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Dendritic cell vaccination as postremission treatment to prevent or delay relapse in acute myeloid leukemia

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Key Points

- *WT1* mRNA-electroporated DCs can prevent or delay relapse in 43% of patients with AML in remission after chemotherapy.
- OS compares favorably with the new survival data from the Swedish Acute Leukemia Registry and correlates with molecular and WT1-specific CD8$^+$ T-cell responses.

Relapse is a major problem in acute myeloid leukemia (AML) and adversely affects survival. In this phase 2 study, we investigated the effect of vaccination with dendritic cells (DCs) electroporated with Wilms' tumor 1 (*WT1*) messenger RNA (mRNA) as postremission treatment in 30 patients with AML at very high risk of relapse. There was a demonstrable antileukemic response in 13 patients. Nine patients achieved molecular remission as demonstrated by normalization of *WT1* transcript levels, 5 of which were sustained after a median follow-up of 109.4 months. Disease stabilization was achieved in 4 other patients. Five-year overall survival (OS) was higher in responders than in nonresponders (53.8% vs 25.0%; $P = .01$). In patients receiving DCs in first complete remission (CR1), there was a vaccine-induced relapse reduction rate of 25%, and 5-year relapse-free survival was higher in responders than in nonresponders (50% vs 7.7%; $P < .0001$). In patients age ≤65 and >65 years who received DCs in CR1, 5-year OS was 69.2% and 30.8% respectively, as compared with 51.7% and 18% in the Swedish Acute Leukemia Registry. Long-term clinical response was correlated with increased circulating frequencies of polyepitope WT1-specific CD8$^+$ T cells. Long-term OS was correlated with interferon-γ$^+$ and tumor necrosis factor-α$^+$ WT1-specific responses in delayed-type hypersensitivity-infiltrating CD8$^+$ T lymphocytes. In conclusion, vaccination of patients with AML with *WT1* mRNA-electroporated DCs can be an effective strategy to prevent or delay relapse after standard chemotherapy, translating into improved OS rates, which are correlated with the induction of WT1-specific CD8$^+$ T-cell response. This trial was registered at www.clinicaltrials.gov as #NCT00965224. (*Blood*. 2017;130(15):1713-1721)

Introduction

Acute myeloid leukemia (AML) still has a dismal prognosis.[1,2] According to the latest data from the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute,[1] 5-year overall survival (OS) of patients with AML is only ~25%. One of the major reasons for this is that a majority of patients relapse even after complete remission (CR) is achieved with standard chemotherapy.[3] Relapse is usually caused by the persistence of a small population of residual leukemic cells, a condition designated as minimal residual disease (MRD).[4] Allogeneic hematopoietic stem-cell transplantation (allo-HSCT), the best established postremission treatment to eradicate MRD, decrease the risk of relapse, and increase survival after chemotherapy, is still beset by substantial morbidity and mortality.[5] As a consequence, allo-HSCT is generally not considered as a therapeutic option in the large group of older patients with AML. For these patients and for younger patients without compatible donors, there is currently no standard adjuvant treatment to prevent postchemotherapy relapse.[4]

The beneficial effect of allo-HSCT against leukemia is mediated in large part by T cells that are capable of recognizing antigens expressed on the leukemia cells and of subsequently mediating AML-cell killing.[6] Stimulation of autologous T cells by in vivo immunization with leukemia-associated antigens is an innovative strategy to combat relapse in AML,[7-11] acting via the reduction or

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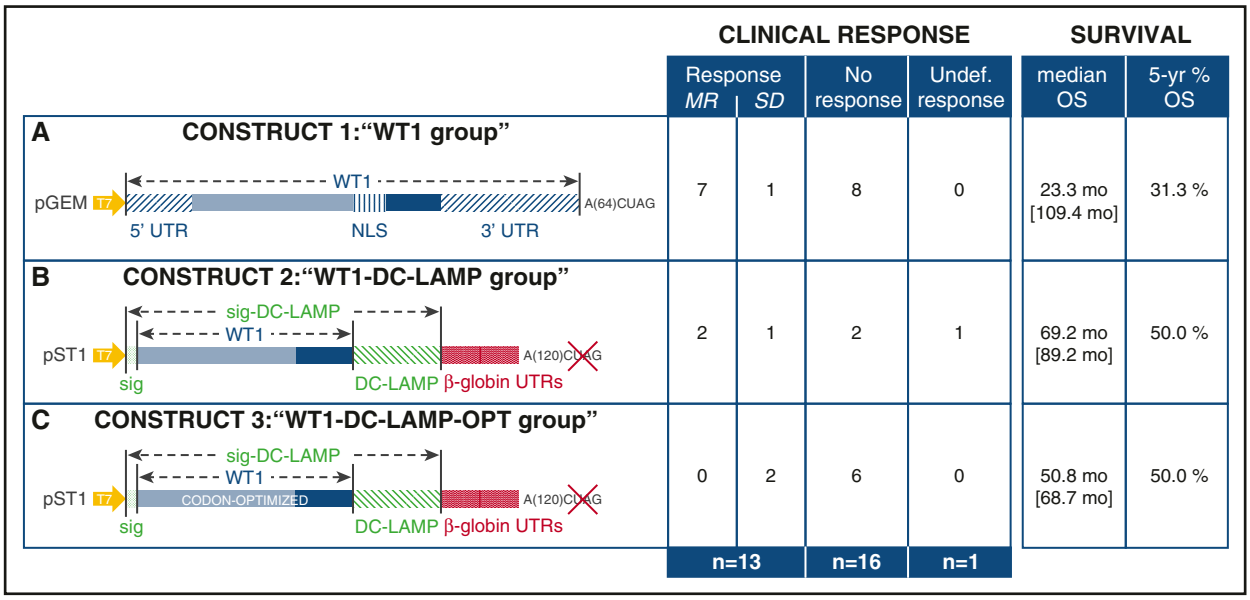

Figure 1. The 3 different *WT1* constructs used to generate mRNA for electroporation into DCs and their corresponding clinical responses and survival outcomes. Construct 1, WT1 (A); construct 2, WT1-DC-LAMP (B); and construct 3, WT1-DC-LAMP-OPT (C). Additional details have been reported by Benteyn et al.[18] Median OS and 5-year OS percentage were calculated from the start of WT1/DC vaccination; values in brackets represent median follow-up. Full-color blue bars represent the remaining coding sequence of *WT1* in constructs 2 and 3. The stable disease (SD) phase in UPN35 started during the administration of DCs electroporated with construct 3 (supplemental Table 1). MR, molecular remission; NLS, nuclear localization signal; undef, undefinable; UTR, untranslated region.

eradication of MRD. Several antigens have been identified to serve as T-cell targets in AML, including the Wilms' tumor protein 1 (WT1), which is highly overexpressed in AML and is also involved in leukemogenesis.[12] In view of their role as the most potent antigen-presenting cells of the immune system, dendritic cells (DCs) are eminently equipped to stimulate antigen-specific T-cell immunity.[13] This explains the strong interest in the use of these cells for cancer vaccination strategies.[14]

The aim of this phase 2 study was to determine the clinical efficacy of DC vaccine therapy in AML and, more specifically, whether this form of immunotherapy can be applied in the adjuvant setting to decrease the risk of relapse after chemotherapy and improve survival. To this end, we vaccinated 30 patients with AML in remission but at very high risk of relapse with autologous DCs loaded with the WT1 antigen by means of messenger RNA (mRNA) electroporation, a technique that allows for human leukocyte antigen (HLA) haplotype-independent, multiple-epitope antigen presentation to T cells.[15,16]

Methods

Patients

Thirty patients with AML were enrolled in this phase 2 study, whereby the first 10 patients were also included in a preceding feasibility, safety, and immunogenicity study (registered at www.clinicaltrials.gov as #NCT00834002).[17] This study was approved by the ethics committee of the Antwerp University Hospital, Edegem, Antwerp, Belgium. Enrollment criteria for the phase 2 study were adult patients with AML (except acute promyelocytic leukemia), diagnosed according to World Health Organization criteria, in remission after ≥1 course of polychemotherapy and at high risk of relapse as defined by: age >60 years or if <60 years without matched sibling donor for allo-HSCT, poor-risk cytogenetic or molecular markers, hyperleukocytosis at presentation, and/or previous relapse.

