Induction of complete and molecular remissions in acute myeloid leukemia by Wilms’ tumor 1 antigen-targeted dendritic cell vaccination

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Active immunization using tumor antigen-loaded dendritic cells holds promise for the adjuvant treatment of cancer to eradicate or control residual disease, but so far, most dendritic cell trials have been performed in end-stage cancer patients with high tumor loads. Here, in a phase I/II trial, we investigated the effect of autologous dendritic cell vaccination in 10 patients with acute myeloid leukemia (AML). The Wilms’ tumor 1 protein (WT1), a nearly universal tumor antigen, was chosen as an immunotherapeutic target because of its established role in leukemogenesis and superior immunogenic characteristics. Two patients in partial remission after chemotherapy were brought into complete remission after intradermal administration of full-length WT1 mRNA-electroporated dendritic cells. In these two patients and three other patients who were in complete remission, the AML-associated tumor marker returned to normal after dendritic cell vaccination, compatible with the induction of molecular remission. Clinical responses were correlated with vaccine-associated increases in WT1-specific CD8+ T cell frequencies, as detected by peptide/HLA-A*0201 tetramer staining, and elevated levels of activated natural killer cells postvaccination. Furthermore, vaccinated patients showed increased levels of WT1-specific IFN-γ-producing CD8+ T cells and features of general immune activation. These data support the further development of vaccination with WT1 mRNA-loaded dendritic cells as a postremission treatment to prevent full relapse in AML patients.

cancer vaccine | active specific immunotherapy | phase I clinical trial

Overall, acute myeloid leukemia (AML) has a bad prognosis; indeed, only 23.8% of patients are alive 5 y after diagnosis (1). One of the main reasons for this poor outlook is that the majority of patients will relapse, despite reaching complete remission with classical polychemotherapy (2–4). Relapse is caused by the persistence of malignant cells, also designated as minimal residual disease, during complete remission. Furthermore, most AML patients are more than 60 y old, and the prognosis in this age group is worse because of comorbidity and higher rates of chemotherapy resistance (5).

One treatment with demonstrable effect on residual disease, relapse rate, and survival is allogeneic hematopoietic stem-cell transplantation, in which donor T lymphocytes exert a graft versus leukemia effect. However, allogeneic hematopoietic stem-cell transplantation is still beset by morbidity and mortality issues that preclude its routine use in older AML patients (3, 4). Thus, except for allogeneic hematopoietic stem-cell transplantation in subsets of younger AML patients, there is no consensus as to which postremission treatment should be applied to prevent relapse (3–6).

Recently, immunotherapeutic strategies have been developed to raise autologous antileukemic immunity to control malignant disease. Vaccination with peptides derived from leukemia-associated antigens has led to clinical and immunological responses in AML (7). These antigens include proteinase 3, from which the immunogenic PR1 peptide is derived (8–10), Wilms’ tumor 1 (WT1) protein (8, 10–12), and the receptor for hyaluronic acid-mediated motility (RHAMM/CD168) (13). Although promising, the peptide-vaccination approach has limitations; in particular, the peptides need to be tailored to the MHC antigens of the patient, and not all MHC-restricted peptides are yet defined. This problem can be circumvented by introducing the full-length antigen into professional antigen-presenting cells, such as dendritic cells (DC), which will present multiple epitopes to T lymphocytes in the context of autologous MHC. We have developed a highly efficient, transient, and clinically safe transfection procedure to introduce mRNA encoding an antigen into DC by electroporation (14), a process that results in efficient antigen presentation (15). DC have been intensively investigated as cellular adjuvants for therapeutic cancer vaccination, and since the first reported trial in lymphoma patients in 1996 (16), DC have shown an unsurpassed capacity to induce in vivo antitumor responses. However, the overall clinical response rate, if any, remains very low (17).

