Cancer–Testis Antigen 7 Expression and Immune Responses Following Allogeneic Stem Cell Transplantation for Multiple Myeloma

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Abstract
Cancer–testis antigen 7 (CT7) is the most frequently and consistently expressed MAGE antigen in multiple myeloma, exhibits tissue-restricted expression, and is an independent negative prognostic factor for multiple myeloma. We sought to characterize CT7 protein expression in the bone marrow of patients with multiple myeloma undergoing allogeneic T cell–depleted hematopoietic stem cell transplantation (alloTCD-HSCT), and to examine the significance of CT7-specific cellular immune responses. We further aimed to determine CT7-derived immunogenic epitopes and their associated allelic restrictions. CT7 protein expression in neoplastic CD138+ plasma cells was evaluated by immunohistochemistry in bone marrow biopsies from 10 patients. CT7 was present in 8 of 10 patients. Longitudinal analyses of the 10 patients revealed an association between CT7 expression and prognosis. Longitudinal monitoring of CT7-specific T cells revealed an association between increased frequencies of CT7-specific T cells and reductions in specific myeloma markers. Epitope-specific reactivity to the nonamer FLAMLKNTV was detected by intracellular IFN-γ assay in peripheral blood (PB) and bone marrow–derived T cells from HLA-A*0201 patients. Serial monitoring of PB CT7-specific T-cell frequencies in 4 HLA-A*0201 patients by HLA-A*0201-CT7 tetramer staining revealed an association with disease course. Phenotypic analyses revealed bone marrow enrichment for central memory CT7-specific T cells, while effector memory cells dominated the PB. Together, these findings support the development of immunotherapeutic strategies that aim to enhance CT7-directed immune responses for the treatment of multiple myeloma. Cancer Immunol Res; 2(6): 1–12. ©2014 AACR

Introduction
Cancer–testis antigen 7 (CT7) or melanoma-associated antigen (MAGE)-C1 is the most frequently and consistently expressed MAGE antigen by malignant plasma cells in patients with multiple myeloma, and it does not seem to be down-regulated during the course of the disease (1–4). Its expression correlates strongly with plasma cell proliferation index, paraprotein levels, and reduced time to relapse, rendering CT7 a significant and independent negative prognostic factor for multiple myeloma (3). Evidence suggests that CT7 may play a gatekeeper function in terms of MAGE coexpression in multiple myeloma (5). CT7 may also play central roles in antiapoptosis, in the dysregulation of cell-cycle control and tumor progression, and in the development of chemotherapy resistance (3, 4, 6, 7). The high and consistent levels of CT7 expression, along with its tissue-restricted expression pattern, make CT7 a potential target for immunotherapeutic strategies against multiple myeloma at all clinical stages, including minimal residual disease (MRD).

Although much is known about the spontaneous development of cancer–testis antigen (CTA), and specifically CT7-specific immune responses in metastatic melanoma, data are very limited about their development in multiple myeloma (8–12). However, the detection of humoral and cellular immune responses against CTAs in patients with multiple myeloma following allogeneic stem cell transplantation (alloSCT), combined with occurrences of donor lymphocyte infusion (DLI)–induced remissions in relapsed patients, suggests that strategies that boost anti-CTA responses might foster long-lasting remissions (13, 14).