DC vaccination

Clinical grade *WT1* mRNA-electroporated DC (WT1/DC) vaccines were prepared and administered intradermally as described previously.[16,17] Three different *WT1* constructs were used to generate mRNA by in vitro transcription: construct 1 (WT1), encoding full-length *WT1*[16,17]; construct 2 (WT1-DC-LAMP), incorporating a Sig-DC-LAMP major histocompatibility complex (MHC) class 2–skewing signal with deletion of the WT1 nuclear localization signal; and construct 3 (WT1-DC-LAMP-OPT), a codon-optimized version of construct 2 (Figure 1; supplemental Table 1, available on the *Blood* Web site).[18]

Molecular tumor marker monitoring and clinical response criteria

Longitudinal monitoring of *WT1* transcripts was performed as described previously using an in-house assay,[17,19] the Ipsogen WT1 ProfileQuant Kit (Qiagen)[20] or the WT1 mRNA OneStep Assay (Otsuka Pharmaceutical Co).[19] *WT1* mRNA levels above background (respectively >1 and 25 copies of *WT1* mRNA per 1000 *ABL* copies in blood and marrow in the in-house assay; according to manufacturers' instructions in the commercial kits) are indicative of MRD and herald full relapse.[21-25] Responders were characterized by MR or SD. MR was defined by normalization of *WT1* mRNA levels in blood and/or bone marrow during WT1/DC vaccination, while reaching or maintaining hematological CR. SD was defined by stable *WT1* blood transcript levels above background, with stable blood values without blasts. The minimum duration of SD was 2 months, after which *WT1* mRNA levels did not increase by more than a factor of 0.5 $\log_{10}$ and ≥1 basic blood value (hemoglobin, thrombocyte count, and/or absolute neutrophil count) was normal. Patients who relapsed without achieving MR or SD status were categorized as nonresponders. The classical definitions of CR, partial remission (PR), relapse, and survival were used.[26] Long-term survivors were defined as surviving for ≥3 years[26,27] and long-term responders as patients with an MR or SD response remaining in CR for ≥3 years after the first dose of WT1/DCs.

Immunomonitoring

Detection and subtyping of anti-WT1 antibodies in pre- and postvaccination plasma samples were performed as described previously.[17,28] The increase in

WT1 immunoglobulin G antibody levels after vaccination was determined by subtraction of the corresponding prevaccination values.

Cytokine plasma levels were determined using the T-helper 1 (Th1)/Th2 multiplex immunoassay (Bender MedSystems). Ex vivo flow cytometric analysis of lymphocyte subsets was performed using directly conjugated monoclonal antibodies (BD Biosciences).

Circulating WT1-specific $CD8^+$ T cells obtained before vaccination and after the fourth dose of WT1/DCs were stained with peptide-HLA-A*0201 tetramers and quantified as described previously.[17,29] Whenever cells were available, they were also analyzed at different time points afterward until relapse and/or progression.

Two weeks after the fourth WT1/DC vaccination, delayed-type hypersensitivity (DTH) was tested against the complete vaccine (ie, keyhole limpet hemocyanin [KLH]–exposed WT1/DCs, except in patients UPN11, 12, and 13, in whom non–KLH-exposed WT1/DCs were used). This was performed by intradermal injection of 0.5×10^6 WT1/DCs on the back of the patient. Forty-eight hours later, erythema and induration at the injection site were measured and skin punch biopsies were taken for culture of DTH-infiltrating lymphocytes (DILs). These DILs were allowed to expand for 2 to 3 weeks in medium with interleukin-2 (100 IU/mL). Expanded DILs were then harvested and tested for antigen specificity as described previously.[17]

Data mining and statistical analysis

Statistical calculations and data graphing were carried out using Prism version 5.01 (GraphPad). A P value $<.05$ was considered statistically significant.

Results

Patients

WT1/DC vaccination as a postremission treatment was evaluated in 30 patients with high-risk AML in remission after chemotherapy. Patient characteristics with baseline evaluation and initial treatments are listed in supplemental Table 1. There were 15 men and 15 women, with a median age at diagnosis of 65 years. Before WT1/DC vaccination, 27 patients had achieved CR after chemotherapy, whereas 3 had PR. Six patients had a preceding hematological disorder: myelodysplastic syndrome (n = 5) or myeloproliferative neoplasia (n = 1). The cytogenetic risk group[30] was adverse in 1, intermediate in 23, and favorable in 6 patients. All patients were at increased risk of relapse, as indicated by the unfavorable prognostic features listed in supplemental Table 1. In particular, *WT1* transcript levels above background postinduction and/or postconsolidation chemotherapy were predictive of relapse[21-25] in 5 of 6, 18 of 23, and 1 of 1 patients at respectively favorable, intermediate, and adverse cytogenetic risk. Of the remaining 6 patients without increased *WT1* transcript levels after chemotherapy, only 1 with erythroleukemia in the intermediate-risk group (UPN22) did not relapse, and his response was categorized as undefinable (Figure 1).

Clinical response

There was a demonstrable antileukemic effect of the WT1/DC vaccination only without any concomitant chemotherapy in 13 of 30 patients, corresponding to a clinical response rate of 43%. Of these 13 patients, 9 went into MR, as demonstrated by the normalization of *WT1* transcript levels in blood and/or bone marrow (UPN01, 06, 08, 10, 11, 14, 15, 16, and 17). Of these 9 patients, 2 went from PR to CR (UPN08 and 16),[17] and 5 were still in CR (UPN06, 08, 10, 14, and 15) with a median duration of 114.5 months and median follow-up after the first WT1/DC vaccination of 109.4 months. Four patients relapsed after reaching MR, with 3 ultimately dying as a result of AML (UPN01, 11, and 16) and 1 achieving long-term CR after undergoing allo-HSCT (UPN17). In all relapsing patients, recurrence of AML was preceded and accompanied by increased *WT1* transcript levels. In the remaining 4 patients (UPN21, 33, 35, and 48), clinical response was characterized by SD, as demonstrated by elevated but stable *WT1* transcript levels in blood and stable blood values without blasts. An example of SD is shown in Figure 2. The stable *WT1* profile seen during SD contrasts with the steeply rising curve that is usually seen when patients with AML are relapsing.[19,23,25]

Only a minority of patients had a molecular MRD marker other than *WT1* mRNA. Overall, there was a corresponding evolution between the *WT1* transcript levels and other markers of MRD, such as the fusion transcripts *RUNX1-RUNX1T1* [translocation t(8;21)] and *CBFB-MYH11* [inversion inv(16)]. For instance, in clinical responder UPN15, the normalization of bone marrow *WT1* expression after 4 WT1/DC vaccinations (from 638 to 4 copies per 1000 *ABL* copies) was paralleled by a decrease in *RUNX1-RUNX1T1* transcript level from 1.43 to 0.0495 copies per 1000 *ABL* copies. In nonresponder UPN28, the increase in already-elevated blood *WT1* transcript level (from 24.1 to 1195 copies per 1000 *ABL* copies) was mirrored by an increase in *CBFB-MYH11* level from 10 to 469 copies per 1000 *ABL* copies.

