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Increased cytotoxic capacity of tumor antigen specific human T cells after *in vitro* stimulation with IL21 producing dendritic cells

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Abstract

Monocyte derived dendritic cells (moDC) electroporated with tumor associated antigen derived mRNA can elicit specific T cells against tumor cells *in vivo*. IL21 has been shown to enhance activation and cytotoxicity in CD8+ T cells. We therefore investigated *in vitro* effects on human CD8+ T cells after stimulation with IL21 mRNA electroporated moDC. Codon modification of the IL21 gene significantly enhanced IL21 production upon electroporation of moDC. Tumor associated antigen specific CTL induction efficiency was significantly enhanced when codon modified IL21 mRNA was co-electroporated with tumor associated antigen mRNA. Tumor associated antigen specific T cells induced by codon modified IL21-DC demonstrated increased cytotoxic capacity and killing compared to control cultures. In conclusion, ectopic expression of codon modified IL21 by moDC enhances the priming efficiency of the DC as well as the cytotoxic potential of the induced CTL.

1. Introduction

In immunotherapy of cancer, dendritic cells (DC) loaded with tumor associated antigens (TAA), are often used to induce anti-tumor effects via the activation of specific T cells. DC can take up, process and present antigens to T cells. The number of DC found in peripheral blood is low. More abundantly present monocytes can also be used to generate DC *in vitro*, thus providing a good supply of professional antigen presenting cells [1,2]. For an effective anti-tumor immune response both cytotoxic and helper T cells should be activated via MHC class I and II respectively [3–7]. DC transfected with full length TAA mRNA can process the translated protein and present potentially immunogenic peptides without prior knowledge of the MHC restriction elements [8] Potentially resulting in a broader TAA-specific T cell response independent of patient's HLA haplotype or predetermined specific epitopes [9].

Under physiological conditions immature DC are better equipped to take up antigen from the extracellular environment than mature DC. A number of groups have used TAA mRNA electroporated monocyte derived dendritic cells (moDC). The DC were either electroporated prior to or after maturation. It was shown that electroporation after maturation resulted in superior CTL induction [10,11]. Others and we have found that the levels of translated protein are highest in the first 24–48 h after electroporation [11–13]. Co-electroporation of TAA with mRNA encoding the DC activating proteins CD40, CD70 and constitutively active TLR-4 was shown to lead to a substantial increase in the numbers of TAA specific T cells [9]. Co-electroporation of IL12 mRNA has been shown to have a positive effect on the percentage and functional avidity of TAA specific T cells [13].

Interleukin 21 (IL21), mainly produced by Th17 cells, is a cytokine that plays a central role in the differentiation and proliferation of T and B cells [14,15]. The receptor for IL21 is present on T cells, B cells, NK cells, and various populations of myeloid cells [16]. IL21 has been shown to induce an effector memory phenotype for CD8+ T cells which is associated with long term tumor immunity [17,18].

In mice it has been shown that IL21 can stimulate CD8+ T cells to proliferate, without concomitant stimulation of regulatory T cells [19]. IL21 has also been shown to reduce tumor growth, to increase the influx of tumor infiltrating lymphocytes (TIL) and tumor specific CD8+ T cells in murine models of renal carcinoma and malignant melanoma [20–23].

In humans, IL21 has been given systemically in three clinical trials in patients with malignant melanoma and renal cell carcinoma, resulting in a small number of complete responses and several partial responses [24–27]. After analyzing the immunological responses in these patients, an increase in the cytotoxic capacity of the CD8+ T cells and NK cells was found [28].

Here we investigated the effects of enforced production of IL21 by mature dendritic cells on the induction of tumor antigen specific T cells. For this purpose we made used of dendritic cells electroporated with mRNA encoding the tumor antigen alone or in combination with wild type or codon modified interleukin 21 mRNA. Effects on overall and antigen specific T cell proliferation as well as cytotoxic capacity and target cell killing were investigated.

2. Materials and methods

2.1. DNA vectors and *in vitro* transcription of mRNA

For *in vitro* transcription (IVT) of mRNA we cloned several DNA sequences in the empty pGEM/4Z vector, kindly provided by Dr. V. van Tendeloo (Antwerp, Belgium). The IL21 sequence was obtained from CD4+ T cell derived cDNA of a healthy donor's blood using the following primers: Forward: TATACCATGGGCTCCAGTCCTGGCAA CATGG and reverse: TCCTCAGGAATCTTCACTTCCGTGTGT. The PCR product was used to generate pGEM/IL21 (referred to as wild type IL21). At a later stage we also used a codon modified version of the IL21 sequence (referred to as codon modified IL21) which was obtained from GeneArt (Regensburg, Germany). Codon modification has been described to increase protein expression by maximizing translation efficacy and stabilizing the mRNA [29]. We also made use of pGEM/eGFP and pGEM/IL12 as negative and positive controls in T cell inductions [13]. A codon modified minigene (Ubi(Mart)$_4$) containing four repeats of the altered peptide ligand ELA- GIGILTV of Mart-1 in a string of beads manner (GeneArt) was used as described before [13]. IVT was carried out according to manufacturer's protocol of the T7 mMessage-mMachine kit (Ambion, Huntingdon, Cambridgeshire, UK) to generate m7G(50)pppG capped mRNA (CAP). mRNA quality was checked by agarose gel electrophoresis. RNA concentration was assayed by spectrophotometrical analysis at OD_{260}.