This poor clinical outcome could be, at least in part, ascribed to the fact that most clinical DC trials to date have been performed in end-stage cancer patients, often with bulky tumor loads and a compromised immune system caused by intensive treatment schedules and/or advanced disease stage (18). These observations plead for a more careful clinical-trial design that selects patients with residual but substantially reduced disease in whom immunotherapeutic interventions could significantly improve the clinical outcome after standard chemotherapy treatment (19). In lymphoma, this strategy used with patient-specific idioype vaccination led to remarkable complete molecular responses (20). However, to our knowledge, such results have never been reported for DC-based cancer vaccines.


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Here, we present the results of a phase I/II vaccination trial using autologous monocyte-derived DC electroporated with WT1 mRNA in AML patients who achieved complete or partial remission after polychemotherapy but remained at high risk of full relapse. WT1 is overexpressed in the vast majority of AML cases (21–25). Furthermore, there is evidence that it plays an important role in the malignant phenotype of AML (24, 26–29). In an immunodeficient mouse model, there is a selective elimination of leukemic stem cells, but not normal human progenitors, and leukemia cells by WT1-specific cytotoxic CD8+ T cells (30–32). Of note, in a prioritization study carried out by the National Cancer Institute, WT1 was selected from 75 defined tumor antigens to rank as the most promising cancer vaccine target (33).

The expression of WT1 mRNA in bone marrow, or preferably, in peripheral blood, has been shown to be a relevant tumor marker in AML (25, 34–38). Especially after treatment, it has a high positive-predictive value as a molecular residual-disease marker (i.e., WT1 mRNA expression levels above background in peripheral blood always herald clinical relapse) (25, 36, 37, 39). Moreover, failure to reduce WT1 transcripts below the threshold limits after chemotherapy invariably predicts relapse in patients with complete remission, which enables the early prediction of treatment outcome and the distinction of patients with continuous remission from those with only apparent complete remission (25, 39).

In this study, we show the immunogenic and antileukemic activity of a WT1-targeted DC vaccine in AML patients, evidenced by the conversion of partial to complete remission and the induction of molecular remission. Importantly, we found WT1-specific and non-specific immunological correlates of these clinical responses.

Results

Clinical Results. The clinical details of the 10 AML patients recruited into this study are summarized in Table S1. Successful vaccine production was obtained in all patients from a single apheresis procedure (10–15 L), and DC vaccination was well-tolerated. In all patients, there was local erythema and induration at the site of injection, starting from the second vaccination. Patient with unique patient number (UPN)09 reported pain at the level of the draining axillary lymph nodes after DC vaccination. In patient UPN016, the platelet count dropped after the first DC injection and normalized 5 wk after the fourth vaccination (Fig. 1); she also experienced a mild flare-up of a preexisting inflammation of the Achilles and foot tendons, which started around the period of the fourth DC vaccination.

The most striking and demonstrable clinical effects were observed in patients UPN08 and UPN16, who were both in partial remission after chemotherapy and reached complete remission after DC vaccination. Before DC vaccination, these two patients were refractory to chemotherapy, and AML disease was not controlled according to classical morphological criteria [i.e., the percentage of myeloblasts in the bone marrow was increased above normal and was higher after the last (consolidation) chemotherapy than after the first (induction) chemotherapy]. After DC vaccination, the myeloblast percentage decreased to normal, from 6% to 1% in UPN08 (Fig. 1A) and from 9% to 0.3% in UPN16 (Fig. 1B). This was confirmed by flow-cytometric and histological examinations of the bone marrow. Concomitant with these changes, there was normalization of elevated WT1 mRNA levels in peripheral blood after DC vaccination (Fig. 1). Patient UPN016 has relapsed in the bone marrow 9 mo after the start of DC vaccination, and this relapse was preceded by an increase in WT1 mRNA expression levels above normal (Fig. 1B).

The return to normal values of the WT1 tumor marker not only confirmed the antileukemic effect of DC vaccination in patients in partial remission, but it also revealed efficacy in some patients in complete remission (UPN01 and UPN06). In these latter subjects, WT1 mRNA expression levels also illustrated the dynamics of minimal residual disease and the temporary nature of DC vaccine-induced control. After normalization of WT1 mRNA expression associated with the initial round of DC vaccinations, this tumor marker increased on different occasions, compatible with molecular relapse. This was reversed by additional rounds of DC vaccination, which were administrated usually on a bimonthly basis (Fig. 2). Patient UPN01 relapsed almost 4 y after starting DC vaccination, and elevated levels of WT1 mRNA in peripheral blood were observed 3 mo before relapse. Thus, as in patient UPN16, molecular relapse preceded morphological and clinical relapse.

