Biologic activity of irradiated, autologous, GM-CSF-secreting leukemia cell vaccines early after allogeneic stem cell transplantation

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Through an immune-mediated graft-versus-leukemia effect, allogeneic hematopoietic stem cell transplantation (HSCT) affords durable clinical benefits for many patients with hematologic malignancies. Nonetheless, subjects with high-risk acute myeloid leukemia or advanced myelodysplasia often relapse, underscoring the need to intensify tumor immunity within this cohort. In preclinical models, allogeneic HSCT followed by vaccination with irradiated tumor cells engineered to secrete GM-CSF generates a potent antitumor effect without exacerbating the toxicities of graft-versus-host disease (GVHD). To test whether this strategy might be similarly active in humans, we conducted a Phase I clinical trial in which high-risk acute myeloid leukemia or myelodysplasia patients were immunized with irradiated, autologous, GM-CSF-secreting tumor cells early after allogeneic, nonmyeloablative HSCT. Despite the administration of a calcineurin inhibitor as prophylaxis against GVHD, vaccination elicited local and systemic reactions that were qualitatively similar to those previously observed in nontransplanted, immunized solid-tumor patients. While the frequencies of acute and chronic GVHD were not increased, 9 of 10 subjects who completed vaccination achieved durable complete remissions, with a median follow-up of 26 months (range 12–43 months). Six long-term responders showed marked decreases in the levels of soluble NK2D ligands, and 3 demonstrated normalization of cytotoxic lymphocyte NK2D expression as a function of treatment. Together, these results establish the safety and immunogenicity of irradiated, autologous, GM-CSF-secreting leukemia cell vaccines early after allogeneic HSCT, and raise the possibility that this combinatorial immunotherapy might potentiate graft-versus-leukemia in patients.

Notwithstanding the GVL effect, patients with advanced myelodysplasia (MDS) or refractory/relapsed acute myeloid leukemia (AML) remain at high risk for eventual disease progression after allogeneic HSCT (6). Although the pathways responsible for treatment resistance remain to be elucidated, therapeutic strategies that augment tumor immunity might render HSCT more efficacious. Among these, cancer vaccines represent one promising approach, as improved tumor antigen presentation by stimulated dendritic cells might enhance donor T cell expansion and function and help focus the immune response toward leukemia cells (7).

The transplant setting presents several potential advantages for cancer vaccination (8). Following drug-induced lymphodepletion, the resultant cytokine milieu, characterized by high levels of IL-7, IL-12, and IL-15, favors the accelerated T cell expansion and function and help focus the immune response toward leukemia cells (7).

In view of these considerations, the initial clinical trials of leukemia vaccines combined with allogeneic HSCT have explored immunization at relatively late time points, when the likelihood of acute GVHD is low and immunosuppressive medications have been withdrawn. In this setting, vaccination with peptides derived from proteinase-3, WT-1, and Bcr-Abl, 3 well-defined leukemia antigens, induced specific immune responses in the absence of GVHD (17–19). Similarly, vaccination with irradiated, leukemia cells engineered to express CD40 ligand and IL-2 stimulated antitumor cellular and humoral responses without significant toxicity (20). Together, these pilot...
investigations suggest that leukemia vaccines administered several months after allogeneic HSCT may be safe and biologically active.

In complementary work, we showed in several murine models that immunization with irradiated tumor cells engineered to secrete GM-CSF early after allogeneic HSCT elicited potent tumor protection without exacerbating GVHD (21, 22). Vaccination evoked CD4+ and CD8+ antitumor T-cell responses that were comparable to reactions generated in immunized, nontransplanted animals. These investigations raise the possibility that cellular vaccines administered early after allogeneic HSCT may also enhance antitumor immunity.

In this context, we previously reported several clinical trials of vaccination with lethally irradiated, autologous tumor cells engineered to secrete GM-CSF in patients with diverse solid malignancies (23–25). These studies established the ability of this immunization scheme to elicit a coordinated cellular and humoral antitumor response that effectuated significant tumor necrosis in a majority of subjects. Based on this experience and the murine transplant experiments, we undertook a Phase I trial of vaccination with lethally irradiated, autologous, GM-CSF-secreting leukemia cells early after allogeneic HSCT in high-risk MDS and AML patients. Our results show that, despite the use of the calcineurin inhibitor tacrolimus as GVHD prophylaxis, immunization in this setting is safe and associated with a potent GVL effect.