Specific T-cell responses to CT7 have been examined in the bone marrow of only 4 patients with multiple myeloma. Minor frequencies of 0.04% and 0.06% CD3⁺ CD8⁺ T cells were detected in the bone marrow of 2 of these patients, following in vitro T-cell expansion (15). This study did not distinguish between CD8⁺ and CD4⁺ T-cell reactivity, nor did it examine the specificity of CT7 reactivity in terms of epitope recognition. Peripheral blood
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Ted epitopes: 971 unable to detect any CD8+ C3 and C3 within the MAGE homology domain (MHD), and 491 those 3 patients who developed CD4+ T-cell reactivity in vitro T-cell expansion, but was unable to detect any CD8+ T-cell reactivity (16). Interestingly, those 3 patients who developed CD4+ T-cell responses to CT7 had been shown in earlier studies to produce a humoral response to the protein, thereby demonstrating a potential concordance in the immune response to CT7, albeit in a very limited number of patients (17). Analyses of the minimal epitopes of CT7 and their restriction elements conducted in monoclonal T-cell lines expanded from those 3 patients with multiple myeloma identified three novel MHC class I-restricted epitopes: 971–990 DDS presented by HLA-DRB1*0701, 991–990 RT presented by HLA-DPB1*1901 [both contained within the MAGE homology domain (MHD)], and 991–1010 LS presented by HLA-DP*0401 [located within the repetitive sequences of the 5′-end of the protein; ref. 16]. Five additional immunogenic epitopes of CT7 have been reported in patients with metastatic melanoma and in healthy donors (9, 18). As the immunodominant epitopes of CT7 that are recognized by healthy donors and patients with metastatic melanoma and multiple myeloma may differ, further research is required to (i) determine whether the reported epitopes are capable of inducing immune responses in patients with multiple myeloma, (ii) identify the MHC class I-restricted multiple myeloma–specific epitopes of CT7, and (iii) evaluate the potential clinical benefit of targeting these epitopes in patients with multiple myeloma.

Spontaneous humoral responses against CTAs have been documented in several studies, often in concordance with T-cell responses (17, 19). Antibody responses to CTAs are detected frequently in allograft recipients, and occur in the absence of bone marrow expression of the antigen, suggesting that they are derived from the allogeneic B-cell responses (14). In one study, CT7 seemed to elicit a dominant humoral response in patients with multiple myeloma (17). The CT7 protein elicited heterogeneous responses in the sera, with several B-cell epitopes of CT7 detected, and preferential targeting of the C-terminus of the CT7 protein (CT7522–601) occurring.

In the present study, we examined the significance of bone marrow expression of CT7, and the development of CT7-specific cellular immune responses in patients undergoing allogeneic T cell–depleted hematopoietic SCT (allogeneic HSCT) and DLI for high-risk and multiply relapsed multiple myeloma. In this study, we also sought to identify CT7-derived immunogenic epitopes and their associated allelic restrictions.

Our results demonstrate a correlation between CT7 protein levels in the bone marrow of patients with multiple myeloma and disease outcome, supporting the prognostic value of CT7 in multiple myeloma. Patients who developed marked populations of CT7-specific T cells had better disease outcomes, whereas patients who failed to expand CT7-specific T cells exhibited worse prognoses. We also identified a novel HLA-A*0201–restricted epitope of CT7, FLAMLKNTV, and demonstrated an association between CT7-specific T-cell frequencies and disease response.

Together, our data provide evidence to support the development of immunotherapeutic approaches targeting CT7 in patients with multiple myeloma. Adoptive immunotherapy with CT7-specific T cells after alloTCD-HSCT may generate levels of myeloma-specific T cells capable of eradicating MRD and preventing relapse in multiple myeloma.

Materials and Methods

Patients

After obtaining written informed consent, we obtained PB and bone marrow samples from patients before and after alloTCD-HSCT as part of a study approved by the Institutional Review Board of the Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY; registered at http://clinicaltrials.gov, ID: NCT01131169). Patients with high-risk cytogenetics presenting with refractory or relapsed disease following autologous HSCT for multiple myeloma were eligible for this study. After cytoreduction therapy with bortezomib, melphalan, fludarabine, and antithymocyte globulin preparative therapy, patients received alloTCD-HSCT from HLA-compatible donors. Patients did not receive immunosuppressive therapy after transplantation. DLIs were generally administered not sooner than 5 months after alloTCD-HSCT, at 5 × 10⁶ CD3/kg for the first and second infusions, with subsequent doses of 1 × 10⁶ CD3/kg administered if required.

Response criteria

Clinical responses to alloTCD-HSCT and DLI were assessed according to criteria of the European Group for Blood and Marrow Transplantation (EBMT; ref. 20).

Assessing CT7 protein expression in the bone marrow by immunohistochemistry

For morphologic in situ protein expression analysis, immunohistochemical stains for CD138 and CT7 were performed. Single- as well as double-staining techniques were used. Formalin-fixed paraffin-embedded samples of bone marrow biopsies were obtained from the archives of the Department of Pathology at MSKCC. Monoclonal antibody (mAb) CT7-33, previously generated by our group, was used for the detection of CT7, and mAb MI-15 (DAKO) served as reagent for the analysis of CD138 (21).