The clinical response rate was 50% in patients with favorable cytogenetic risk (MR in 3 of 6 patients). Among the 22 treatment response–evaluable patients with intermediate cytogenetic risk, 6 experienced an MR and 4 achieved SD, corresponding to a clinical response rate of 45%. Response to WT1/DC vaccination, grouped according to the *WT1* construct used, is indicated in Figure 1 and supplemental Table 1. Of the 29 patients with a definable response or nonresponse, there was an effect in 8 of 16 in the WT1 group, 3 of 5 in the WT1-DC-LAMP group, and 2 of 8 in the WT1-DC-LAMP-OPT group. Of the 3 patients receiving DCs prepared without KLH (UPN11, 12, and 13), 1 achieved MR (UPN11).

Overall, 6 of 30 patients (UPN06, 08, 10, 14, 15, and 22) had not yet relapsed and were still in first CR (CR1), with a median duration of 107.6 months and median follow-up of 101.8 months after the first dose of WT1/DCs. Of the remaining 24 of 30 patients, 1 did not reach CR1 (UPN20), 1 died presumably as a result of a lung adenocarcinoma without morphological evidence of AML relapse (UPN07), 3 had a second or third relapse (UPN02, 16, and 34), and 19 had a first relapse. Four of these 19 patients with AML in first relapse received supportive care (UPN01, 11, 21, and 38), whereas the remaining 15 were treated with salvage therapy (chemotherapy ± allo-HSCT; supplemental Table 1). The CR2 rate in this group was 73.3% (11 of 15 patients: UPN05, 12, 13, 17, 28, 30, 35, 36, 46, 47, and 48); the remaining 4 patients had progressive disease (UPN03, 09, 29, and 33).

Survival

Survival data are shown in Figures 1 and 3, Table 1, and supplemental Table 1. Five-year relative OS data compared favorably with those reported by the SEER program of the National Cancer Institute.[1] OS and RFS were significantly higher in responders as compared with nonresponders, irrespective of age category. OS data were not influenced significantly by allo-HSCT, which was carried out in some patients who relapsed during WT1/DC vaccination (data not shown).

Of the patients in CR1 (Table 1) who received WT1/DCs, survival from diagnosis compared favorably with that of patients from the SALR, a comprehensive population-based AML database.[31] Five-year relative RFS of patients with AML in CR1 treated with WT1/DCs was similar to that of patients in the SALR. It should be noted that the risk of relapse in patients treated with WT1/DCs was most probably higher than that of patients in the SALR, some of whom (overall 32.1%) did not relapse after 5 years (Table 1). In contrast, all but 1 of the patients who were vaccinated with WT1/DCs were predicted to relapse based

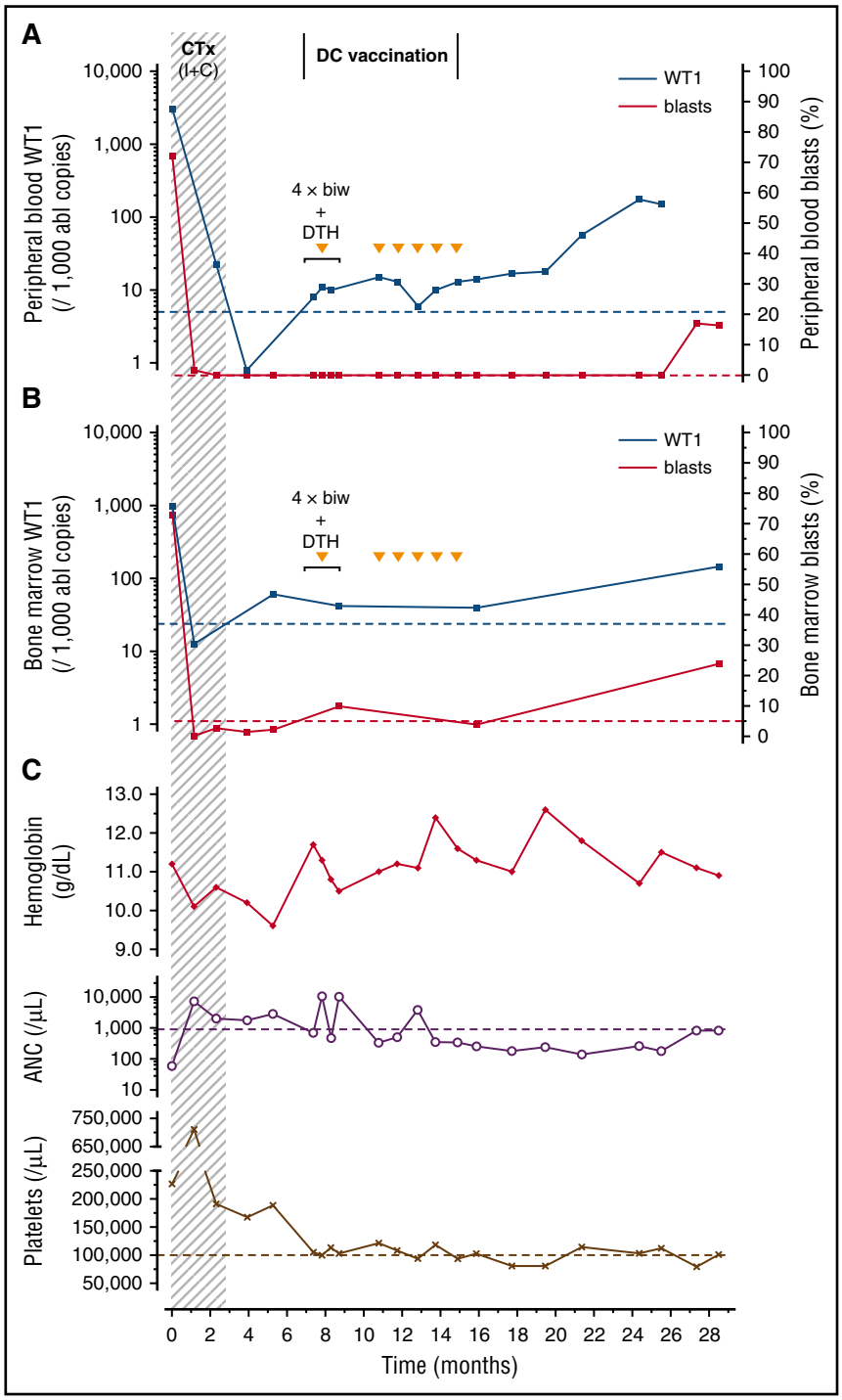

Figure 2. SD in patient UPN33 during (arrows) and after WT1/DC vaccination. *WT1* transcript levels (determined by the Ipsogen WT1 ProfileQuant Kit) in blood (A) and bone marrow (B) were above background (indicated by the dotted blue line) but remained stable, and the bone marrow blast count normalized (normal value indicated by the dotted pink line). (C) Blood values showed pancytopenia at the start of DC vaccination but a normal hemoglobin level (without transfusions) at the end of the SD period (at 19 months); neutropenia was treated with granulocyte colony-stimulating factor. 4 × biw + DTH, period of the first 4 biweekly WT1/DC vaccinations and DTH; ANC, absolute neutrophil count; CTx (I + C), polychemotherapy (induction + 2 consolidations).