**Results**

**Clinical Findings.** The design of the clinical protocol is illustrated in Fig. 1. Twenty-eight advanced MDS or high-risk AML patients with a median age of 62 (range, 41–71 years) were enrolled onto the study. In all cases the disease was not in remission and the median marrow blast content was 20% (range, 6–91%). Four subjects were withdrawn before HSCT because of rapidly progressive disease or uncontrolled infection. Of the 24 subjects who underwent HSCT, 13 received matched grafts from unrelated donors and 11 from matched sibling donors. Fifteen transplanted subjects began vaccination with irradiated, autologous myeloblasts engineered by adenoviral mediated gene transfer to secrete GM-CSF between days 30 and 45 after HSCT. Those who were ineligible to start included 4 with marked leukemia progression that required hospice before day +30, 3 with grade II to IV acute GVHD before day +30, 1 who died of sepsis and multiorgan failure on day +23, and 1 who died of an idiopathic pneumonia syndrome on day +47.

The disease characteristics and outcomes of the 15 subjects who began immunization are shown in Supporting information (SI) Table S1. Ten subjects completed the full course of 6 vaccines; 3 were removed early because of rapidly progressive disease, 1 because of an unrelated Clostridium difficile related colitis and appendicitis (that was treated with surgery and antibiotics), and 1 because of grade II skin acute GVHD that responded to systemic steroids.

A total of 74 immunizations were administered on the trial.

The median vaccine cell dose was 1.0 × 10^7 cells (range, 0.1–1.0 × 10^7), the mean GM-CSF secretion rate was 52 ng/10^6 cells every 24 h, and the average prefreeze viability was 98%. Four subjects developed mild, grade 1 injection site reactions characterized by induration, erythema, and pruritus, and these were the only adverse events definitely attributable to immunization. No autoimmune toxicities were evident.

Among the 15 subjects who received at least 1 vaccination, only a single subject manifested grade II skin acute GVHD during immunization, as indicated above. One subject developed grade II skin GVHD on day +200 at the end of weaning tacrolimus, 3 months after vaccine completion, and this resolved with prednisone. An additional subject manifested grade II acute GVHD, with intestinal and skin involvement, at day +182 in the setting of a rapid tacrolimus taper several months after finishing immunization. The GVHD resolved with systemic corticosteroids and the acute leukemia entered a durable complete remission. The cumulative incidence of grade II to IV acute GVHD for subjects who received at least 1 vaccine was 20%.

Seven of the 15 immunized subjects developed chronic GVHD (6 mild, and 1 moderate) that in each case responded to corticosteroids. The primary organs involved were mouth (3 subjects), eyes (3 subjects), and liver (1 subject). The cumulative incidence of chronic GVHD for patients who received at least 1 vaccine was 47%, and for subjects who completed all 6 vaccines was 60%. Overall, the rates of acute and chronic GVHD in this study are comparable to those of MDS and AML patients treated at our institution with nonmyeloablative HSCT alone (26).

Of the 15 subjects who started vaccination after transplant, 9 are alive and in continuing complete remission, with a median follow up of 25.5 months. Among the 5 subjects who initiated but did not complete all 6 immunizations, 4 died from relapsed disease. One subject who stopped therapy after 2 vaccines because of grade II skin GVHD generated a sustained remission, but died from complications of pneumonia and chronic lung disease 13.5 months after HSCT. Nine of the 10 subjects who completed all 6 immunizations remain in complete remission, with a median follow-up of 26 months (range 12–43 months). Three of these long-term survivors manifested hematologic relapse or progression early after HSCT, but achieved complete responses after vaccination and tacrolimus taper.

The Kaplan-Meier estimate for 2-year overall survival (OS) for patients who received at least 1 immunization was 57 ± 14%. The clinical results were superior among subjects who completed all 6 vaccines, where the Kaplan-Meier estimate of 2-year OS was 88 ± 12%. OS for the entire cohort of 24 transplanted subjects, including those not vaccinated, was 35 ± 10%.