2.2. Dendritic cell generation and phenotyping

Healthy donor derived peripheral blood mononuclear cells (PBMC) were isolated from an HLA-A2.1 positive buffycoat (Sanquin, Amsterdam, The Netherlands) by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. Monocytes were isolated by positive selection using a MACS column (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). PBMCs were stained with anti CD14 beads (Miltenyi Biotec) followed by MACS sorting according to the manufacturer's protocols. Subsequently, the monocytes were cultured with 100 ng/mL recombinant human granulocyte colony stimulating factor (rhGM-CSF; Sagramostim, Berlex, Seattle, WA, USA) and 10 ng/mL interleukin-4 (IL4; R&D systems) for 6–7 days in IMDM medium supplemented with 8% fetal calf serum (FCS; HyClone, Logan, UT, USA), 2 mM L-glutamine and antibiotics (100 IE/mL penicillin and 100 lg/mL streptomycin, Life Technologies, Carlsbad, California, USA). The immature DC were then matured by culturing them with medium containing 25% monocyte conditioned medium (MCM) (generated as previously described [30]) and 50 ng/mL tumor necrosis factor (TNF)α (Miltenyi Biotec). DC were analyzed phenotypically to determine the maturation status using flow cytometry. The following antibodies were used for phenotypic analysis: CD80, CD83, HLA-DR (BD Biosciences, Heidelberg, Germany), CD86, CD40 (Pharmingen, San Diego, CA, USA) and isotype control IgG1 (BD Biosciences) labeled with Phycoerythrin (PE). Mean fluorescence index was calculated as follows: MFI-Index = (mean fluorescence intensity marker)/(fluorescence intensity isotype).

2.3. mRNA electroporation and IL21 ELISA

The production of IL21 protein by APCs was measured with an IL21 ELISA (BioLegend, Inc., San Diego, USA). DC were electroporated according to manufacturers protocol (Cell

Projects Ltd., Kent, UK). Briefly, moDC were washed with IMDM medium without serum and subsequently washed with RNAse free wash buffer. They were electroporated with 10 lg of IL21 mRNA in 200 µL electro-buffer in 4 mm cuvets at 300 V and 150 µF (Easyject plus, Equibio, Kent, UK). Supernatants were harvested 24 h after electroporation. The cells were subsequently washed thoroughly and incubated for another 24 h in fresh medium. This was repeated for 5 days to monitor IL21 production. Supernatants were stored at -20 ^{o}C until an IL21 ELISA was performed according to manufactures' protocol (BioLegend).

2.4. Allogeneic T cell stimulations

T cells were isolated from healthy donor blood (Sanquin, Amsterdam, The Netherlands) employing the Miltenyi Macs system. All cells were mycoplasm free and were maintained at 37 ^{o}C in humidified air containing 5% CO_2. T cells were cultured in Yssel's medium [31] supplemented with 1% pooled human serum (Sanquin, Amsterdam, The Netherlands) and antibiotics (100 IE/mL penicillin and 100 µg/mL streptomycin, Life Technologies). One million CD8β positive T cells were stained with 1 lM CarboxyFluorescein Succinimidyl Esters (CFSE) and incubated with 1 x 10^5 - wtIL21, cmIL21 or eGFP mRNA electroporated allogeneic moDC. After 5 days the T cells were measured by flow cytometry to analyze the percentage of CFSE low T cells.

2.5. K32 T cell expansions

T cell expansion experiments were performed by stimulating CD8β positive T cells with genetically modified myeloid K562 cells, stably transfected with the low-affinity Fcgamma receptor CD32 (K32). Irradiated (10,000 Rad) K32 were loaded with anti-CD3 (Orthoclone OKT3 (OrthBiotech Products, Raritan, NJ)). CD8β T cells (1 x 10^6) were cultured with anti-CD3 loaded K32 cells (1 x 10^5) in Yssels medium, supplemented with 10 U/mL IL7 (R&D Systems). Recombinant IL21 (final concentration 50 ng/mL (eBioscience, San Diego, USA)) or no additional cytokine were added to the cultures. On day 7 the T cells were harvested, stained for granzyme B (clone: CLB-GB11, Sanquin, Amsterdam, The Netherlands) and used in a re-directed killing assay and measured by flow cytometry (described in paragraph 2.7).

2.6. Induction of Mart-1 peptide-specific CD8+ T cells

Isolation of resting CD8β positive T cells from healthy donor PBMC was performed by positive selection using a MACS column (Miltenyi Biotec) as previously described [32]. For this purpose, total PBL were stained with anti-CD8b mAb (clone: 2ST8.5H7, Beckman Coulter, Inc., Marseille, France) and microbead-conjugated anti-mouse IgG Abs (Miltenyi Biotec), followed by MACS sorting according to the manufacturer's protocol. Mature DC were electroporated with Ubi-(Mart)4 mRNA alone or in combination with wild type IL21 mRNA, codon modified IL21 mRNA or eGFP mRNA. IL12 mRNA co-electroporation has been previously described [13] and was used as a positive control in T cell induction experiments. One hour after electroporation DC were washed and multiple mini-cultures set up in Yssel's medium supplemented with 1% hAB and 10 U/mL IL7 and containing 0.5–1 x 10^6 CD8β T cells, 0.5–1 x 10^5 mRNA-transfected DC and 0.25–0.5 x 10^6 irradiated autologous CD4+ T cells. After 10 days and the following weeks, T cells were analyzed for tetramer binding using PE- and/or APC-labeled HLA-A*0201 tetramers

(Tm) presenting the Mart-$1_{26–35}$A27L epitope. Tetramer staining was performed in PBS supplemented with 0.1% BSA and 0.01% natrium azide for 15 min at 37 °C. On day 10 the bulk cultures were re-stimulated with mRNA electroporated DC and the next day 20 U/mL IL2 (R&D systems, Oxon, UK) was added. Bulk cultures were re-stimulated weekly as described above.

2.7. Functional assays

To determine cytotoxic potential, T cells were stained with granzyme B-PE (clone: CLB-GB11, Sanquin, Amsterdam, The Netherlands) after a rest period of at least 7 days. To visualize Mart-1 specific T cells tetramer staining were used. To determine T cell avidity, an IFN-γ peptide titration assay was performed on the stimulated T cells of two donors. T cells were stimulated with moDC loaded with decreasing amounts of Mart-1 26L peptide for 4 h in the presence of Golgi apparatus blocker. The T cells were stained with PE-labeled anti-IFN-γ (BD Biosciences) and tetramer before flow cytometric analyses. An estimation of T cell avidity was obtained through stimulation of T cells with suitable target cells pre-incubated with decreasing amounts of synthetic peptide. The percentage of IFN-γ producing cells found at the highest concentration of peptide (1 μM) used, was set to one. Relative T cell avidity was compared at half maximal IFN- γ positivity [33].