on increased prevaccination *WT1* transcript levels and/or did in fact relapse. RFS of responding patients was markedly better than RFS of patients in the SALR. Of the 25 response-evaluable patients in CR1, 5 had not yet relapsed (UPN06, 08, 10, 14, and 15, all of whom were responders), compatible with a vaccine-induced relapse reduction rate of 25%. The median duration of CR1 in these 5 patients is indicated in "Clinical response." There were 16 of 30 patients in the long-term survivor category (UPN01, 06, 08, 10, 11, 12, 14, 15, 17, 21, 22, 28, 29, 33, 34, and 35).

Long-term survival was observed in 11 of 13 responders and in 4 of 16 nonresponders; the difference was significant (Fisher's exact test $P = .0025$), correlating long-term survival with response to WT1/DCs. Long-term survival was noted in 5 of 6 and 10 of 22 response-evaluable patients at respectively favorable and intermediate cytogenetic risk; the difference between the 2 groups was not significant ($P = .20$). There were no significant differences in OS outcome between the different *WT1* constructs used (Figure 1; WT1 vs WT1-DC-LAMP, $P = .48$; WT1 vs WT1-DC-LAMP-OPT, $P = .44$; WT1-DC-LAMP vs WT1-DC-LAMP-OPT, $P = .84$; WT1 vs WT1-DC-LAMP + WT1-DC-LAMP-OPT, $P = .34$).

As shown in supplemental Table 1, as of 31 December 2016, 11 of 30 patients were alive in CR, with a median OS from diagnosis of

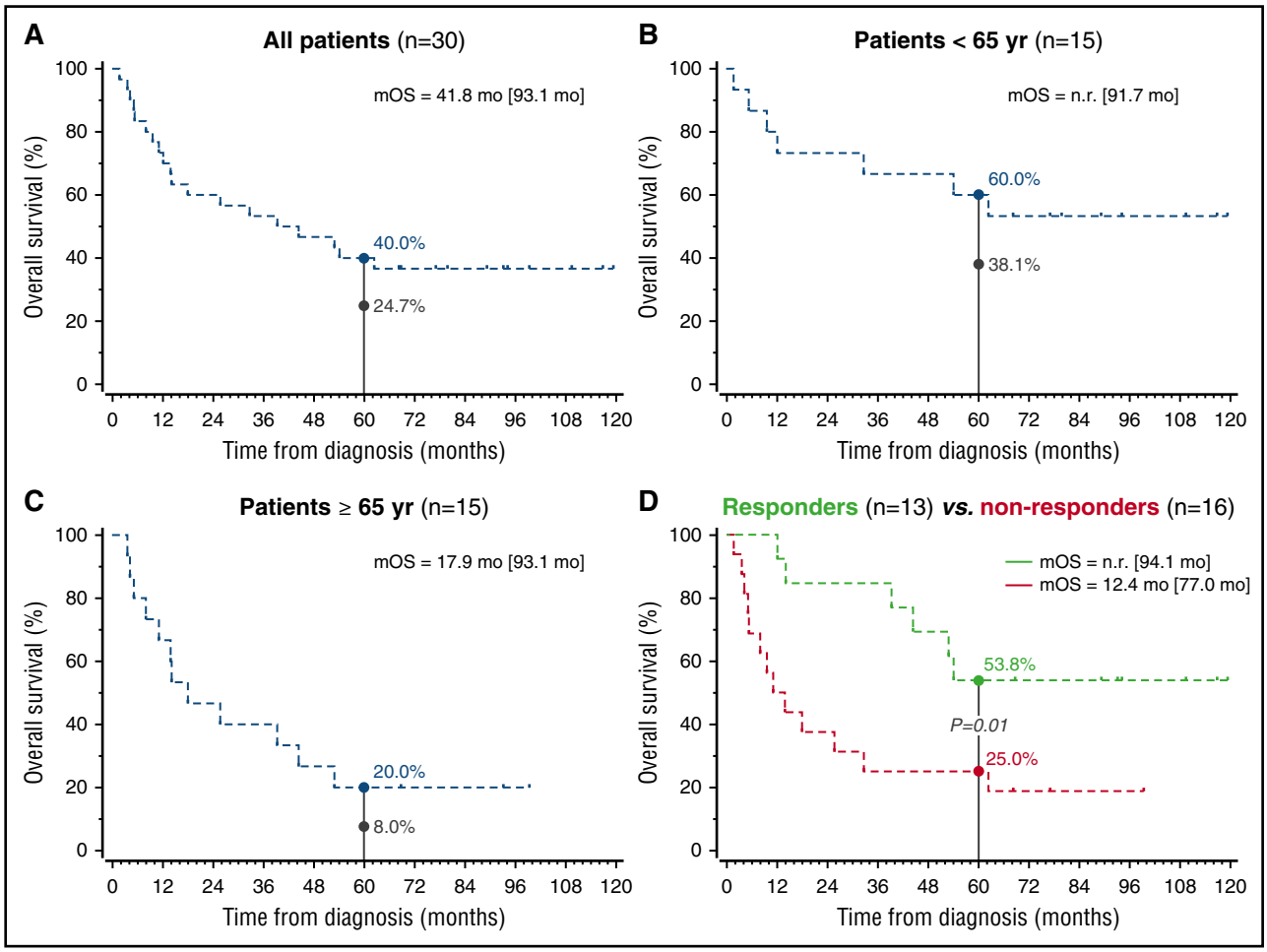

Figure 3. Kaplan-Meier curves of the OS data. The values on the curves are 5-year relative survival from the start of WT1/DC vaccination; the values underneath in gray (A-C) are 5-year relative survival data from SEER (observed survival of newly diagnosed patients with AML included in SEER*Stat database,[50] whereby the following case selection criteria were applied: age [minimum age, 30 years; maximum age, 79 years], race [white], and year of diagnosis [2005-2012]; the patient with an undefinable response [UPN22] was not included in panel D). For median OS (mOS), values in brackets represent median follow-up. n.r., not reached.

99.4 months (range, 72.6-125.5 months). Of these 11 patients, 6 were alive in continuing CR1 (UPN06, 08, 10, 14, 15, and 22). Five other surviving patients who relapsed after WT1/DC vaccination were brought back into CR by chemotherapy followed (UPN17 and 28) or not (UPN12, 34, and 35) by allo-HSCT. Remarkably, patients UPN12 and 34 were alive in continuing CR respectively >7 and 4 years after achieving CR2 and CR3 with chemotherapy alone.