As of May 2010, three patients are in continuous complete remission with a normal peripheral blood picture. Of those three patients, two had increased WT1 mRNA levels in blood that normalized post-DC vaccination; WT1 expression normalized after the second DC vaccination in patient UPN08 (Fig. 1A) and after an additional fifth DC vaccine administered 2 mo after the initial cycle of four biweekly DC vaccinations (UPN06) (Fig. 2). In the third patient (UPN10), we observed a normalization of bone-marrow WT1 mRNA expression from an increased value before DC vaccination to background levels postvaccination.

![Fig. 1. Induction of complete remission by DC vaccination in patients UPN08 (A) and UPN16 (B).](https://example.com/fig1.png)
of the WT1 mRNA tumor marker seen after DC vaccination is indicative of molecular remission. In the two other patients who responded to DC vaccination but eventually relapsed, WT1 mRNA expression in blood initially normalized after the first DC vaccination (UPN06) (Fig. 1B) and after the second DC vaccination (UPN01). As of now, 5 of 10 patients can be considered as clinical responders (UPN01, UPN06, UPN08, UPN09, and UPN16) and 3 of them as long-term responders (UPN01, UPN06, and UPN08), based on a complete remission status lasting for at least 3 y. Seven patients died, six because of relapse of AML, and the seventh had a progressively deteriorating clinical condition, presumably caused by adenocarcinoma of the lung (UPN07). In these seven patients, WT1 mRNA expression either did not normalize postvaccination in blood (UPN03, UPN07, and UPN09) or bone marrow (UPN02 and UPN05) or returned to pathologically increased values after initial normalization (UPN01 and UPN16).

**Immunomonitoring.** Immunomonitoring was performed on peripheral blood mononuclear cells (PBMC) and plasma samples obtained before and after DC vaccination. Immunophenotypic analyses revealed no significant changes in the circulating frequencies or absolute numbers of lymphocyte subsets [CD4+ and CD8+ T cells, B cells, and natural killer (NK) cells]. Notably, DC vaccination did not affect the relative frequencies of regulatory CD4+ T (Treg) cell subsets, including naturally occurring CD25+FoxP3+ Treg and induced IL-10+ and/or TGF-β+ Treg. Furthermore, the relative frequencies of naive (CD45RA−CD62L+), terminally differentiated effector (CD45RA−CD62L−), effector memory (CD45RA−CD62L−), and central memory (CD45RA−CD62L+) subsets within the CD3+CD4+ and CD3+CD8+ T cell compartments remained unchanged. However, a significant increase in circulating levels of plasma IL-2 and activated HLA-DR+CD4+ T cells was observed postvaccination, irrespective of clinical response (Fig. S1). More importantly, we found a significant correlation ($P = 0.01$) between clinical responses and the presence of high numbers of activated NK cells postvaccination (i.e., more than 40% HLA-DR+ cells within the total NK cell population in four of five clinical responders and zero of five nonresponders). Furthermore, the level of HLA-DR+ NK cells in all evaluable patients postvaccination was significantly higher compared with healthy volunteers [32.25 ± 16.8% vs. 19.9 ± 10.9%, respectively (mean ± SD; n = 10); $P = 0.04$], whereas the prevaccination levels were not significantly increased [26.8 ± 17.7% vs. 19.9 ± 10.9% (mean ± SD; n = 10); $P = 0.25$].

WT1-specific immune responses were evaluated using anti-WT1 antibody analysis, WT1 peptide/HLA-A*0201 (pHLA-A*0201) tetramer staining, and functional intracellular cytokine assays. Analysis of humoral anti-WT1 responses showed relatively low, but not significantly different, concentrations of WT1 antibodies in the plasma before and after vaccination. Subclass analysis of WT1 antibodies showed a predominant T helper 1-associated IgG2 subtype.

