (2,5-OAS) ([27] and unpublished data). PKR is known to inhibit protein synthesis and 2,5-OAS activates RNAse L, that degrades single-stranded RNA. Therefore, the mRNA expression levels of these genes induced by the different maturation cocktails were measured. As shown in Fig. 2c, cocktails containing poly(I:C) strongly increased the expression of MDA-5, 2,5-OAS, PKR and RIG-I, whereas cytokine cocktail or R848 alone did not. Expression levels were upregulated 48 h after the induction of maturation ($t = 0$ h), the time point at which the cells were electroporated, and remained upregulated compared to cDC and R848-DC up to at least 24 h after electroporation. Western blot analysis corroborated our findings by qPCR and demonstrated elevated protein levels of RIG-I, PKR and MDA-5 following exposure to poly(I:C) (Fig. 2d) at the time of electroporation and up to 24 h later. Furthermore, confocal microscopy analysis showed an increased expression of MDA-5 in the cytoplasm of DC stimulated with a mixture containing poly(I:C) (Fig. 2e). Collectively, these data show that the presence of poly(I:C) in the maturation cocktail prevents effective expression of TAA after electroporation of DC with mRNA. The upregulation of genes involved in the induction of an antiviral state in the DC and regulating protein expression levels is uniquely associated with the presence of poly(I:C) in the maturation cocktail and might be an explanation for this phenomenon.

Discussion

In this study we show that CEA, gp100 and tyrosinase proteins are expressed already 30 min after mRNA electroporation. Furthermore, electroporated DC present dominant epitopes derived from the encoded tumor Ag to specific CTL. Importantly, we show that the presence of poly(I:C) in the maturation cocktail prevents efficacious protein expression after electroporation of mRNA encoding TAA and subsequent CTL recognition. This correlates with upregulation by poly(I:C) of genes regulating protein expression levels by inducing degradation of mRNA and inhibition of protein synthesis.

Dendritic cell vaccination trials have shown that DC vaccines can induce immune responses in cancer patients. However, as reviewed in [28], many variables need to be optimized and standardized to further improve the efficacy of DC vaccination. One of these parameters is the way of loading the DC with Ag. Electroporation of mRNA is an efficient way of antigen loading [15, 16]. We used synthetic mRNA encoding well-characterized TAA as a source to transfect DC. This has the advantage that mRNA can be produced in batches for a large number of patients and it allows proper immune monitoring and comparison with peptide-loaded DC.

It is now generally accepted that mature DC should be applied for vaccination purposes [29, 30]. Currently,