Of the 19 patients with AML who were in first relapse after WT1/DC vaccination, 52.6% were alive at 3 years and 36.8% at 5 years from diagnosis. Of the latter patients, 4 achieved CR2 after chemotherapy (UPN12, 17, 28, and 35), and the other 3 had a remarkably long period of CR1 before relapsing (UPN01, 11, and 29; respectively 47, 51.6, and 59.8 months after the start of WT1/DC vaccination).

WT1/DC vaccine–induced immune responses

Immunomonitoring was performed on peripheral blood mononuclear cells, DILs, and plasma samples obtained before and after WT1/DC vaccination. There were no significant changes after vaccination with respect to: frequencies or absolute numbers of circulating lymphocyte subsets ($CD4^+$ and $CD8^+$ T cells, B cells, and natural killer cells); relative frequencies of naïve, terminally differentiated effector, effector memory, and central memory subsets within the $CD4^+$ and $CD8^+$ T-cell compartments; relative frequencies of regulatory T-cell subsets, or relative frequency of myeloid-derived suppressor cells. Th1/Th2 cytokine levels and anti-WT1 immunoglobulin G titers in plasma were also unchanged after WT1/DC vaccination.

WT1-specific T-cell responses were evaluated using pHLA-A*0201 tetramer staining and intracellular cytokine assays. Increased (>1.5-fold) frequency of WT1-specific tetramer$^+$ $CD8^+$ T cells was observed after vaccination in 6 of 12 evaluable (HLA-A*0201$^+$) patients. A significant positive correlation (P = .018) was found between long-term clinical response (UPN01, 08, and 35) and increased circulating frequency of polyepitope WT1-specific tetramer$^+$ $CD8^+$ T cells (supplemental Table 2). Of the 9 HLA-A*0201$^+$ nonresponder patients, only UPN30 had an increased number of polyepitope WT1-specific $CD8^+$ T cells. The other non–long-term responders showed either no (6 of 9 patients) or monoepitope (2 of 9 patients) WT1-specific $CD8^+$ T-cell response to WT1/DC vaccination. WT1-specific tetramer$^+$ $CD8^+$ T cells were also assessed in 7 HLA-A*0201$^+$ patients (UPN16, 17, 30, 34, 35, 38, and 47) at relapse or disease progression; frequency was not lost in any patient upon relapse. In all but 1 patient (UPN16), we observed an increase in frequencies of ≥2 epitopes as compared with the postfourth WT1/DC vaccination sample and in all patients a maintenance and/or increase for the other epitope(s) examined (data not shown).

Table 1. OS and RFS in patients with AML in CR1 enrolled in this study compared with patients in SALR

		*This study**			*SALR*†
		All	**Responders‡**	**Non-responders**	
All ages		**n=26**	**n=12**	**n=13**	**n=2495**
OS	*median*	56.1 mo	n.r. [101.7 mo]	21.1 mo	29.9 mo
	5-yr %	50.0 %	75.0 %	23.1 %	37.9 %
			P=0.0019		
RFS	*median*	14.2 mo	59.6 mo	8.7 mo	16.7 mo
	5-yr %	30.8 %	50.0 %	7.7 %	32.1 %
			P<0.0001		
≤65 years		**n=13**	**n=6**	**n=6**	**n=1542**
OS	*median*	n.r. [101.7 mo]	n.r. [115.9 mo]	29.1 mo	74.8 mo
	5-yr %	69.2 %	100 %	33.3 %	51.7 %
			P=0.0179		
RFS	*median*	58.0 mo	n.r. [114.8 mo]	9.4 mo	30.3 mo
	5-yr %	46.2 %	66.7 %	16.7 %	43.9 %
			P=0.0059		
>65 years		**n=13**	**n=6**	**n=7**	**n=953**
OS	*median*	32.2 mo	59.2 mo	21.1 mo	18.2 mo
	5-yr %	30.8 %	50.0 %	14.3 %	18.0 %
			P=0.0270		
RFS	*median*	11.5 mo	41.0 mo	6.4 mo	10.6 mo
	5-yr %	15.4 %	33.3 %	0.0 %	14.9 %
			P=0.0043		

OS calculated from time of diagnosis; RFS calculated from CR1. Values in square brackets indicate median follow-up time. *P* values (Gehan-Breslow-Wilcoxon test) indicate survival comparison between responders and nonresponders.

n.r., not reached; RFS, relapse-free survival; SALR, Swedish Acute Leukemia Registry.

*All newly diagnosed patients with AML in CR1 enrolled in the DC vaccination studies NCT00834002 and NCT00965224; 3 patients with AML who were in CR2 or CR3 (UPN02, 16, and 34) and 1 patient with AML who did not reach CR1 (UPN20) were excluded from analysis.

†Observed survival of newly diagnosed patients with AML in CR1 enrolled in SALR; diagnosis from 1997-2014.

‡All study patients who responded to DC vaccination; patient UPN22 had an undefinable response and was excluded from analysis.

Because KLH was shown in our previous study[17] to skew T cells toward a Th2 profile, which could be detrimental for a cytotoxic antitumoral response, it was omitted from the preparation of the DCs in 3 patients (UPN11, 12, and 13). In those patients, there was no local immunoreactivity at the site of DC injection, nor was there any DTH reactivity, and it was thus impossible to assess in vivo the quality of the DCs for their capacity to migrate to the lymph nodes or elicit T-cell response. For this reason, it was therefore decided to reincorporate KLH into the DC vaccine preparation in all other patients. All the KLH-exposed patients showed a DTH response; in 13 of these patients, DILs were obtained for immune response assessment. Functional analysis of DILs restimulated with autologous WT1-loaded DCs demonstrated WT1-specific $CD8^+$ but not $CD4^+$ T-cell responses after vaccination, as shown by significant increases in WT1-specific interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) but not interleukin-5 production. This vaccine-specific $CD8^+$ T-cell response was present in the long-term but not in the non–long-term survivor group (Figure 4A-C). Notably, significant WT1-specific bifunctional TNF-α^+/IFN-γ^+ $CD8^+$ T-cell responses were also detected in the long-term survivor group (Figure 4D). In some long-term responders (UPN14) or survivors (UPN17, 34, and 35), the proportion of IFN-γ^+ and/or TNF-α^+ WT1-specific $CD8^+$ DILs was very high (range, 5%-50%). IFN-γ^+ and TNF-α^+ WT1-specific $CD8^+$ but not $CD4^+$ DIL responses were significantly higher in patients vaccinated with the *DC-LAMP–*containing *WT1* constructs as compared with those vaccinated with the wild-type *WT1* construct (data not shown).