Immunohistochemistry (IHC) was performed as described previously (22, 23). For the single-stain technique, slides were subjected to a heat-based antigen retrieval procedure (30 minutes, 98°C) before the application of the primary antibody. Primary incubation with mAb CT7-33 or mAb MI-15 was done overnight at 4°C. A biotinylated horse anti-mouse antibody (1:200; Vector Laboratories) was used as a secondary reagent, followed by an avidin–biotin complex system using peroxidase as a reporter enzyme (ABC-elite kit; Vector Laboratories); 3,3′-diaminobenzidine tetrahydrochloride (liquid DAB; Biogenex) served as chromogen. For double staining, a sequential staining technique was used.

Double staining commenced with the detection of CD138. After completion of the first immunohistochemical staining step with mAb MI-15, a second round of antigen retrieval
was performed, followed by an avidin–biotin blocking procedure (VECTORSTAIN Elite ABC Kit; Vector Laboratories) to prevent cross-reactivity between the two detection steps. The second primary antibody CT7-33 to CT7 was then applied for 1 hour at 20°C. The biotinylated horse anti-mouse antibody served as a secondary reagent, followed by an alkaline phosphatase–linked streptavidin (Boehringer). A new fuchsin-based chromogen (Vector Red; Vector Laboratories) was used for the visualization of CT7. Morphologic evaluation of antigen coexpression was based on the membranous expression pattern of CD138 and the cytoplasmic/nuclear presence of CT7 in myeloma cells. To control for appropriate double staining, each case was also subjected to a conventional staining protocol using anti-CT7 and anti-CD138 on separate slides. Appropriate negative controls omitting the primary reagent were included for each case. The extent of CT7 staining was estimated on the basis of CD138-positive tumor cells and graded as follows: focal, approximately <5%; +, 5%–25%; ++, >25%–50%; ++++, >50%–75%; and +++++, >75%.

CT7 peptides

Overlapping peptides ranging from 20 to 26 amino acids in length and spanning the entire CT7 protein were purchased from Research Genetics (Bio-Synthesis Inc.). Peptides were manufactured to meet the specifications of validity of sequence, 95% purity and sterility. A total pool of 110 synthetic pentadecapeptides spanning the entire 1,142 amino acid sequence of the CT7 protein, with 11 amino acid overlaps, was prepared and stored. The nonamer FLAMLKNTV was manufactured to 98% purity and sterility (GenScript).

Quantitation of functional CT7- and FLAMLKNTV-specific T cells by intracellular IFN-γ analyses

The proportion of T cells producing IFN-γ in response to overnight stimulation with the CT7 total pool or the FLAMLKNTV peptide loaded onto autologous PB mononuclear cells (PBMC) were measured by fluorescence-activated cell sorting (FACS) analysis, as previously described (24, 25).

Determination and characterization of CT7 peptide-specific frequencies by MHC-tetramer analyses

CD8⁺ CT7-specific T-cell frequencies were quantified at multiple time points before and after transplantation in patients expressing HLA-A’0201 by staining with the HLA-A’0201-CT71086–1095 MHC-tetramer. The HLA-A’0201-CT71086–1095 MHC-tetramer complex was generated by the Ludwig Institute (Lausanne, Switzerland) and used as previously described (25–27). Immunophenotyping was concurrently performed. PBMC and bone marrow mononuclear cells (BMMC) were stained with phycoerythrin (PE)-labeled tetrameric complex, monoclonal anti-CD3 phycoerythrin-cyanin-7 (PE-Cy7), anti-CD8 PerCP, anti-CD45RA allophycocyanin (APC), and anti-CD62L fluorescein isothiocyanate (FITC; all BD Bioscience) for 20 minutes at 4°C. Appropriate control stains with HLA-mismatched cytomegalovirus (CMV) tetramers were also performed.

**Flow-cytometric analyses**

Data acquisition was performed with a FACSCalibur flow cytometer with triple lasers for 10-color capability using BD FACSDiva Software (BD Biosciences). Data analyses of T-cell frequencies were performed using FlowJo software (TreeStar Inc.).