**Immunologic Activity.** Injections of irradiated, autologous, GM-CSF-secreting leukemia cells elicited local reactions in 14 assessable subjects, and the intensity of these responses typically increased with subsequent inoculations. Clinically, the vaccine sites were characterized by erythema, induration, and pruritus that gradually resolved within 48 to 72 h. Pathologic analysis of punch biopsies revealed significant accumulations of dendritic cells, macrophages, neutrophils, eosinophils, and lymphocytes (Fig. 2). Strong reactions included endothelial cell activation and damage to the superficial venules. A few biopsies also showed minor interface changes, consistent with changes seen in mild GVHD, although there were no clinical manifestations of GVHD in these subjects. Immunohistochemistry disclosed the presence of abundant CD1a+ dendritic cells and CD4+ and CD8+ T cells, with some scattered CD20+ B cells. The vaccine responses were judged by pathologic criteria to be of moderate to strong intensity in 4 of 14 biopsies obtained with the first injection and in 5 of 7 biopsies taken after the fifth inoculation. While the frequency of intense reactions in this trial was...
somewhat less than in our earlier studies of solid tumor patients (23–25), the immune responses were qualitatively similar, despite the administration of tacrolimus.

To examine whether the local reactions engendered systemic immunity, we injected irradiated, autologous, nontransduced myeloblasts before and after vaccination. Examination of the initial delayed-type hypersensitivity (DTH) responses revealed either trace or absent infiltrates in all 13 subjects who underwent biopsies. However, when tested again at the time of the fifth vaccine, while still on immune suppression or thereafter, 7 of 8 subjects developed significant DTH responses. Pathologic examination disclosed brisk CD4 and CD8 T-cell infiltrates, with rare CD20 B cells (Fig. 3). Eosinophils were prominent in 4 subjects, but fewer CD1a dendritic cells were evident compared to the vaccine sites, which probably reflects the GM-CSF production by the transduced leukemia cells. Because GVHD was not evident clinically or pathologically at this time, the robust DTH reactions raise the possibility that vaccination may have enhanced systemic antileukemia immunity. Indeed, the 7 responding subjects achieved long-term clinical remissions.

Fig. 2. Irradiated, GM-CSF secreting, autologous leukemia cells elicit local vaccine reactions early after allogeneic HSCT. (Top Left) Dermal cellular infiltrates, magnification 200× (H&E). (Top Right) Eosinophil degranulation and lymphocyte infiltrates, magnification 500× (H&E). (Middle and Bottom) CD4, CD8, CD1a, and CD20, magnification 250×. Arrows denote dendritic cells.

In solid tumor subjects immunized with irradiated, GM-CSF-secreting tumor cells, examination of distant metastases resected after therapy often reveals the development of dense intratumoral T cell and eosinophil infiltrates (23–25). Serial bone marrow biopsies suitable for this type of analysis were available from 1 subject (MYTX-14) who achieved an ongoing complete remission (29+ months). A specimen obtained shortly after finishing immunization demonstrated the presence of abundant CD3 T cells that were associated with malignant myeloblasts and frequent eosinophils distributed through the marrow (Fig. 4). Three months later, no leukemia was evident in the bone marrow, which instead displayed normal hematopoietic differentiation. Two additional subjects who had complete responses after vaccination similarly showed increased eosinophils in the remission marrows, although staining for CD3+ T cells in earlier samples was technically not feasible. Together, these findings are consistent with the idea that, as in solid tumor patients, vaccination early after HSCT can provoke host reactions in distant sites of disease.

In previous studies of solid tumor patients who responded to GM-CSF-secreting tumor cell vaccines, we identified a correlation between immune-mediated tumor destruction and decreases in the levels of circulating soluble MHC class I chain-related protein A (MICA) (27). MICA and the closely related MHC class I chain-related protein B (MICB), as well as several UL16-binding proteins (ULBP), are ligands for NKG2D, an activating receptor expressed on natural killer (NK) cells and CD8+ T lymphocytes that contributes to antitumor cytotoxicity (28–30). Tumor cells may escape from NKG2D-mediated immune destruction, however, through the shedding of surface MICA and possibly other ligands (31–33).