Because patient accrual in our trial was independent of HLA haplotype caused by the polyepitope full-length antigen strategy, only five patients (UPN01, UPN05, UPN09, UPN09, and UPN16) were evaluable by pHLA-A*0201 tetramer analysis. Four pHLA-A*0201 tetramers specific for different HLA-A*0201-restricted WT1 epitopes were used: WT137–45, WT1126–134, WT1187–195, and WT1235–243. Increased (>1.5-fold) frequencies of WT1-specific tetramer+CD8+ T cells (11) were observed in UPN01 and UPN08 compared with UPN05, UPN09, and UPN16 (Table 1). A significant positive correlation ($P = 0.025$) was found between long-term responders (UPN01 and UPN08) and an increase in WT1-specific tetramer+CD8+ T cells. Interestingly, this association was observed for more than one of the epitopes studied (i.e., WT1187–195 and WT1235–243 in UPN01 and all four epitopes examined in UPN08), suggesting a clinically relevant polyepitope-specific T cell response (Table 1). In patient UPN16, who responded to DC vaccination acutely but not in the long term, there was no increase postvaccination in the frequency of WT1-specific CD8+ T cells, but the frequency of HLA-DR+ NK cells nearly doubled compared with prevaccination levels (from 29% to 57%); this observation suggests that the short-term antileukemic effect seen in patient UPN16 was mediated, at least in part, by NK cells rather than by CD8+ T cells.

**Table 1. Overview of WT1-specific tetramer+CD8+ T cell frequencies pre- and post-DC vaccination in the peripheral blood of HLA-A*0201+ AML patients**

<table>
<thead>
<tr>
<th>Percent WT137–45</th>
<th>Percent WT1126–134</th>
<th>Percent WT1187–195</th>
<th>Percent WT1235–243</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-DC</td>
<td>Post-DC</td>
<td>Pre-DC</td>
<td>Post-DC</td>
</tr>
<tr>
<td>UPN01*</td>
<td>0.016</td>
<td>0.002</td>
<td>0.016</td>
</tr>
<tr>
<td>UPN05</td>
<td>ND</td>
<td>ND</td>
<td>0.017</td>
</tr>
<tr>
<td>UPN08*</td>
<td>0.015</td>
<td>0.097</td>
<td>0.021</td>
</tr>
<tr>
<td>UPN09</td>
<td>0.078</td>
<td>0.003</td>
<td>0.102</td>
</tr>
<tr>
<td>UPN16</td>
<td>0.212</td>
<td>0.040</td>
<td>0.204</td>
</tr>
<tr>
<td>UPN01</td>
<td>0.004</td>
<td>0.009</td>
<td>0.004</td>
</tr>
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ND, not determined.

*Long-term responders.
Detection of functional WT1-specific T cells was performed using a 1-wk in vitro antigen rechallenge protocol followed by intracellular cytokine staining. This approach revealed a significant increase in WT1-specific IFN-γ-producing CD8+ T cells postvaccination (Fig. 3) compared with both prevaccination and medium controls. However, there was no correlation between antileukemic clinical responses and the frequency of WT1-specific IFN-γ+CD8+ T cells. Moreover, by assaying simultaneously for CD107a mobilization and intracellular IL-2, IFN-γ, TNF-α, and IL-10 production on a single-cell level after in vitro restimulation with WT1 mRNA-electroporated or WT1 protein-loaded DC, polyfunctional WT1-specific T cells were not identified in any substantial numbers. Keyhole limpet hemocyanin (KLH)-specific T cell responses were readily detected in a 3-d in vitro restimulation assay, revealing a partially T helper 2 (Th2)-skewed cytokine profile characterized by secretion of IL-5, IL-10, and TNF-β.