A re-directed killing assay was used to determine T cell killing capacity. For this assay 15,000 irradiated (10,000 Rad) K32 (target) cells stained with CFSE and loaded with OKT3 were incubated with 15,000 or 30,000 T (effector) cells. After 4 or 8 h the CFSE positive cells were stained with 7-AAD (BD Biosciences, Heidelberg, Germany) to determine cell death.

2.8. Statistical analysis

Data points showing a Gaussian distribution were analyzed using the paired Student's *t*-tests or one-way ANOVA tests with a Tukey post to determine statistical differences. In case of a non-Gaussian distribution, the Mann–Whitney *U* test or Kruskall–Wallis test was used, followed by Dunn's post test. Findings were considered statistically significant when *p*-values were below 0.05. Statistical analyses were performed using GraphPad Prism (Version 5 (2007), GraphPad Software, Inc., La Jolla, California, USA).

3. Results

3.1. IL21 production by mRNA electroporated dendritic cells

In vitro transcribed mRNA was used to transfect DC by means of electroporation as described before [13,34]. To facilitate the production of IL21 by DC we used the construct pGEM/wtIL21 and as a control pGEM/eGFP. The electroporation efficiency was measured by analyzing the percentage of GFP positive cells, 24 h after electroporation, using flow cytometry. The percentage of GFP positive cells was about 90% when using the pGEM/eGFP construct (Supplementary Fig. 1A shows data from one donor). IL21 production was measured in the supernatant of electroporated DC by ELISA and was calculated to reach about 600 pg/1 x 10^6 cells/24 h, after which it dropped rapidly (Supplementary Fig. 1B and C).

3.2. Lack of negative effects on DC phenotype of IL21 production

To investigate potential negative effects of IL21 electroporation (as suggested by an earlier report [35]) on mature DC we monitored the expression of DC (co-stimulatory) markers after electroporation of mRNA encoding eGFP as a control and of mRNA encoding IL21. We determined the percentage and the mean fluorescence intensity (MFI) of the markers CD40, CD80, CD83, CD86, and HLA-DR after electroporation of mature moDC. Negligible differences were found between wtIL21-DC or eGFP-DC. Supplementary Fig. 2A shows data of one representative donor out of four donors tested.

Next, the CD8+ T cell stimulatory capacity of wtIL21-DC and eGFP-DC was analyzed employing an allogeneic T cell proliferation assay. Cells from four different donors were used, resulting in three DC-T cell combinations in this assay. Although the expression levels of co-stimulatory markers were comparable, wtIL21-DC, showed a small, but non significant increase in the proliferation of allogeneic CD8+ T cells compared to control eGFP-DC (Supplementary Fig. 2B).

3.3. Effects of IL21 mRNA transfected DC on TAA specific T cells

We next investigated antigen specific stimulatory effects of wtIL21-DC using primary CD8+ T cell cultures. To this end PBMCs from 3 different healthy donors were incubated with autologous mature moDC electroporated with either Ubi-$(Mart)_4$ mRNA alone, or in combination with wtIL21 mRNA. Per donor, the experiments were set up in multiple mini-cultures (6–9 separate cultures per condition, depending on cell numbers available). For one of the donors we also included the combination of Ubi-$(Mart)_4$ and IL12 electroporated DC as a comparison. Fig. 1(A) shows the results of tetramer staining of T cell cultures obtained after the second *in vitro* stimulation. Compared to control cultures on eGFP-DC, donor 2 showed significantly higher percentages of tetramer positive T cells in the wtIL21-DC condition. This was, however, not found for donor 1 and donor 3. In comparison, IL12-DC more efficiently enhanced the outgrowth of Mart-1 specific T cells in donor 3.

T cell cultures resulting from donor 3 were used to determine T cell avidity. T cells were stimulated with JY cells exogenously loaded with a range of synthetic peptide concentrations and subsequently used in an interferon-γ production assay. From the data, presented in Fig. 1(B), it is clear that resulting T cells from the wtIL21-DC cultures were