To explore whether this pathway might be operative in the HSCT setting, we measured levels of soluble NKG2D ligands in the 15 immunized subjects. Although none of these subjects had significant levels of soluble ULBP-1–3 upon study entry, 13 showed high levels of shed MICA and MICB. Moreover, long-
To determine whether declines in soluble NKG2D ligands are generally linked to clinical response, we examined serial serum samples obtained from 34 patients with hematologic malignancies who achieved complete remissions as a function of allogeneic HSCT alone. Included in this cohort were subjects who underwent either nonmyeloablative or ablative conditioning regimens and who developed either mild or no chronic GVHD. Among this group, only a minority showed high pretreatment levels of soluble MICA in a range similar to the vaccinated subjects, perhaps indicative of the advanced disease-risk status of the immunized patients. Nonetheless, none of the control subjects who did not derive sustained clinical benefits from treatment failed to display persistent decreases in sMICA/MICB (not shown).

To investigate the role of the NKG2D pathway in more detail, we used flow cytometry to characterize NKG2D surface expression on circulating NK and CD8\(^+\) T cells from the long-term responders to vaccination after HSCT. One subject with elevated sMICB at study entry showed diminished NKG2D expression and reduced the amount of shed ligand (Fig. 6A and not shown). However, NKG2D staining was not decreased in the other long-term responders with elevated sMICA/MICB, which suggests that factors in addition to shed ligands may also modulate receptor levels. Consistent with this idea, the 2 long-term responders without detectable sMICA/MICB showed diminished NKG2D staining on NK cells (but not CD8\(^+\) T lymphocytes) upon study entry, whereas therapy restored expression (Fig. 6B and not shown).

**Discussion**

Although allogeneic HSCT is one of the most striking examples of successful cancer immunotherapy (34), disease relapse remains a major problem for patients with advanced MDS and recurrent or refractory AML (6). The clinical data presented here demonstrate that immunization with lethally irradiated, autologous leukemia cells engineered to secrete GM-CSF early after allogeneic HSCT is feasible, safe, and biologically active. Despite immune suppression for GVHD prophylaxis, vaccination elicited significant local and systemic antileukemia reactions, suggesting that some protective mechanisms might function through calcineurin-independent pathways. Long-term clinical responders generated, as a function of treatment, DTH reactions to nontransduced, autologous leukemia cells and, in a limited number of cases available for study, bone marrow eosinophil and T-lymphocyte infiltrates. Marked decreases in the levels of soluble NKG2D ligands and normalization of NKG2D expression on NK cells and CD8\(^+\) T lymphocytes were also associated with leukemia cell destruction. Overall, these immunologic characteristics are similar to those previously observed in vaccine studies of nontransplanted, solid tumor patients (23–25), and suggest that the period early after allogeneic HSCT may be particularly favorable for immune manipulation in patients.

A major challenge in this work is to delineate the specific contribution of vaccination to the overall GVL effect. Donor lymphocytes may react to leukemia-associated gene products or
than the 56% for subjects who received at least 1 vaccination. The 2-year OS for this group was 21%, which is significantly lower.

The HSCT conditioning regimen was administered from days –5 to –2 and consisted of i.v. fludarabine 30 mg/m²/day × 4 and busulfan 0.8 mg/kg × 8. Unmanipulated, G-CSF mobilized peripheral blood stem cells were infused on day 0. The GVHD prophylaxis was tacrolimus starting at day –3 and i.v. methotrexate 5 mg/m² on days +1, 3, 6, and 11. Recombinant GM-CSF protein (250 mg/m²) was administered daily beginning on day +1 until the absolute neutrophil count exceeded 1,000 cells/μl. The infection prophylaxis included acyclovir, trimethoprim-sulfamethoxazole or atovaquone, and a quinolone. A preemptive treatment strategy for cytomegalovirus was pursued.