In addition to these in vitro T cell responses, all patients exhibited in vivo delayed-type hypersensitivity (DTH) skin reactions (indurations of ≥2 mm) after four vaccinations to the vaccine (i.e., KLH-exposed WT1 mRNA-electroporated DC) and to KLH separately as well as to nontransfected or WT1 mRNA-transfected DC not exposed to KLH.

Discussion
In this phase I/II DC vaccination study, we have shown that WT1 mRNA-electroporated autologous DC are immunogenic and that they induce a measurable antileukemic effect in AML patients treated with polychemotherapy but at high risk of full relapse. DC vaccination was associated with the achievement of molecular remission in 5 of 10 patients who exhibited elevated WT1 mRNA expression levels before therapy. Moreover, in two of these patients, partial remission with demonstrable AML disease was converted to complete remission by the four biweekly DC vaccinations. Three of these five patients, including patient UPN08 who was in partial remission before vaccination, are currently alive and well with normal blood counts and normal blood WT1 mRNA levels. It should be noted that all of the five responding patients were expected to experience a full-blown relapse based on partial remission status indicating morphologically demonstrable AML disease (3) and/or increased WT1 mRNA levels after chemotherapy (25, 36, 37, 39). Of note, two of five responding patients eventually relapsed. These relapses were preceded by loss of control of WT1 mRNA expression levels, confirming the value of this parameter as a predictive tumor marker for AML relapse. Loss of clinical and molecular response has also been described in WT1 peptide-vaccination studies (10–12).

The close temporal relationship between DC vaccination and the antileukemic effect is a strong argument for a causal association. More importantly, clinical responses were correlated with both innate (i.e., superior levels of activated NK cells in responders) and adaptive (i.e., increased WT1 tetramer+CD8+ T cell frequencies in long-term responders only) immune responses postvaccination, indicating that the vaccine elicited clinically relevant and vaccine-specific immunity. To date, only a few DC vaccine studies have attempted to evaluate the nonspecific, yet potentially clinically relevant, NK cell response to immunization (40), but there is an increasing body of evidence that DC–NK interactions during the early phase of innate immunity can impact the quality and magnitude of the subsequent adaptive immune response (41). In our trial, it can be hypothesized that the effect of DC vaccination in clinical responders is, at least in part, mediated initially by a (nonspecific) innate immune response but that a subsequent adaptive WT1-specific T cell response is necessary to mediate a long-lasting (defined as a remission period of more than 3 y) (42) clinical benefit, as observed in UPN01 and UPN08. This hypothesis fits with the observation that patient UPN16, who showed a strong initial clinical response immediately after vaccination associated with extremely high levels of activated NK cells (57% of all NK cells) yet without any evidence of increased WT1-specific CD8+ T cell immunity, relapsed 9 mo after the start of DC vaccination and succumbed 3 mo later. Moreover, we observed that WT1-specific CD8+ T cell populations in these long-term clinical responders targeted multiple HLA-A*0201-restricted WT1 epitopes (43), providing in vivo human proof of concept that single-antigen mRNA-based loading of DC results in polyepitope-specific T cell responses. The ex vivo peripheral blood WT1-specific tetramer+CD8+ T cell populations identified in the current trial were small but clearly identifiable and are consistent with the data of a recent study showing a lower level of WT1-specific CD8+ T cells in blood compared with bone marrow (44).