Discussion

In this phase 2 study, we demonstrated clinical activity of autologous *WT1* mRNA-electroporated DC vaccination in patients with AML in remission and showed that this form of cancer vaccine therapy may offer OS benefit that is linked to the induction of WT1-specific $CD8^+$ T-cell immunity. The clinical response rate that was obtained in this study (43%) is of considerable interest, with 30% of patients achieving MR and 13% SD. The high rate of patients achieving MR (9 of 30 patients) is an important finding, because these patients were otherwise likely to relapse based on their increased prevaccination *WT1* transcript levels.[19,21-25] In the SD response group (4 of 30 patients), WT1/DC vaccination was effective in temporarily halting AML progression (as characterized by stabilization of *WT1* transcript levels and stable blood values without blasts), thereby delaying the occurrence of florid relapse. SD is an unexpected response category in the context of AML, where relapses are normally characterized by exponentially increasing *WT1* transcript levels at a constant doubling time.[19] Similar observations of disease stabilization have been made in WT1 peptide vaccine trials in AML,[7,8,32] indicating that SD should be included as a separate category in the response assessment of immunotherapeutic

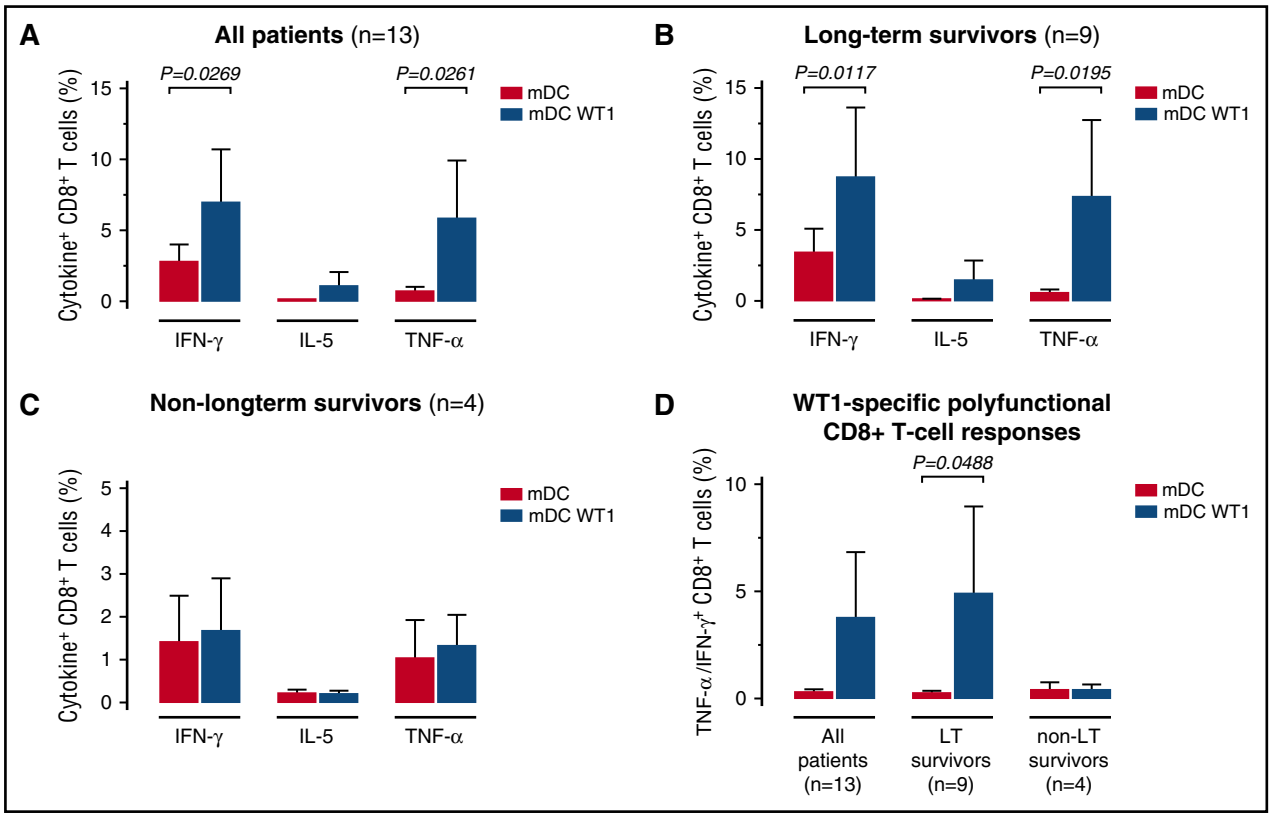

Figure 4. Intracellular cytokine staining of CD8$^+$ DILs after restimulation with mature DCs alone (mDC) or *WT1* mRNA-electroporated DCs (mDC WT1). The WT1-specific T-cell cytokine response was evaluated by comparing mDC WT1 with mDC (all patients examined: UPN03, 05, 06, 08, 14, 17, 21, 22, 29, 30, 34, 35, and 47; long-term [LT] survivors: UPN06, 08, 14, 17, 21, 22, 29, 34, and 35; non-LT survivors: UPN03, 05, 30, and 47). IFN-γ^+, interleukin-5$^+$ (IL-5$^+$), or TNF-α^+ CD8$^+$ T cells are shown for all patients (A), LT survivors (B), and non-LT survivors (C). Polyfunctional TNF-α^+/IFN-γ^+ CD8$^+$ T cells are shown in the same patients (D).

interventions in hematological malignancies, as is now routinely the case in the field of solid tumor immunotherapy.[33] Altogether, our data indicate that WT1-targeted DC vaccination can be an effective strategy to prevent or delay relapse in AML, without the toxicity of allo-HSCT. This is an important finding in light of the growing number of elderly patients with AML who are generally not considered candidates for allo-HSCT because of toxicity considerations.[5] On the basis of these results, WT1/DC vaccination could also have a role among younger patients with AML who do not proceed to allo-HSCT after standard chemotherapy because of refusal or lack of suitable donors.

OS data in our study compared favorably with current and new data from SEER and SALR, respectively. Importantly, our comparatively longer OS was observed not only in younger patients (≤65 years) but also in the poor-prognosis older-age category (>65 years). Long-term OS was seen in both favorable and intermediate cytogenetic risk groups. OS and RFS also correlated with clinical response to WT1/DCs. These observations further validate the use of *WT1* transcript levels as a suitable marker for leukemic residual disease and for monitoring the effect of therapy in AML. Increased OS in AML was recently found to correlate with reduced *WT1* mRNA levels and WT1-specific CD8$^+$ T-cell responses in a cohort of patients with AML after chemotherapy and allo-HSCT.[34] The comparatively longer OS observed in patients with AML vaccinated with WT1/DCs is consistent with a meta-analysis indicating that DC vaccine therapy can offer OS benefit in patients with solid malignancies, including melanoma, prostate cancer, glioblastoma multiforme, and renal cell cancer.[14]

Intriguingly, 5-year relative OS but not RFS in patients vaccinated with WT1/DCs compared favorably with SALR data. Our data suggest that 1 major reason for the OS advantage was the unexpectedly high clinical response rate and long survival in patients who had relapsed after WT1/DC vaccination. For patients with AML in first relapse, the probability of achieving CR2 with salvage treatment (ie, chemotherapy and/or allo-HSCT) has been reported to be 46%,[35] whereas the CR2 rate in our study was 73.3%. Likewise, the 5-year OS rate of WT1/DC-vaccinated patients with AML after first relapse was 36.8% in our study, which compares favorably with the 11% 5-year OS rate for patients with AML in first relapse that has been described in the literature.[35] These data suggest that WT1/DC vaccination can potentiate the response to subsequent treatment, providing an explanation for how vaccination contributes to prolongation of survival. A similar scenario has been reported for solid tumors, where improved clinical outcomes have been documented in patients who received chemotherapy after the seeming failure of immunotherapy as compared with patients who received chemotherapy alone.[36-44] This outcome may reflect synergism between immunotherapy and chemotherapy, the latter having not only antiproliferative but also immunostimulatory effects.[6,13] The precise mechanisms underlying the anticancer synergy between tumor vaccines and chemotherapy are currently being investigated[41]; 1 potential mechanism involves the release of cytokines (eg, TNF-α) by vaccine-induced CD8$^+$ T cells, which in turn enhances the ability of chemotherapy to induce apoptotic tumor-cell death.[45]