**Correlating CT7-specific T-cell frequencies with disease status**

The ability of CT7-specific T cells to mediate in vivo anti-myeloma cytotoxicity was measured indirectly by correlating T-cell emergence with myeloma markers, determined at diagnosis and followed in patients after alloTCD-HSCT. Absolute numbers of CT7-specific T cells/µL PB were calculated by multiplying the percentage of IFN-γ-producing or tetramer-positive (tet⁺) FLAMLKNTV-specific T cells by the absolute numbers of CD8⁺ and CD4⁺ T cells, derived from the absolute lymphocyte count (ALC). Levels of standard myeloma markers in the PB, M-spike gamma, or immunoglobulin A (IgA), were monitored and used to determine disease response or progression.

**Results**

**CT7 expression by malignant plasma cells changes over the course of disease**

Although it has been shown that CT7 mRNA is expressed frequently in myeloma patients with advanced-stage disease, the course of CT7 protein expression in individual patients has not been examined in detail. To evaluate the longitudinal expression of CT7 protein in the bone marrow of patients with multiple myeloma, a dual-staining immunohistochemical approach was developed to examine the expression of CT7 (clone CT7-133; red) in neoplastic CD138⁺ plasma cells (clone MI15; brown). CT7 protein expression was monitored longitudinally in bone marrow specimens from 10 patients with myeloma, who were at least 2 years out from alloTCD-HSCT. Representative immunohistochemical stains of archival paraffin-embedded bone marrow specimens with varying levels of plasma cell infiltration are shown in Fig. 1A–C.

Overall, CT7 protein expression was detected in 8 of 10 patients. Pretransplant biopsy specimens were available from 9 of the 10 patients, and CT7 expression was detected in 6 of those 9 patients’ pretransplant specimens. Two of the 10 patients were negative for CT7 expression at all time points tested.

Fluctuations in CT7 protein expression levels in the bone marrow were observed in each of the 8 CT7-expressing patients following alloTCD-HSCT and DLI. Durable reductions in bone marrow CT7 protein expression were detected in 4 patients (UPN7/8/4/1), and coincided with the achievement of complete remission (CR) or very good partial response (VGPR; Fig. 1D–G, respectively). UPN7 showed focal expression of CT7 protein in CD138⁺ plasma cells in the marrow before alloTCD-HSCT, but was negative for CT7 at all time points tested thereafter (Fig. 1D).

Increases in CT7 protein expression ultimately occurred in the remaining 4 patients (UPN3/10/5/6), with concurrent progression of disease (POD) observed (Fig. 1H–K, respectively). The
expression of CT7 protein in >5% of CD138⁺ plasma cells (grade + or higher) preceeded disease relapse in several cases (Fig. 1E, I–K), supporting previous reports that CT7 may serve as an early marker for multiple myeloma relapse. The expression of CT7 protein was low (only 5% of the CD138⁺ multiple myeloma cells) at the time point preceding relapse in UPN3 (Fig. 1H).

The maximum level of CT7 protein expression detected in the bone marrow before or after transplant, as determined by IHC grading, seemed to be associated with disease outcome (Table 1). The maximum level of CT7 expression detected in the 4 patients who achieved durable CR in response to allotransplant and DLI was ++ (2 graded negative, 2 focal, and 1 + + +). One patient with maximal + + + disease achieved stable VGPR, but only upon reduction of CT7 protein expression to focal levels (<5% of CD138⁺ multiple myeloma cells; UPN1; Fig. 1G) following alloTCD-HSCT and DLI. Three patients with maximal CT7 disease ranging from ++ to ++++ achieved only partial response (PR; UPN5/6) or VGPR (UPN10), persisting for...
only 2 to 3 months, when DLIs were administered in addition to or following chemotherapy (Fig. I–K, respectively). The short durations of these PIs suggest that responses to DLI are inferior in patients with higher levels of CT7 expression.

The persistent presence of the CT7 protein in a high percentage of CD138<sup>+</sup> tumor cells and graded as follows: focal, approximately <5%; +, 5%–25%; ++, >25%–50%; ++++, >50%–75%; and +++++, >75%.

Patients received DLI in combination with or following chemotherapy, due to POD.