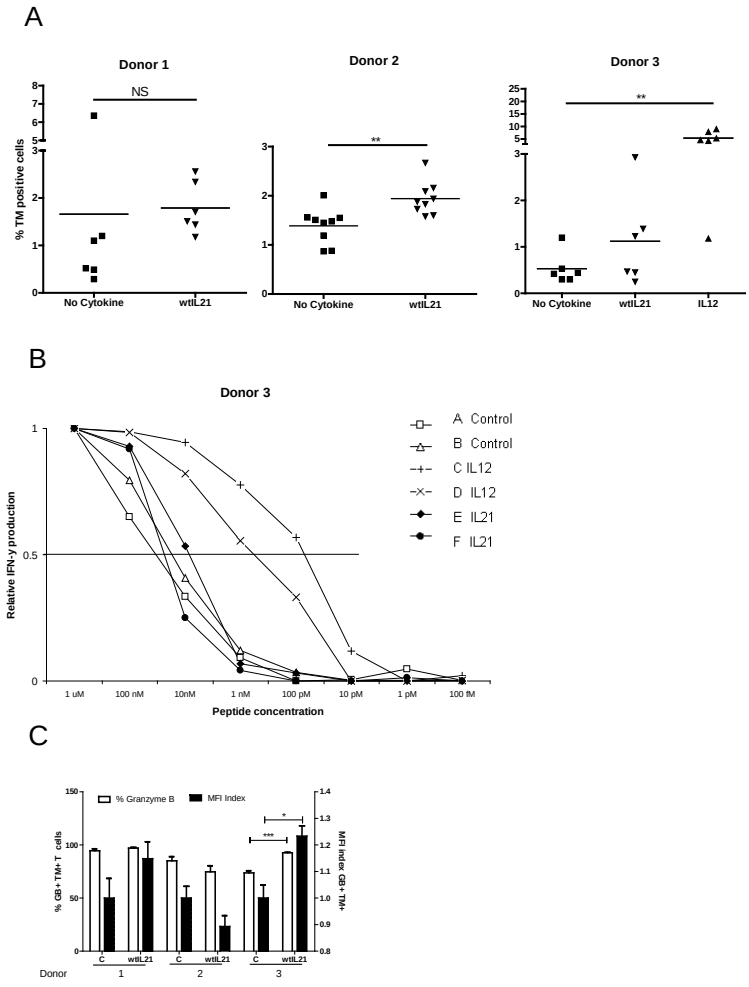

Fig. 1. Effects of IL21-DC on TAA specific T cells. *Dendritic cells were electroporated with mRNA encoding Ubi-Mart alone or in combination with wild type IL21 (or IL12 in donor 3). Autologous CD8 positive T cells were stimulated twice with these DC, and resulting cultures were examined for tetramer binding and the production of IFN-γ.* ***(A)*** *The percentage Mart-1 tetramer positive T cells is indicated for three donors. Statistical analyses performed for donor 1: Mann–Whitney U, not significant; for donor 2: student t test, $p < 0.01$; for donor 3: Kruskall–Wallis test, with Dunn's post test, $p < 0.01$ no cytokine versus IL12.* ***(B)*** *Resulting T cells from donor 3 were used in a peptide titration assay measuring IFN-γ. The maximum percentage of IFN-γ tetramer positive T cells was set to 1. Relative IFN-γ production of two different cultures of TAA specific T cells stimulated with either Ubi-Mart alone (open symbols), Ubi-Mart/IL21 (closed symbols) or Ubi-Mart/IL12 (symbols: plus and cross).* ***(C)*** *The percentage of granzyme B positive T cells in the Mart-1 tetramer positive population is shown in the left Y-axis. MFI index of the granzyme B tetramer positive population is shown in the right Y-axis. No significant difference (student t test) in Granzyme B was found for donor 1 and 2. In donor 3, a significantly higher percentage of granzyme B positive T cells ($p < 0.001$) with a higher MFI index ($p < 0.05$) was found.*

of equal avidity when compared to T cells derived from control cultures with eGFP-DC. As expected, T cells from the IL12-DC cultures were of higher avidity when compared to controls. Interestingly, however, the granzyme B expression in T cell cultures derived from the wtIL21-DC were higher when compared to controls in this donor (Fig. 1C).

3.4. Higher amounts of IL21 increase granzyme B content and killing capacity of T cells

In view of the marginal effects of wild type IL21-DC on T cells we performed an experiment using recombinant IL21 at a concentration of 50 ng/mL, focusing on the effects of IL21 on the cytotoxic potential of T cells. The increase in expression of granzyme B (Supplementary Fig. 3A), seen in T cells cultured in the presence of recombinant IL21, clearly correlated with increased killing ability of these T cells (Supplementary Fig. 3B) compared to controls without IL21.

3.5. Codon modification of IL21 leads to higher IL21 production

To improve on mRNA stability and increase translation into protein, the wild type IL21 DNA sequence was codon optimized for expression in human cells. We compared the presence of IL21 in the supernatant of moDC electroporated with either wild type (wt) IL21 or codon modified (cm) IL21 mRNA for two different donors (in three separate mini cultures per donor per condition). The amount of IL21 protein, measured by ELISA, appeared to be 5–7 times higher in the supernatant of the cmIL21-DC compared to wtIL21-DC (Supplementary Fig. 3C).

DC electroporated with cmIL21 mRNA were also analyzed for the presence and level of expression of co-stimulatory markers as described above (Results Section 3.2). Despite the increase in IL21 production by cmIL21-DC, we again only found small differences in the expression of co-stimulatory markers (shown in Supplementary Fig. 2A). The proliferative effects of IL21 producing DC on allogeneic CD8+ T cells are shown in Supplementary Fig. 2B. From these data it is clear that wtIL21-DC were capable of increasing T cell proliferation moderately, whereas the cmIL21- DC increased T cell proliferation significantly when compared to eGFP-DC.

3.6. Effects of IL21 codon modification on the induction of tumor antigen specific T cells

Next, we stimulated T cells with autologous mature DC electroporated with Ubi-(Mart)4 in combination with either wild type or codon modified IL21 mRNA, or with control eGFP mRNA. DC-T cell stimulations were set up for three different donors, to compare the effects of eGFP-DC, wtIL21-DC and cmIL21-DC. For donor 4 the T cell stimulations with wtIL21-DC were omitted. After three rounds of *in vitro* stimulation the presence of tetramer positive T cells was analyzed and plotted for each separate mini-culture (Fig. 2A). In three out of four donors tested, the percentage of Mart specific T cells was increased significantly in the cmIL21-DC conditions compared to control eGFP-DC. In three out of three donors tested we observed a significant increase of Mart specific T cells in the cmIL21-DC condition compared to wtIL21-DC. In three out of three donors tested we noted near equal percentages of specific T cells comparing control eGFP-DC versus wtIL21-DC conditions. Compared to control eGFP-DC conditions, the total number of T cells was significantly increased in the cmIL21-DC condition (data not shown).