In this study, clinical response and survival were found to be correlated with induction of WT1-reactive CD8$^+$ T-cell immunity by DC vaccination, providing a mechanistic basis for the antileukemic activity of WT1/DCs. First, we found a correlation between long-term clinical response and increased circulating frequency of polyepitope WT1-specific tetramer$^+$ CD8$^+$ T cells. Maintenance of or increase in the frequency of WT1-specific CD8$^+$ T cells at relapse or progression points

toward antigen-driven immune activation associated with increasing exposure to the WT1 antigen at relapse[46] and with the continuation of the WT1/DC vaccination. This suggests that antigen-specific T-cell numbers may be necessary but not sufficient to ultimately control AML. Second, we found a correlation between WT1-specific IFN-γ^+ and/or TNF-α^+ DTH-infiltrating $CD8^+$ T lymphocytes and long-term OS. This suggests that $CD8^+$ T-cell function is needed for long-term control of AML, at least in the immunotherapy setting. Because DTH was not performed in this study at the time of relapse, we cannot exclude that despite maintenance of or increase in WT1-specific cell numbers, their function may be deficient at that time.

In line with preclinical data,[18] 3 different *WT1* constructs were used in this study: a native full-length *WT1* construct, a *WT1* construct incorporating the lysosomal targeting signal of *DC-LAMP*, and a codon-optimized version of the latter construct. Theoretically, the *DC-LAMP*–containing constructs would facilitate MHC class 2 antigen presentation and subsequent $CD4^+$ T-cell stimulation. Contrary to expectations, no statistical evidence for induction of WT1-specific $CD4^+$ T-cell immunity was found in the patients in whom the *DC-LAMP*–containing constructs were used. This is in contrast with a recently published study in melanoma, which used DCs loaded with melanoma antigen-encoding mRNA linked to a similar *DC-LAMP* construct.[47] The apparent lack of $CD4^+$ T-cell stimulation in our study may have resulted from the fact that the strongly immunogenic $WT1_{332\text{-}347}$ MHC class 2 epitope[48,49] is not encoded by the *DC-LAMP*–containing constructs because they lack the *WT1* nuclear localization signal.[18] Nevertheless, even without significantly increased stimulation of WT1-specific $CD4^+$ T cells, the *DC-LAMP*–containing constructs did induce higher frequencies of WT1-specific $CD8^+$ T cells as compared with the wild-type *WT1* construct, presumably as a consequence of higher cytoplasmic WT1 expression and MHC class 1 epitope presentation.[18] This, however, did not translate into superior clinical response rates or improved survival outcomes.

In summary, WT1-targeted DC vaccination can elicit antileukemia T-cell immunity in patients with AML at very high risk of relapse. The induction of functional WT1-specific $CD8^+$ T cells is likely a mechanism to help eliminate residual leukemic cells, decrease the likelihood of AML relapse, and improve survival. Vaccination with WT1/DCs can therefore be considered as a nontoxic, postremission strategy to prevent or delay relapse of AML in the adjuvant setting.

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Conflict-of-interest disclosure: V.F.V.T. and Z.N.B. are coinventors of a patent covering the messenger RNA electroporation technique (WO/2003/000907; improved transfection of eukaryotic cells with linear polynucleotides by electroporation). Z.N.B. is a member of the Scientific Advisory Board of ExoCyte Therapeutics. The remaining authors declare no competing financial interests.

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References

1. Howlader N, Noone AM, Krapcho M, et al. SEER Cancer Statistics Review, 1975-2012. Bethesda, MD: National Cancer Institute. http://seer.cancer.gov/csr/1975_2012/browse_csr.php?sectionSEL=13&pageSEL=sect_13_table.16.html. Accessed 31 December 2016.
2. Dinmohamed AG, Visser O, van Norden Y, et al. Treatment, trial participation and survival in adult acute myeloid leukemia: a population-based study in the Netherlands, 1989-2012. *Leukemia*. 2016;30(1):24-31.
3. Ravandi F. Relapsed acute myeloid leukemia: why is there no standard of care? *Best Pract Res Clin Haematol*. 2013;26(3):253-259.
4. Anguille S, Lion E, Smits E, Berneman ZN, van Tendeloo VF. Dendritic cell vaccine therapy for acute myeloid leukemia: questions and answers. *Hum Vaccin*. 2011;7(5):579-584.
5. van Besien K. Allogeneic transplantation for AML and MDS: GVL versus GVHD and disease recurrence. *Hematology Am Soc Hematol Educ Program*. 2013;2013:56-62.
6. Berneman ZN. Autologous T cells on the attack against AML. *Blood*. 2012;120(6):1151-1152.
7. Oka Y, Tsuboi A, Taguchi T, et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci USA*. 2004;101(38):13885-13890.
8. Rezvani K, Yong AS, Mielke S, et al. Leukemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. *Blood*. 2008;111(1):236-242.
9. Maslak PG, Dao T, Krug LM, et al. Vaccination with synthetic analog peptides derived from WT1 oncoprotein induces T-cell responses in patients with complete remission from acute myeloid leukemia. *Blood*. 2010;116(2):171-179.
10. Qazilbash MH, Wieder E, Thall PF, et al. PR1 peptide vaccine induces specific immunity with

clinical responses in myeloid malignancies. *Leukemia*. 2017;31(3):697-704.