The emergence of CT7-specific T-cell frequencies following alloTCD-HSCT and DLI coincides with disease regression

To date, CT7-specific T-cell responses have only been described in a very small number of patients with multiple myeloma, following in vitro T-cell expansion methods. In this study, the development of CT7-specific T-cell responses was evaluated following alloTCD-HSCT and DLI in all 10 patients in whom CT7 bone marrow expression was examined. The clinical significance of the CT7-specific T-cell responses was further explored by correlating T-cell emergence with myeloma disease load. Absolute numbers of CD8<sup>+</sup> and CD4<sup>+</sup> T cells specifically producing IFN-γ in response to overnight stimulation with the total pool of CT7 peptides were quantified in freshly isolated PBMCs, and compared with each patient’s relevant marker of disease, either M-spike gamma or IgA (Fig. 2).

Increments in CT7-specific T-cell frequencies were observed in all patients following DLI, and were associated with reductions or stabilization of myeloma load of varying durations. Clear temporal associations between peak CT7-specific T-cell responses and disease regression were observed in 6 of the 10 patients monitored (UPN7/4/8/9/10/6; Fig. 2A–D, F, and H, respectively). In UPN7, a marked T-cell response of 16 CT7-specific T cells/µL of blood occurred 8 weeks after the administration of the first therapeutic DLI, and coincided with disease regression to continuous CR (Fig. 2A). Persistent low CT7-specific T-cell frequencies were detected at several time points thereafter, and the patient developed a second peak of CT7-specific T-cell response at a later time point, as shown in Fig. 2A. Findings from molecular chimerism studies conducted on isolated T cells 4 weeks after alloTCD-HSCT indicate that the CT7-specific T cells are of donor origin. A pronounced increase in the CT7-specific T-cell population that occurred following UPN7’s second DLI was also associated with the stabilization of the M-protein in an otherwise very aggressive myeloma (Fig. 2E).

Conversely, declining CT7-specific T-cell populations were temporally associated with elevated myeloma markers and disease progression in several patients (UPN10/3/6/2; Fig. 2F–I). Moreover, patients who failed to develop marked frequencies of CT7-specific T cells, despite repeated DLI, generally exhibited progressive disease courses (UPN10/3/5; Fig. 2F, G, and J).

Identification of a novel HLA-A<sup>0201</sup>-restricted immunogenic epitope of CT7

To further evaluate the specificity of the CD8<sup>+</sup> CT7-specific T cells detected in our patients with multiple myeloma, we sought to identify the immunogenic epitopes to which these CT7-specific T cells were responding. Reverse immunology was used to predict the epitopes of CT7 that were most likely to have high affinity for, bind to, and be presented by the commonly expressed HLA allele A<sup>0201</sup>. The top hit found in this search, the nonamer FLAMLKNTV, was located at position 1087 of the CT7 protein. A BLAST search revealed this nonamer to be unique to the CT7 protein. Reactivity to this protein was examined in 10 HLA-A<sup>0201</sup>-expressing patients, who had undergone alloTCD-HSCT followed by DLI by intracellular IFN-γ assays. FLAMLKNTV-specific IFN-γ production was observed to varying degrees in all patients examined (mean, 3.17%; range, 0.179%–15.49% CD8<sup>+</sup> T cells). Representative FACS plots from 7 patients are shown in Fig. 3A–G. Overall, significantly greater IFN-γ production was observed in response to autologous PBMC targets pulsed with the FLAMLKNTV peptide compared with control nonpulsed autologous PBMC (P = 0.0156; Fig. 3H).

Because of the consistent and reproducible detection of FLAMLKNTV-specific IFN-γ production in all HLA-A<sup>0201</sup>- patients, we proceeded to generate the cognate tetramer, HLA-A<sup>0201</sup>-CT7-1087. The generation of this tetramer facilitated the longitudinal monitoring of FLAMLKNTV-specific CD8<sup>+</sup> T-cell frequencies in PB and bone marrow samples from HLA-A<sup>0201</sup>- patients. Multiparametric analyses of samples stained with the tetramer and cell surface markers CD45RA and CD62L enabled phenotypic classification of CT7-specific T cells. Figure 4 shows representative FACS plots of staining performed on PBMC and BMMC isolated from UPN7 approximately 3 years after transplant, while the patient was in durable CR. Phenotypic analyses indicate the accumulation of effector memory T cells (TEM) specific for CT7 in the PB, and the selective accumulation of central memory T cells (T<sub>CM</sub>) specific for CT7 in the bone marrow.