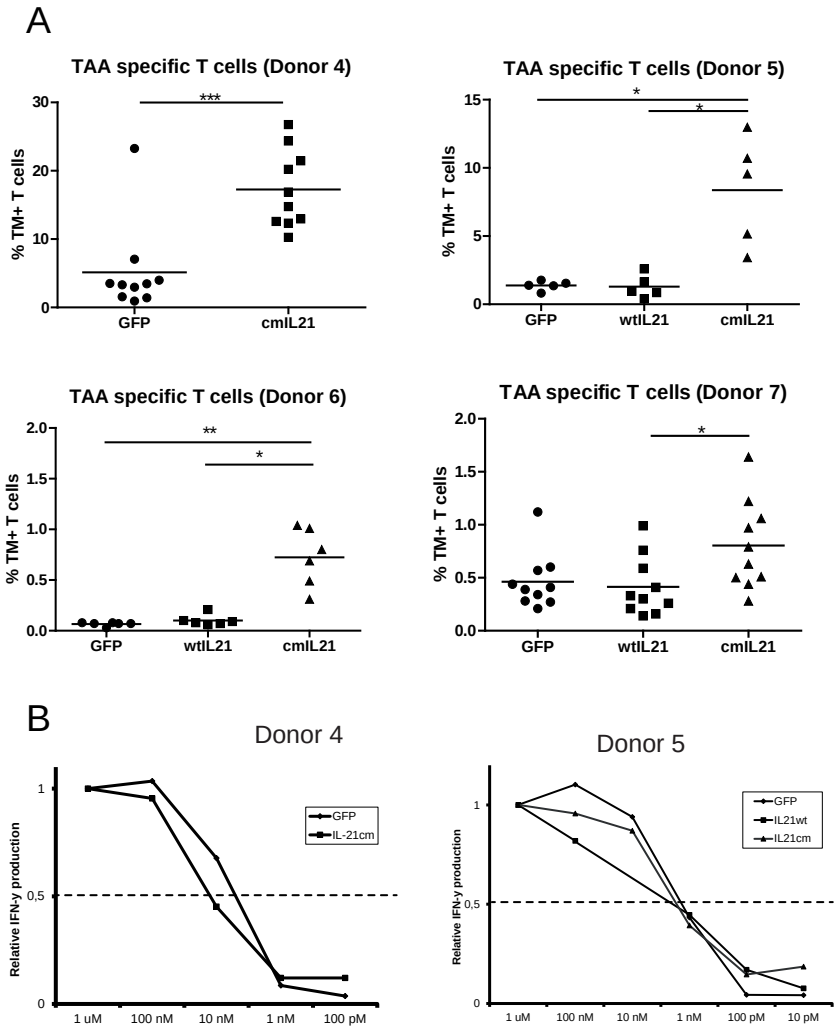

Fig. 2. Effects of codon modified IL21-DC on TAA specific T cell proliferation avidity. *Dendritic cells were electroporated with mRNA encoding Ubi-Mart plus GFP, Ubi-Mart plus wtIL21or Ubi-Mart plus cmIL21 mRNA. Autologous CD8+ T cells were stimulated three times with these DC and resulting cultures were examined for tetramer binding and IFN-γ production.* ***(A)*** *The percentage of Mart-1 tetramer binding T cells after stimulation with autologous moDC electroporated with Ubi-Mart and GFP, wtIL21 or cmIL21 mRNA for four donors. A Mann–Whitney U test was performed for donor 4 ($p < 0.001$). A Kruskal–Wallis test with Dunn's post test was performed for donors 5, 6 and 7; significant differences were found between cmIL21 and GFP (donors 5 and 6: $p < 0.05$) and cmIL21 and wtIL21 (donors 5, 6 and 7: $p < 0.05$).* ***(B)*** *Resulting T cells were used in a peptide titration assay measuring IFN-γ of the tetramer positive population from donors 4 and 5. The maximum percentage of IFN-γ tetramer positive T cells was set to 1. Relative IFN-γ production of TAA specific T cells stimulated with either Ubi-Mart/eGFP (symbol: closed diamond), Ubi-Mart/wtIL21 (symbol: closed square) or Ubi-Mart/ cmIL21 (symbol: closed triangle).*