Notably, we also consistently observed signs of general immune stimulation postvaccination in the whole patient population, regardless of clinical outcome, as evidenced by significant increases in plasma IL-2 levels and frequencies of circulating activated CD4+ T cells. Notwithstanding the fact that these observations were based on direct ex vivo blood analyses, in vitro antigen rechallenge assays failed to reveal significant increases in either WT1- or KLH-specific IL-2–secreting CD4+ T cells postvaccination. However, there was a significant increase in the peripheral blood postvaccination of WT1-specific IFN-γ+CD8+ T cells in the patient population as a whole, but this was not correlated with clinical benefit. This lack of correlation has been reported previously in a WT1 peptide-vaccine trial in cancer patients (11). Thus, whereas numerous reports already indicated that T cell responses after DC vaccination in humans can be more complex than initially expected (45–48), our findings underscore the need for more appropriate functional T cell assays that provide a more global view of the vaccine-specific T cell repertoire (e.g., by detection of transient CD137 expression on CD8+ T cells on antigen [re]challenge, which is independent of cytokine profile) (49). Furthermore, our data reiterate the importance of a comprehensive immunomonitoring approach that includes disease-localized tissue sampling. The positive DTH reaction to the vaccine and its single-antigen components, indicative of an in vivo cell-mediated immune response, indirectly points to a migration of at least...
part of the DC vaccine to the lymph nodes, where it presumably elicited antivaccine T cell responses. However, further investigation is warranted, because it is possible that the inclusion of KLH as a noncognate CD4+ T helper antigen might have negatively influenced the immunological and clinical outcome of the WT1-targeted DC vaccination in some patients, which a partially Th2-skewed KLH-specific T cell response might suggest. The antileukemic effect of the first round of biweekly DC vaccinations was transient in some patients, and additional bi-monthly DC administrations were needed to induce repeated normalizations of the WT1 mRNA tumor marker. Similar booster effects of DC vaccinations have been observed in melanoma patients (45). The reason for the occasionally transient effect of DC vaccination, which is also observed in WT1 peptide-vaccine patients (45), is not fully understood. However, KLH as a noncognate CD4+ T cell stimulation postvaccination was most probably caused by cytoplasmic antigen expression after mRNA electroporation, leading to predominant MHC class I antigen presentation. One way to tackle this limitation and potentially improve the efficacy of DC vaccination is to introduce MHC class II-skewing signals into the mRNA construct [e.g., dendritic cell lysosome-associated membrane glycoprotein (DC-LAMP)] (50).

In contrast to peptide-vaccination studies, which have shown clinical activity in AML (9–13), DC vaccination in AML patients to date has been ineffective or inconclusive (51–53). The clinical efficacy of our DC approach may be caused by several factors. First and similar to analyses conducted in WT1 peptide-vaccination studies (10–12), the use of the sensitive WT1 mRNA residual disease marker revealed a clinical effect in some complete remission patients with increased WT1 mRNA expression levels indicative of ongoing leukemic activity (54). Second, we were able to obtain DC from blood CD14+ monocytes in all cases from AML patients in remission, in contrast to the generation of DC from primary AML cells, which was only successful in a minority of patients tested (53). Third, the choice of the WT1 antigen (33), rather than leukemic cell lysates or AML-derived DC (51–53), may also be advantageous, because WT1 is already recognized immunologically in AML patients (8, 55, 56); furthermore, WT1-targeted vaccine approaches have been successful in increasing the specific immune response in such a way that they can control AML, at least in some patients, as evidenced here and in other studies (10–12). Fourth, our unique clinical trial design (57), which is in stark contrast to current DC trials focusing on end-stage cancer patients, may have contributed to the demonstrable clinical effects observed (58).

In conclusion, vaccination with WT1 mRNA-electroporated DC exhibits antileukemic activity in AML patients and elicits both innate and adaptive immune responses correlated with clinical benefit. These findings support the further development of vaccination with WT1-loaded DC as an immunotherapeutic strategy to prevent relapse in AML. Finally, WT1 is overexpressed in a majority of malignancies (24, 56), and WT1 peptide vaccination has led to clinical responses in patients with various solid tumors (11, 56). Thus, the promising data presented here suggest that WT1 mRNA-electroporated autologous DC might serve as a platform model for the development of a universal cancer vaccine in the adjuvant setting.