GM-CSF-secreting leukemia cell vaccines were manufactured using an adenoviral vector encoding GM-CSF, as previously reported (24, 25), with doses ranging from 1 × 10⁶ to 1 × 10⁷ myeloblasts (harvested prior to HSCT) depending on the overall cell yield. Vaccination was initiated between days 30 and 45 after HSCT if no grade II to IV acute GVHD, no systemic corticosteroid therapy, no uncontrolled acute infection; adequate hematologic recovery and nonhematologic toxicity less than grade 3. Immunosuppression was administered intradermally and subcutaneously weekly times 3, followed by every other week times 3, as described (24, 25). DTH testing with 1 × 10⁶ irradiated, autologous, nontransduced leukemia cells injected intradermally was performed with the first, fifth, and fourth weeks after the sixth vaccine.

Patients were maintained on therapeutic levels of tacrolimus until 4 weeks after completing the immunizations, when tapering was begun. Acute GVHD was graded according to modified Glucksberg criteria.

In conclusion, this Phase I clinical trial has revealed the safety and biologic activity of lethally irradiated, autologous, GM-CSF-secreting leukemia cells early after allogeneic HSCT. Future investigations will explore the interplay of vaccination with myeloablative conditioning regimens, donor lymphocyte infusions, and a reduction in immune suppression for GVHD prophylaxis. Overall, our results suggest that combinatorial immunotherapies in the setting of HSCT are a promising strategy to intensify GVL.

Materials and Methods

**Clinical Protocol.** This Phase I trial received approval from the Dana-Farber/ Harvard Cancer Center institutional review board and biosafety committees, the National Institute of Health Recombinant DNA Advisory Committee, and the Food and Drug Administration. The eligibility requirements were: appropriate for nonmyeloablative HSCT; age ≥18; MDS-RAEB or AML not in remission (defined as ≥5% marrow blast); available 6 out of 6 matched donor (by high-resolution typing) at HLA-A, B, and DRB1, and Eastern Cooperative Oncology Group performance status 0 to 2. Uncontrolled infection, active CNS leukemic involvement, HIV positivity, or inadequate organ function were exclusion criteria.

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Patients were maintained on therapeutic levels of tacrolimus until 4 weeks after completing the immunizations, when tapering was begun. Acute GVHD was graded according to modified Glucksberg criteria.
Pathology. Skin biopsies were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, and stained with H&E. Immunohistochemistry was performed using standard techniques with monoclonal antibodies to CD4, CD8, CD20, and CD1a. Bone marrow biopsies were formalin fixed, paraffin embedded, and stained with Giemsa and anti-CD3 antibodies.

Vaccine and DTH responses were considered strong when the following were present: mononuclear cells admixed with eosinophils and basophils accumulated around blood vessels; endothelial cells were swollen or necrotic, often with vessel luminal occlusion; dermal edema and fibrin exudation were present.

NGK2D Pathway. Soluble MICA, MICB, and ULBP-1-3 levels were measured with ELISA using recombinant protein ligands and anti-ligand monoclonal antibodies (R&D Systems), and NGK2D expression on circulating NK cells (CD3+ CD56−) and CD8+ T cells (CD3+ CD56−) was determined by flow cytometry, as previously reported (27, 33).

Statistics. Descriptive statistical analysis was performed to assess patient baseline characteristics, disease, disease stage, GVHD, sMICA/MICB levels, and toxicity data. Cumulative incidence of acute or chronic GVHD was calculated reflecting time to onset of GVHD and time to death without GVHD as competing risks. Overall survival was calculated using the Kaplan-Meier method.

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Table S1. Features and clinical outcomes of subjects who started vaccination after HSCT