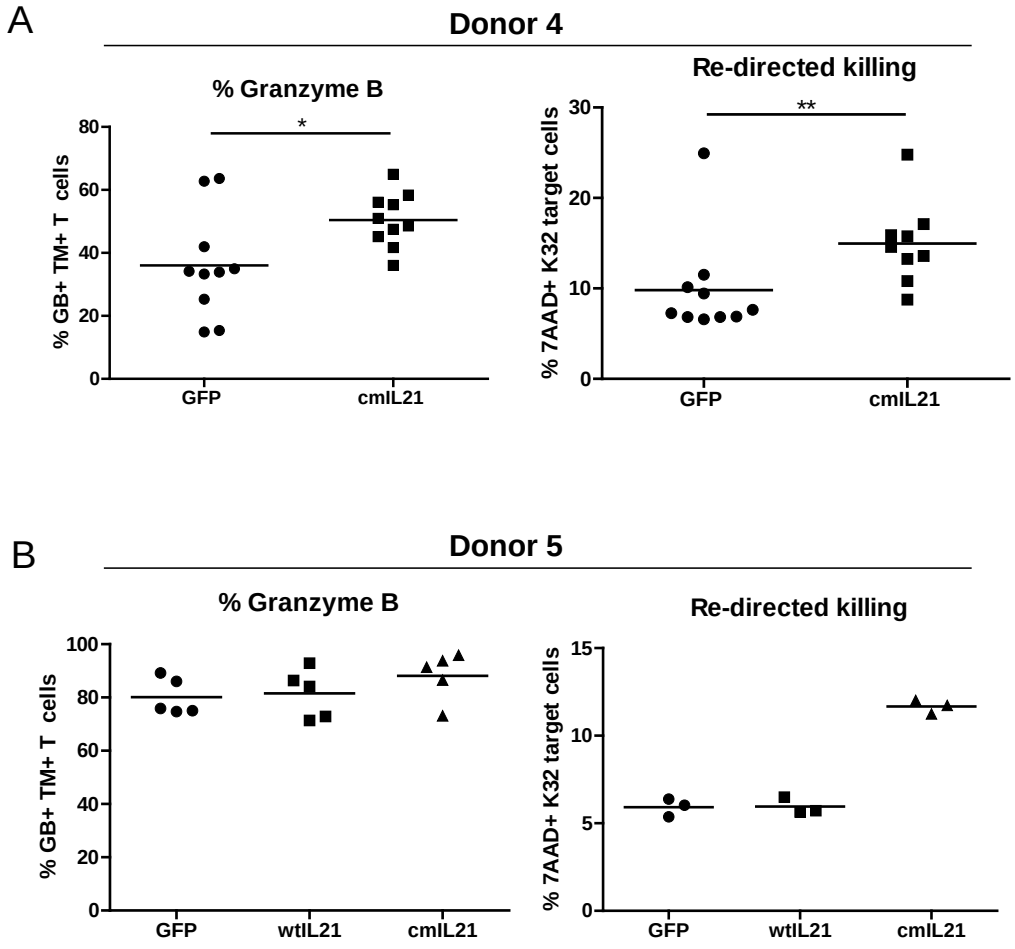

Fig. 3. Effects of wild type versus codon modified IL21-DC on cytotoxic capacity. *T cells resulting from Mart-1 specific inductions from donor 4 and 5 were analyzed for granzyme B and killing capacity.* ***(A)*** *The percentage of granzyme B positive T cells in the Mart-1 tetramer positive population (left panel) and % target kill (right panel) from donor 4. A student t test was performed and revealed significant differences for the percentage of granzyme B positive cells ($p < 0.05$). The T cells were used in a re-directed killing assay shown in the right panel. Irradiated, OKT3 loaded and CFSE labeled K32 cells were used as loaded targets. The percentages of 7-AAD positive K32 cells are shown. A Mann–Whitney U test was performed and showed significant differences ($p < 0.01$).* ***(B)*** *The percentage of granzyme B positive T cells in the Mart-1 tetramer positive population (left panel) and % target kill (right panel) from donor 5. The T cells were used in a re-directed killing assay shown in the right panel. Irradiated, OKT3 loaded and CFSE labeled K32 cells were used as loaded targets. The percentages of 7-AAD positive K32 cells are shown.*

In accordance with the experiments shown in Fig. 1, where we used wtIL21, we found no differences in T cell avidity after stimulation with cmIL21-DC compared to the eGFP-DC (Fig. 2B). In the tetramer positive population we found significantly higher percentages of granzyme B positive T cells after stimulation with cmIL21-DC compared to the eGFP-DC (Fig. 3(A) and (B) left hand panels). Similar effects on granzyme B expression were observed in the tetramer negative T cell population (data not shown). Subsequently we used the T cells in a killing assay. From the results, shown in Fig. 3(A) and (B) right hand panels, it is clear that the T cells arising after stimulation with cmIL21-DC have a higher killing capacity compared to those from eGFP-DC controls.

4. Discussion

For the induction of tumor antigen specific T cells, dendritic cells loaded with tumor antigens and equipped to produce appropriate cytokines are in use for pre-clinical investigations which will hopefully lead to clinical application. The cytokine interleukin 21 has a promising T cell activation profile. Recombinant IL21 has been used in *in vitro* experiments for the induction of virus and tumor specific human T cells [18,36–38].

In order to facilitate ectopic production of IL21 by mRNA electroporated dendritic cells, we made use of two pGEM-constructs; one containing the unmodified wild type sequence of the IL21 open reading frame and another in which the sequence was codon modified for high level expression in mammalian cells. In both cases, IL21 was produced by electroporated DC. The amount of IL21 produced from the wild type construct ranged from 400 to 600 pg/10^6 cells/24 h. The amount of IL21 produced by cmIL21-DC was 5–7 times higher compared to wtIL21-DC derived from the same donor, although the amounts varied substantially between donors. In our experimental wells the concentration of IL21 in the wild type conditions ranged between 40 and 60 pg/mL and in the codon modified conditions between 200 and 400 pg/mL. This is considerably lower compared to the amounts of recombinant IL21 used by others in *in vitro* stimulations [18,36–38].

Several studies on the (direct) effects of IL21 on dendritic cells have been reported in the literature (reviewed in [39,40]). Strengell *et al*., showed that pretreatment of immature DC with recombinant human IL21 inhibited LPS-stimulated DC maturation and expression of the DC marker CD86 and of HLA class II [35,41]. In contrast to this we did not find a difference in cell surface expression of the DC markers CD40, CD80, CD83, CD86 and HLA-DR between mature control eGFP-DC, wtIL21-DC or cmIL21-DC. Apparently immature DC responds differently to IL21 than mature DC. Furthermore, we employed a different method based on TNF-α and MCM instead of LPS, for the maturation of dendritic cells. In how far differences in IL21 concentration in the DC cultures play a role is unclear. In our *in vitro* stimulation experiments we have always used TNF-α and MCM matured DC.

Li *et al*., documented that T cell induction in the presence of re- combinant IL21 shows a dose response effect; concentrations up to 30 ng/mL were increasingly favorable, whereas concentrations of 100 ng/mL were deleterious [36]. In our experiments we used DC transfected with either wtIL21 or cmIL21 mRNA. The IL21 concentration in the supernatant of the wtIL21-DC cultures was comparable to the lower concentrations of recombinant IL21 used by Li *et al*. This however did not result in a significant induction of Mart-1 specific T cells above controls lacking IL21 in any of the three donors shown in Fig. 1. Enhancing translation of the IL21 mRNA by codon modification of the IL21 open reading frame resulted in more pronounced effects on T cells. This resulted in a clear and significant increase in the percentage of tetramer positive T cells in the multiple mini bulk cultures shown in Fig. 2(A). Furthermore it resulted in an increased percentage of granzyme B positive T cells and increased killing capacity (data shown in Fig. 3). Despite the notion that the concentrations we found in the supernatants were lower than the optimal concentrations defined by Li *et al*., one could argue that the local concentration of IL21, at the site of T cell-DC interaction, may have been higher than the overall concentration measured in the supernatant. On the other hand the data by Li *et al*., suggests there may be room for

further improvement if we are to apply cmIL21-DC for clinical application.

In a number of cases we were able to determine the functional avidity of T cells obtained after multiple *in vitro* stimulations with IL21-DC or controls. From the data shown in Figs. 1(B) and 2(B), using interferon production as read-out parameter, it is clear there is no apparent difference in avidity of Mart specific T cells resulting from either wild type IL21, codon modified IL21 or eGFP-DC stimulations. This seems to be in contrast to the results of Li *et al*., who showed differences in lytic activity, measured as chromium release, after DC stimulation in the presence of recombinant IL21 of T cells derived from one healthy donor and one melanoma patient. In re-directed killing assays, we have found that T cells arising from IL21-DC cultures more efficiently lysed target cells compared to control T cells. We also showed that the presence of IL21 led to an increase the percentage of T cells positive for granzyme B, which is a marker often used as an indicator for killing capacity. Moreover we showed increased killing capacity in T cells stimulated with IL21-DC compared to controls. These findings are in agreement with published data employing recombinant IL21 [21,36], where the cytolytic machinery of the cells is measured instead of IFN-γ production.