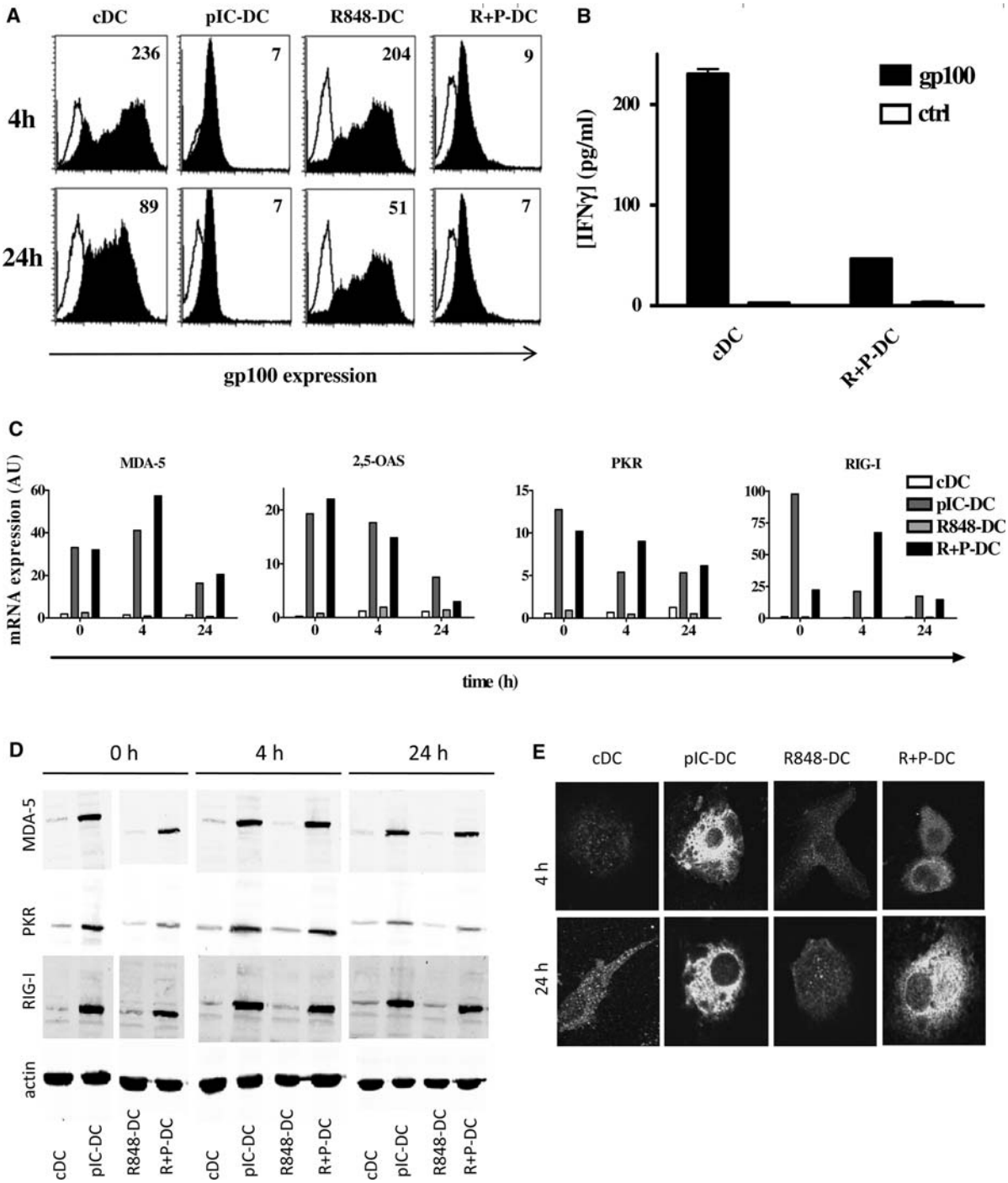

Fig. 2 Polyinosinic–polycytidylic acid stimulation prevents effective protein expression after mRNA electroporation and increases expression of both viral sensors and effector molecules. DC were matured with the conventional cytokine cocktail (TNFα, IL-1β and IL-6; cDC), poly(I:C) (pIC-DC), R848 (R848-DC) or poly(I:C)/R848 (R + P-DC), all with addition of PGE_2. DC were electroporated with gp100-encoding mRNA 48 h later. **a** Histograms of intracellular staining with specific antibody (*filled curves*) or with isotype control Ab (*thick-lined curves*) 4 and 24 h after electroporation. *Numbers* shown in the figures are median fluorescence intensity of gp100-staining (after subtracting median fluorescence intensity of isotype control staining). **b** 2.5×10^4 TIL1200 cells were co-incubated with 400 DC 24 h after electroporation and [IFNγ] was measured in supernatants 24 h later by specific ELISA. **c** mRNA levels of MDA-5, 2,5-OAS, PKR and RIG-I were determined using qPCR at several time points after electroporation (0 h is the time point of electroporation). **d** Protein expression of MDA-5, PKR and RIG-I was analyzed by western blot assay at different time points after electroporation. **e** Four and twenty-four hours after electroporation, DC were stained with antibodies specific for MDA-5 and DC-SIGN, followed by Alexa 568- and Alexa 488-conjucated secondary Ab, respectively, and analyzed by confocal microscopy as described in "Materials and methods." For DC-SIGN a cell surface staining was performed, whereas MDA-5 was stained intracellularly

ex vivo generated monocyte-derived DC, matured with a cocktail containing MCM, TNFα and PGE_2 or IL-1β, IL-6, TNFα and PGE_2, are used commonly in clinical studies. A disadvantage of this maturation cocktail is the poor IL-12p70 production by the DC. We recently proposed a novel clinical grade maturation protocol in which TLR-L poly(I:C) and R848 are combined with PGE_2 to generate DC with both high migratory capacity and IL-12p70 production upon T cell encounter [6]. For peptide loading of DC this seems to be a good choice. However, we show here that protein expression after mRNA electroporation of DC matured for 48 h by cocktails containing poly(I:C) is very ineffective. These results are in agreement with recently published data showing that protein expression after mRNA electroporation of DC matured for 24 h with a cocktail containing poly(I:C) is very inefficient [7]. It is therefore necessary to test maturation cocktails and loading methods of DC individually to find the optimal combination. We propose to mature the DC with R848 and PGE_2 if electroporation of mRNA is the Ag loading method.

The observed interference with effective transgene expression by the presence of poly(I:C) in the maturation cocktail might be explained by the upregulation of genes involved in the innate anti-viral response. Poly(I:C) is a synthetic dsRNA and it has been shown that dsRNA inhibits protein synthesis in cell-free systems prepared from IFN-treated cells [31]. Interferons trigger the development of a defensive response led by the enzymes 2,5-OAS and PKR. 2,5-OAS is activated by dsRNA and on its turn activates the latent ribonuclease RNAse L, which degrades single-stranded RNA. PKR inhibits initiation of protein synthesis. These two pathways regulate protein expression levels [32] and hereby impair the replication of viruses. We here show that incubation of DC with poly(I:C) induces expression of these two dsRNA-dependent enzymes: PKR and 2,5-OAS. These genes and the corresponding proteins are highly expressed at the time of electroporation and up to at least 24 h thereafter. Highly efficient induction of CTL responses by DC co-electroporated with poly(I:C) and mRNA [18] are not in disagreement with our data, but might be explained by translation of the mRNA into protein before the anti-viral response induced by poly(I:C) is full blown.

The data provided here show that electroporation of DC with mRNA encoding TAA is an efficient way of Ag loading, which is feasible and can be performed under cGMP conditions. We propose the use of TLR-L poly(I:C) and R848 in combination with PGE_2 as maturation cocktail for peptide loading of DC and omission of poly(I:C) from the cocktail for mRNA electroporation. Future clinical studies will demonstrate the efficacy of these mRNA-loaded DC vaccines in cancer patients.

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