11. Rosenblatt J, Stone RM, Uhl L, et al. Individualized vaccination of AML patients in remission is associated with induction of antileukemia immunity and prolonged remissions. *Sci Transl Med*. 2016;8(368):368ra171.
12. Anguille S, Van Tendeloo VF, Berneman ZN. Leukemia-associated antigens and their relevance to the immunotherapy of acute myeloid leukemia. *Leukemia*. 2012;26(10):2186-2196.
13. Anguille S, Smits EL, Bryant C, et al. Dendritic cells as pharmacological tools for cancer immunotherapy. *Pharmacol Rev*. 2015;67(4): 731-753.
14. Anguille S, Smits EL, Lion E, van Tendeloo VF, Berneman ZN. Clinical use of dendritic cells for cancer therapy. *Lancet Oncol*. 2014;15(7): e257-e267.
15. Van Tendeloo VF, Ponsaerts P, Lardon F, et al. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. *Blood*. 2001;98(1):49-56.
16. Van Driessche A, Van de Velde AL, Nijs G, et al. Clinical-grade manufacturing of autologous mature mRNA-electroporated dendritic cells and safety testing in acute myeloid leukemia patients in a phase I dose-escalation clinical trial. *Cytotherapy*. 2009;11(5):653-668.
17. Van Tendeloo VF, Van de Velde A, Van Driessche A, et al. Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. *Proc Natl Acad Sci USA*. 2010; 107(31):13824-13829.
18. Benteyn D, Anguille S, Van Lint S, et al. Design of an optimized Wilms' tumor 1 (WT1) mRNA construct for enhanced WT1 expression and improved immunogenicity in vitro and in vivo. *Mol Ther Nucleic Acids*. 2013;2:e134.
19. Ogawa H, Tamaki H, Ikegame K, et al. The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. *Blood*. 2003;101(5): 1698-1704.
20. Cilloni D, Renneville A, Hermitte F, et al. Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute myeloid leukemia: a European LeukemiaNet study. *J Clin Oncol*. 2009; 27(31):5195-5201.
21. Inoue K, Sugiyama H, Ogawa H, et al. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood*. 1994;84(9):3071-3079.
22. Inoue K, Ogawa H, Yamagami T, et al. Long-term follow-up of minimal residual disease in leukemia patients by monitoring WT1 (Wilms tumor gene) expression levels. *Blood*. 1996;88(6):2267-2278.
23. Cilloni D, Gottardi E, De Micheli D, et al. Quantitative assessment of WT1 expression by real time quantitative PCR may be a useful tool for monitoring minimal residual disease in acute leukemia patients. *Leukemia*. 2002;16(10): 2115-2121.
24. Trka J, Kalinová M, Hrusák O, et al; For Czech Paediatric Haematology Working Group. Real-time quantitative PCR detection of WT1 gene expression in children with AML: prognostic significance, correlation with disease status and residual disease detection by flow cytometry. *Leukemia*. 2002;16(7):1381-1389.
25. Cilloni D, Messa F, Arruga F, et al. Early prediction of treatment outcome in acute myeloid leukemia by measurement of WT1 transcript levels in peripheral blood samples collected after chemotherapy. *Haematologica*. 2008;93(6): 921-924.
26. Cheson BD, Bennett JM, Kopecky KJ, et al; International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. Revised recommendations of the International Working Group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia [published correction appears in *J Clin Oncol*. 2004;22(3):576]. *J Clin Oncol*. 2003; 21(24):4642-4649.
27. de Lima M, Strom SS, Keating M, et al. Implications of potential cure in acute myelogenous leukemia: development of subsequent cancer and return to work. *Blood*. 1997;90(12):4719-4724.
28. Oji Y, Hashimoto N, Tsuboi A, et al. Association of WT1 IgG antibody against WT1 peptide with prolonged survival in glioblastoma multiforme patients vaccinated with WT1 peptide. *Int J Cancer*. 2016;139(6):1391-1401.
29. Rezvani K, Brenchley JM, Price DA, et al. T-cell responses directed against multiple HLA-A*0201-restricted epitopes derived from Wilms' tumor 1 protein in patients with leukemia and healthy donors: identification, quantification, and characterization. *Clin Cancer Res*. 2005; 11(24 Pt 1):8799-8807.
30. Grimwade D, Walker H, Harrison G, et al; Medical Research Council Adult Leukemia Working Party. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. *Blood*. 2001;98(5): 1312-1320.
31. Juliusson G, Lazarevic V, Hörstedt AS, Hagberg O, Höglund M; Swedish Acute Leukemia Registry Group. Acute myeloid leukemia in the real world: why population-based registries are needed. *Blood*. 2012;119(17):3890-3899.
32. Keilholz U, Letsch A, Busse A, et al. A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. *Blood*. 2009;113(26): 6541-6548.
33. Wolchok JD, Hoos A, O'Day S, et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res*. 2009;15(23):7412-7420.
34. Casalegno-Garduño R, Schmitt A, Spitschak A, et al. Immune responses to WT1 in patients with AML or MDS after chemotherapy and allogeneic stem cell transplantation. *Int J Cancer*. 2016; 138(7):1792-1801.
35. Breems DA, Van Putten WL, Huijgens PC, et al. Prognostic index for adult patients with acute myeloid leukemia in first relapse. *J Clin Oncol*. 2005;23(9):1969-1978.
36. Wheeler CJ, Das A, Liu G, Yu JS, Black KL. Clinical responsiveness of glioblastoma multiforme to chemotherapy after vaccination. *Clin Cancer Res*. 2004;10(16):5316-5326.
37. Gribben JG, Ryan DP, Boyajian R, et al. Unexpected association between induction of immunity to the universal tumor antigen CYP1B1 and response to next therapy. *Clin Cancer Res*. 2005;11(12):4430-4436.
38. Arlen PM, Gulley JL, Parker C, et al. A randomized phase II study of concurrent docetaxel plus vaccine versus vaccine alone in metastatic androgen-independent prostate cancer. *Clin Cancer Res*. 2006;12(4):1260-1269.
39. Antonia SJ, Mirza N, Fricke I, et al. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. *Clin Cancer Res*. 2006;12(3 Pt 1): 878-887.
40. Gulley JL, Arlen PM, Tsang KY, et al. Pilot study of vaccination with recombinant CEA-MUC-1-TRICOM poxviral-based vaccines in patients with metastatic carcinoma. *Clin Cancer Res*. 2008; 14(10):3060-3069.
41. Chiappori AA, Soliman H, Janssen WE, Antonia SJ, Gabrilovich DI. INGN-225: a dendritic cell-based p53 vaccine (Ad.p53-DC) in small cell lung cancer: observed association between immune response and enhanced chemotherapy effect. *Expert Opin Biol Ther*. 2010;10(6):983-991.
42. Schlom J, Arlen PM, Gulley JL. Cancer vaccines: moving beyond current paradigms. *Clin Cancer Res*. 2007;13(13):3776-3782.
43. Madan RA, Gulley JL, Fojo T, Dahut WL. Therapeutic cancer vaccines in prostate cancer: the paradox of improved survival without changes in time to progression. *Oncologist*. 2010;15(9): 969-975.
44. Gulley JL, Madan RA, Schlom J. Impact of tumour volume on the potential efficacy of therapeutic vaccines. *Curr Oncol*. 2011;18(3):e150-e157.
45. van der Sluis TC, van Duikeren S, Huppelschoten S, et al. Vaccine-induced tumor necrosis factor-producing T cells synergize with cisplatin to promote tumor cell death. *Clin Cancer Res*. 2015; 21(4):781-794.
46. Brayer J, Lancet JE, Powers J, et al. WT1 vaccination in AML and MDS: a pilot trial with synthetic analog peptides. *Am J Hematol*. 2015; 90(7):602-607.
47. Wilgenhof S, Corthals J, Heirman C, et al. Phase II study of autologous monocyte-derived mRNA electroporated dendritic cells (TriMixDC-MEL) plus ipilimumab in patients with pretreated advanced melanoma. *J Clin Oncol*. 2016;34(12): 1330-1338.
48. Fujiki F, Oka Y, Kawakatsu M, et al. A WT1 protein-derived, naturally processed 16-mer peptide, WT1(332), is a promiscuous helper peptide for induction of WT1-specific Th1-type CD4(+) T cells. *Microbiol Immunol*. 2008;52(12): 591-600.
49. Anguille S, Fujiki F, Smits EL, et al. Identification of a Wilms' tumor 1-derived immunogenic CD4(+) T-cell epitope that is recognized in the context of common Caucasian HLA-DR haplotypes. *Leukemia*. 2013;27(3):748-750.
50. National Cancer Institute: Surveillance, Epidemiology, and End Results Program: Incidence – SEER 18 Regs Research Data + Hurricane Katrina Impacted Louisiana Cases, Nov 2014 Sub (1973-2012 varying). https://seer.cancer.gov/data/seerstat/nov2014/. Accessed 31 December 2016.