In murine studies IL21 seems to be an ideal candidate as an adjuvant in immunotherapy of cancer. IL21 has the potential to stimulate cytotoxic T cells but not T regulatory cells [42–44]. In mice it has been shown that intratumoral injection of IL21 resulted in enhanced CD8 positive T cell mediated antitumor immunity compared to subcutaneous IL21 injections, thus stressing the importance of local administration of IL21 for the tumor microenvironment [45]. Clinical trials with IL21 have shown moderate results [24–27] as already described in the introduction. IL21 has been shown to have anti-tumor potency, however, it can hamper DC maturation and thereby inhibiting T cells responses [46]. Furthermore, IL21 has been associated with autoimmune disease (e.g., lupus and rheumatoid arthritis), therefore administering high doses of IL21 might constitute a risk [47,48]. Therefore local production of IL21 may be preferred over systemic administration.

We have used autologous moDC electroporated with mRNA to accomplish IL21 release locally. With this method local high levels of IL21 can be reached for instance in the lymph nodes where many DC/T cell interactions take place. From the perspective of clinical application it would also be attractive to use DC from a non-autologous, unlimited source. DC derived from the human acute myeloid leukemia (AML) cell line MUTZ-3 closely resembles moDC in terms of phenotype and T cell stimulatory capacity [49]. IL21 could be stably transduced in a cell line like this ensuring IL21 locally for a prolonged time.

In summary we have shown that dendritic cell delivered interleukin 21 contributes to the induction of tumor antigen specific T cells, by enhancing T cell proliferation and cytotoxic potential. We have shown that these effects depend on the amount of interleukin 21 present. Codon modification of the interleukin 21 open reading frame greatly enhanced the production of the cytokine and could be considered for general application in messenger RNA electroporated DC-based vaccines.

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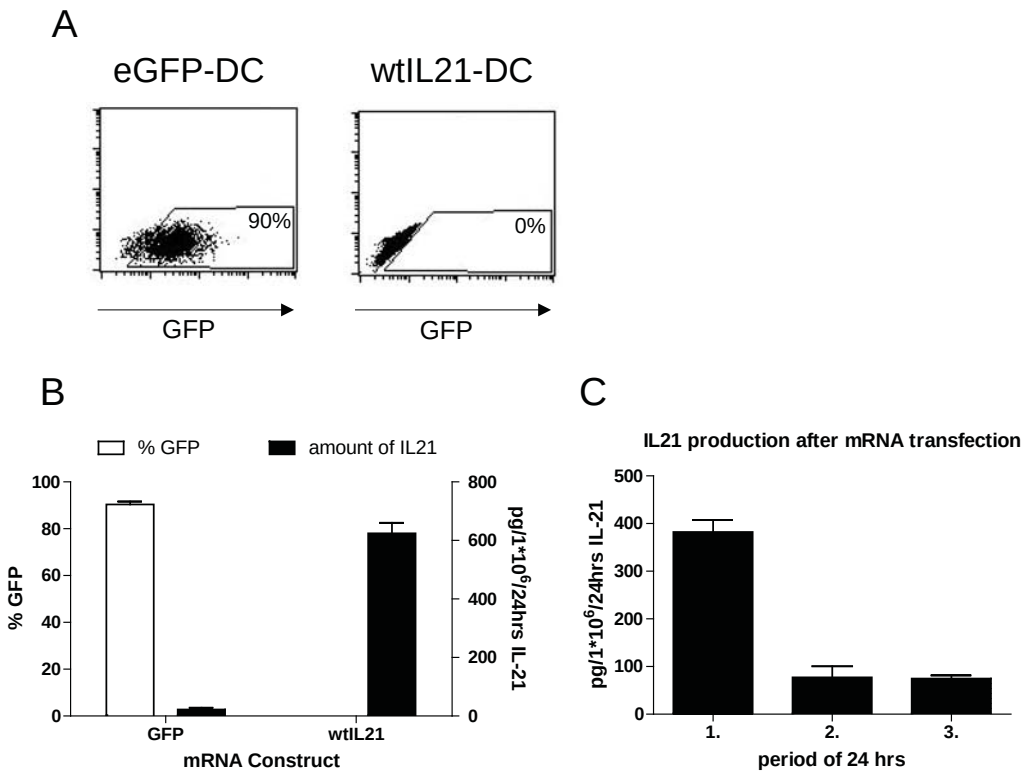
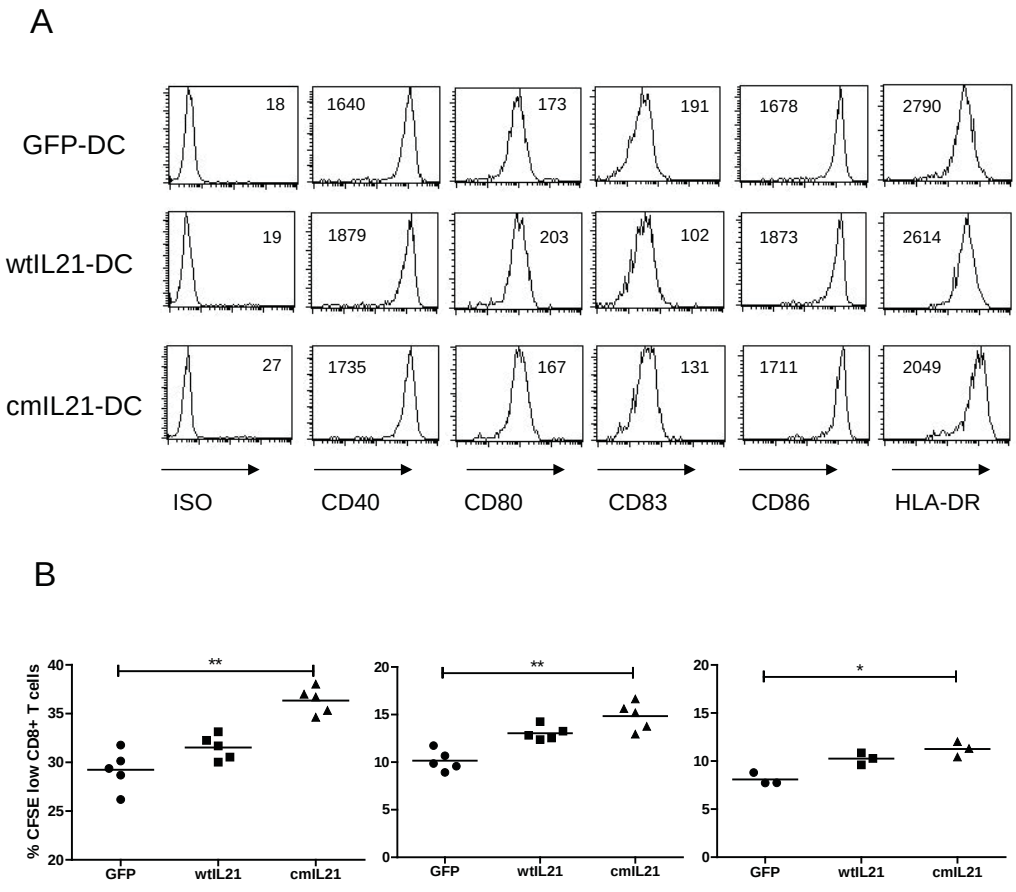
Appendix A. Supplementary data