The clinical significance of the CT7-specific CD8<sup>+</sup> T-cell frequencies detected by MHC-tetramer analyses after alloTCD-HSCT and DLI was again measured indirectly in

Table 1. Maximal CT7 protein expression in the bone marrow and disease outcome

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NOTE: CT7 expression determined by immunohistochemical analysis. The extent of CT7 staining was estimated on the basis of CD138<sup>+</sup> tumor cells and graded as follows: focal, approximately <5%; +, 5%–25%; ++, >25%–50%; ++++, >50%–75%; and +++++, >75%.

*Patients received DLI in combination with or following chemotherapy.
4 HLA-A*0201+ patients by correlating absolute numbers of PB-derived CT7-specific CD8+ tet+ T cells with each individual’s myeloma-specific disease marker. UPN7 experienced a disease recurrence approximately 7 months after alloTCD-HSCT that coincided with the loss of the TCM CT7-specific T cells in the PB. Shortly after the administration of the first DLI, an increase in TEM CT7-specific T cells occurred and the patient entered CR (Fig. 5A). The elevated frequencies of CT7-specific T cells observed later in UPN7, following the achievement of durable CR, may reflect an immune response to subclinical levels of myeloma. Indeed, MRD was detected in UPN7 in later analyses. Emergence of CT7-specific TEM was also observed in UPN4 following the administration of the second DLI, and again coincided with the patient entering a durable CR (Fig. 5B).

In contrast, UPN10 and UPN5 (Fig. 5C and D, respectively) failed to develop marked CT7-specific CD8+ T-cell frequencies, despite the administration of multiple DLI with and without preceding chemotherapy. Both patients exhibited progressive disease courses.

Discussion

CT7 is an X-linked CTA that was originally identified in melanoma cell lines by the technique of serologic analysis of recombinant cDNA expression libraries (SEREX) and by
representational difference analyses (28, 29). Further investigation revealed that CT7 is additionally expressed in tumors of various histologic types, including carcinomas of the liver, mammary glands, and ovary, non–small cell lung carcinoma, metastatic melanoma, and multiple myeloma. Its expression in normal adult tissues is restricted to testicular germ cells, which

Figure 3. FLAMLKNTV is an immunogenic epitope of CT7 that induces IFN-γ production by T cells isolated from patients with HLA-A*0201+ multiple myeloma. Intracellular IFN-γ production was measured in freshly isolated PBMC from 7 HLA-A*0201–expressing patients. Greater IFN-γ production by CD8+ T cells is observed in response to autologous PBMC pulsed with the HLA-A*0201–restricted immunogenic epitope FLAMLKNTV compared with nonpulsed control autologous PBMC targets. UPN4 (A), UPN5 (B), UPN7 (C), UPN10 (D), UPN11 (E), UPN12 (F), and UPN13 (G). H, FLAMLKNTV-induced IFN-γ production is significantly greater than the IFN-γ production to nonpulsed control targets \((P = 0.0156, \text{Wilcoxon matched pairs signed-rank test})\).
Our results expand on these findings by demonstrating a parallel relationship between CT7 expression at the protein level by IHC and disease course. Patients with low-level expression of or negative for CT7 entered CR or showed stable low-level disease. Furthermore, reductions in bone marrow CT7 protein expression levels were observed in 4 of 8 measurable patients following alloTCD-HSCT and DLI, and coincided with the achievement of CR in 3 patients and VGPR in the remaining patient. Increases in CT7 protein expression were observed in the remaining 4 patients, coincident with disease progression. Patients with high levels of CT7 protein experienced worse outcomes. Indeed, the 2 patients with persistently high levels of CT7 succumbed to progressive disease. The detection of CT7 protein both before transplant and after alloTCD-HSCT and DLI provides further evidence that CT7 is a viable target for immunotherapeutic approaches at all clinical stages. Longitudinal monitoring of protein expression may provide a more reliable method of CT7 detection compared with techniques targeting mRNA levels. Weak levels of CTA mRNA expression have been reported, whereas CTA protein expression seems to be potent and stable (3). Although these data are limited by the small number of patients monitored longitudinally, they are suggestive of an association between CT7 expression in the bone marrow and myeloma disease course. Further studies will be required to conclusively determine whether a true correlation exists between CT7 protein expression in the bone marrow and the myeloma disease burden.