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age</th>
<th>Sex</th>
<th>Disease status at collection/HSCT</th>
<th>Marrow blast at HSCT %</th>
<th>Disease Status at day 30, prior to VAX</th>
<th>No. vaccines given</th>
<th>Reason for stopping VAX</th>
<th>VAX response</th>
<th>Current status/survival from HSCT (month)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46</td>
<td>M</td>
<td>Relapsed refractory AML</td>
<td>91%</td>
<td>CR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>Acute appendicitis/ C. diff colitis</td>
<td>Relapse</td>
<td>DOD/3.5</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>M</td>
<td>Untreated secondary MDS/RAEB-II</td>
<td>12%</td>
<td>CR</td>
<td>6</td>
<td>Complete</td>
<td>Relapse</td>
<td>DOD/17.5</td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>M</td>
<td>Refractory CML myeloid blast crisis</td>
<td>57%</td>
<td>PD</td>
<td>1</td>
<td>Disease/Imatinib added</td>
<td>NR</td>
<td>DOD/6.2</td>
</tr>
<tr>
<td>4</td>
<td>61</td>
<td>M</td>
<td>Relapsed AML</td>
<td>50%</td>
<td>CR</td>
<td>6</td>
<td>Complete</td>
<td>CCR</td>
<td>NED/43</td>
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<tr>
<td>5</td>
<td>57</td>
<td>M</td>
<td>Untreated MDS/AML</td>
<td>20–25%</td>
<td>CR</td>
<td>6</td>
<td>Complete</td>
<td>CCR</td>
<td>NED/38</td>
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<tr>
<td>6</td>
<td>66</td>
<td>M</td>
<td>Untreated RAEB-I</td>
<td>6–10%</td>
<td>CR</td>
<td>6</td>
<td>Complete</td>
<td>CR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NED/38</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>M</td>
<td>Untreated M6 AML, complex cytogenetics</td>
<td>11%</td>
<td>CR</td>
<td>2</td>
<td>Grade II skin aGVHD</td>
<td>CCR</td>
<td>Died pneumonia/13.5</td>
</tr>
<tr>
<td>8</td>
<td>64</td>
<td>F</td>
<td>Relapsed refractory AML, inv 11q23, active leukemia cutis</td>
<td>49%</td>
<td>PD</td>
<td>6</td>
<td>Complete</td>
<td>CCR</td>
<td>NED/31</td>
</tr>
<tr>
<td>9</td>
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<td>F</td>
<td>Relapsed AML</td>
<td>14%</td>
<td>PD</td>
<td>4</td>
<td>Disease/hospice</td>
<td>NR</td>
<td>DOD/2.5</td>
</tr>
<tr>
<td>10</td>
<td>62</td>
<td>M</td>
<td>Untreated MDS/RAEB-II</td>
<td>11%</td>
<td>CR</td>
<td>6</td>
<td>Complete</td>
<td>CCR</td>
<td>NED/28.5</td>
</tr>
<tr>
<td>11</td>
<td>61</td>
<td>M</td>
<td>Induction failure AML</td>
<td>20%</td>
<td>CR</td>
<td>4</td>
<td>Disease/chemo added</td>
<td>NR</td>
<td>DOD/13</td>
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<td>12</td>
<td>66</td>
<td>M</td>
<td>Relapsed refractory AML, del 9q trisomy 8</td>
<td>17%</td>
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<td>Complete</td>
<td>CCR</td>
<td>NED/26</td>
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<tr>
<td>13</td>
<td>58</td>
<td>M</td>
<td>Relapsed AML, complex cytogenetics</td>
<td>23%</td>
<td>CR</td>
<td>6</td>
<td>Complete</td>
<td>CR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NED/24</td>
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<tr>
<td>14</td>
<td>49</td>
<td>M</td>
<td>Secondary MDS, RAEB-1 complex cytogenetics</td>
<td>9%</td>
<td>CR</td>
<td>6</td>
<td>Complete</td>
<td>CCR</td>
<td>NED/15</td>
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<tr>
<td>15</td>
<td>58</td>
<td>M</td>
<td>MDS/AML</td>
<td>21%</td>
<td>CR</td>
<td>6</td>
<td>Complete</td>
<td>CCR</td>
<td>NED/12</td>
</tr>
</tbody>
</table>

<sup>a</sup>This subject had a morphologic CR, but not cytogenetic remission.

<sup>b</sup>These 2 subjects had hematologic relapse during or shortly after vaccination, but entered spontaneous CR after tacrolimus taper.

CCR, continued complete remission (applicable to subjects who were in a CR at vaccine initiation and who never relapsed); CML, chronic myelogenous leukemia; CR, complete remission (applicable to subjects who had persistent or relapsed disease after HSCT, but achieved CR after vaccine or withdrawal of tacrolimus); DOD, died of disease; NED, no evidence of disease; NR, no response; PD, persistent disease; RAEB, refractory anemia with excess blasts.