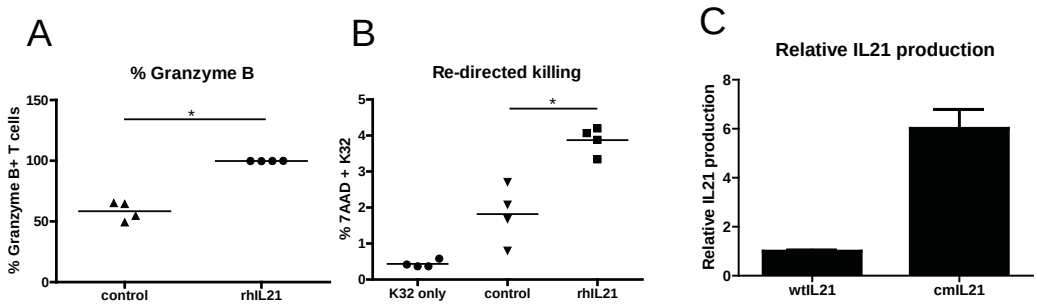
Supplementary Figure 1) GFP expression and cytokine production by mRNA electroporated dendritic cells.
Mature dendritic cells electroporated with mRNA were analyzed for GFP expression and IL21 production. ***A)*** *Mature DC were electroporated with mRNA encoding either GFP or IL21. One hour after electroporation the cells were incubated at 1*10^6/ml for 24 hours. Representative dot plots are shown for the percentage of GFP positive cells after DC transfection with eGFP mRNA (left panel) and IL21 (right panel).* ***B)*** *Indicated is the percentage of GFP positive cells (Y-axis left hand side, closed bars) and the amount of IL21 produced by 1*10^6 DC per ml for 24 hours (Y-axis right hand side, open bars) as measured by ELISA.* ***C)*** *Production of IL21 was measured over a period of 5 days after electroporation of mature DC with mRNA encoding unmodified IL21. DC were electroporated and incubated at 1*10^6/ml for 24 hours. After this period the supernatant was harvested, the cells were washed and incubated in fresh medium for another 24 hrs. This procedure was repeated for five consecutive days. Production of IL21 is shown for the first 3 days. The level of production at days 2-5 did not exceed the lower detection level of around 100pg.*



Supplementary Figure 2) Proliferative effects and co-stimulatory markers of eGFP-DC , wtIL21-DC and cmIL21-DC

A) *Mature dendritic cells from four different donors were transfected with mRNA encoding eGFP, wtIL21 or cmIL21. The presence of the following markers was measured 24 hours after transfection: IgG1 (Iso), CD40, CD80, CD83, CD86 and HLA-DR. The presence of these markers has been depicted in histograms with the mean fluorescence intensity for one representative donor after transfection with eGFP, wtIL21 or cmIL21.*

B) *Dendritic cells electroporated with mRNA encoding eGFP, wild type IL21 or codon modified IL21 were co-cultured with allogeneic CFSE loaded CD8+ T cells for five days. The percentage of proliferating cells was determined by measuring the CFSE low CD8+ T cells. Kruskall-Wallis test, with Dunn's post test were performed to determine statistical significance: eGFP versus cmIL21 p<0.01 (left panel), eGFP versus cmIL21 p<0.01 (middle panel), eGFP versus cmIL21 p<0.05 (right panel). No statistical differences were found between wtIL21 and eGFP or wtIL21 and cmIL21 in all three experiments.*



Supplementary Figure 3) Cytotoxic capacity and killing of T cells co-cultured with recombinant IL21 and increased IL21 production after codon modification.
T cells stimulated with OKT3 loaded K32 cells were cultured in the presence or absence of IL21. After 5 days the T cells were harvested and analyzed for granzyme B expression and killing capacity. ***A)*** *The percentage of granzyme B positive T cells is shown for the T cells cultured with or without IL21. A Mann-Whitney U test was performed revealing significant differences ($p < 0.05$).* ***B)*** *K32 cells were loaded with OKT3 and stained with CFSE. The percentage of 7-AAD positive K32 cells is shown after co-culture with T cells cultured with or without IL21. A Mann-Whitney U test was performed revealing significant differences ($p < 0.05$) for control cultures versus IL21.* ***C)*** *Production of IL21 was measured in two different donors comparing the wild type construct to the codon modified IL21 construct. Relative IL21 production is shown, wtIL21-DC was set to 1, for cmIL21-DC the fold increase of IL21 production is shown.*