Materials and Methods

Patients. After informed written consent was obtained, patients with AML (except acute promyelocytic leukemia) diagnosed according to World Health Organization (WHO) criteria were enrolled in a phase III trial approved by the Ethics Committee of Antwerp University Hospital (ClinicalTrials.gov number NCT00834002). All patients were in hematologic remission after at least one prior antileukemic chemotherapy regimen and were not enrolled until 1 mo after polychemotherapy. Complete remission was defined by the absence of blasts in blood and by less than 5% blasts in marrow. Partial remission was defined as a ≥50% decrease in marrow blasts with normalization of blood counts. Inclusion criteria were the absence of a matched donor for allogeneic hematopoietic stem cell transplantation (allogeneic hematopoietic stem cell transplantation) if ≤60 y and high risk of full-blown relapse as defined by (i) insufficient disease control as in partial remission status (more than 5% blasts in the bone marrow) (3), and/or (ii) WT1 mRNA levels increased above background after chemotherapy (35, 39), and/or (iii) age ≥ 61 y (3–5), and/or (iv) previous relapse (3), and/or (v) hyperleukocytosis at presentation (white blood cell count > 20,000/μL) (2), and/or (vi) poor risk cytogenetic or molecular markers at presentation (2–4) (Table S1).

DC Vaccination. Clinical grade DC vaccines were prepared after leukapheresis of nonmobilized blood (Cobe Spectra) and immunomagnetic selection using CliniMACS (Miltenyi Biotec) (57). Monocyte-derived WT1 mRNA-loaded DC vaccines were generated as described previously (57). DC vaccines were administered intradermally at biweekly intervals as previously reported (57).

Immunomonitoring. Detection and subtyping of anti-WT1 antibodies in pre- and postvaccination plasma samples were performed as previously described (59) (more details in SI Materials and Methods). Cytokine plasma levels were determined with the Th1/Th2 multiplex immunoassay (Bender MedSystems). Direct ex vivo analysis for lymphocyte subsets and activation markers was performed by flow cytometry using directly conjugated monoclonal anti-CD3 (Biocell). Intracellular cytokine-stimulation assays and detection of antigen-specific cytokine responses were performed as described in SI Materials and Methods.

For detection of circulating WT1-specific CD8+ T cells, pHLA-A*0201 tetramers were used. To minimize inter- and intrapatient variability, all tetramer analyses were performed on the same day by the same operator on a validated flow cytometer using identical reagents and instrument settings. Thawed peripheral blood mononuclear cells (PBMCs) (1 × 10^6 per experimental condition) obtained before vaccination and after the fourth vaccination were washed and stained for 15 min at 37 °C with phycoerythrin (PE)-labeled pHLA-A*0201 tetramers refolded with the following WT1 peptides: WT37–243 (43). Cells were then washed and stained with the following mAbs: anti–CD8–Pacific Blue (Dako), anti–CD3–allophycocyanin (APC), anti–CD14–FITC, and anti–CD19–FITC (BD Biosciences). Cells were excluded using propidium-iodide staining (1 μg/mL Sigma-Aldrich), and the FITC channel was used to exclude B cells and monocytes. For each sample, at least 10^5 viable CD3+CD8+ T cells within a standard lymphocyte gate were acquired using a CyFlow ML flow cytometer (Partec), and data were analyzed using FlowJo software (Tree Star). Because of the low frequencies of WT1-specific tetramer–CD8+ events detected in peripheral blood (Table 1), all tetramer data were reanalyzed in an independent and blinded fashion to ensure consistent interpretation as described previously (44).

To assess cell-mediated immunity in vivo, DTH skin testing was performed 2 wk after the fourth DC vaccination by intradermal injection, and measurement of induration was 48 h later.

Molecular Tumor-Marker Monitoring. Longitudinal tumor-marker monitoring in blood and bone marrow was performed by qRT-PCR for WT1 gene expression as described previously (25, 35, 39). Values above 2 and 25 copies of WT1 mRNA per 1,000 ABL copies in blood and marrow, respectively, were considered to be above normal background and thus, indicative of residual disease. More details are in SI Materials and Methods.

Data Mining and Statistical Analysis. Flow-cytometric data analysis was performed using FlowJo version 8.4.A (TreeStar). GraphPad Prism 4.0 software (GraphPad Software) was used for graphical data representations and statistical computations. Statistical analysis was performed using Student t test or one-way ANOVA, where appropriate. Correlations between immunological and clinical responses were examined with a two-sided χ2 test. Any P value <0.05 was considered statistically significant.

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