Very limited data exist about the development of cellular immune responses to CT7. In myeloma in particular, specific T-cell responses to CT7 have only been examined in the PB of 18 patients and the bone marrow of 4 patients, with minor responses detected following in vitro T-cell expansion methods in 16.7% and 50% of patients, respectively (15). In our studies, existing frequencies of CT7-specific T cells were identified in the PB and bone marrow of all 10 patients with multiple myeloma examined, by intracellular IFN-γ and/or MHC-tetramer analyses. We were able to detect these CT7-specific T cells and quantify their absolute numbers in the PB without the requirement for in vitro T-cell expansion.

Our studies did not reveal a correlation between the absolute number of CT7-specific T cells and disease burden, but instead demonstrated a temporal association between CT7-specific
T-cell frequencies and myeloma disease. The minimum number of CT7-specific T cells required to induce a clinical response differs by patient. The “T-cell threshold” required to induce an anti-myeloma response is likely dependent on multiple factors, including (but not limited to) the extent of the myeloma disease, the amount of CT7 expressed by myeloma cells, and the host immune environment.

Longitudinal monitoring of CT7-specific T-cell populations in the PB demonstrated the predominant presence of cells of an effector memory phenotype, and revealed an association with the myeloma disease course. The CT7-specific T-cell responses described herein exhibited similar patterns of T-cell emergence, expansion, magnitude, and contraction as the WT1-specific T-cell responses detected in those patients previously reported (23).

Two patients in whom CT7 protein expression was not detected in the bone marrow developed measurable CT7-specific T-cell responses (UPN2 and UPN9). The CT7-specific T-cell responses detected in these patients could possibly result from previous expression of CT7 protein in the bone marrow, which was present before our analyses. It is also possible that these patients expressed CT7 during the course of our studies, but we were unable to detect it. The bone marrow aspirates obtained and analyzed reflect only a small percentage of the total bone marrow compartment, which may contain low frequencies of malignant cells not captured within the aspirate sample.

We further sought to examine potential epitopes of CT7 to which the T cells detected in our patients with multiple myeloma may be responding. Reverse immunology predicted the HLA-A*0201–restricted epitope FLAMLKNTV, which induced peptide-specific IFN-γ production in all 10 HLA-A*0201+ patients examined. Generation of the novel HLA-A*0201-CT71087–1095 tetramer facilitated serial monitoring of the CT7-specific T-cell frequencies and their phenotypes over the course of the disease in 4 HLA-A*0201+ patients >2.5 years out from transplant. CT7 tetramer–positive T cells were detected in both the PB and bone marrow of patients. Phenotypic analyses revealed the preferential targeting of central memory CT7-specific CD8+ T cells to the bone marrow, whereas effector memory CT7-specific CD8+ T cells were predominantly found in the PB. Although we did not have sufficient bone marrow samples to assess a correlation between bone marrow–derived CT7-specific T-cell frequencies and myeloma control/antigen suppression, we expect that such a correlation exists. Trafficking to the active site of disease, the bone marrow, is likely required for CT7-specific T cells to elicit an anti-myeloma effect.

Longitudinal monitoring of CT7-specific T-cell frequencies by both intracellular IFN-γ and MHC-tetramer analyses showed that patients who developed marked frequencies of CT7-specific T cells after alloTCD-HSCT and DLI had better outcomes, often achieving CR or VGPR. However, those patients who failed to expand CT7-specific T cells, despite repeated DLI administered in combination with or following chemotherapy, experienced progressive disease courses and worse outcomes. These findings corroborate that which we have previously observed in terms of the emergence of T cells specific for WT1 (23).
CT7-specific T-cell frequencies have also been monitored in a subset of patients with multiple myeloma before and following autologous SCT, in addition to the post-alloTCD-HSCT and DLI analyses presented herein. Escalations in the frequencies of CT7-specific T-cell populations occurred following autoSCT, suggesting that autologous transplantation may boost CT7-specific immune responses. The greatest frequencies of CT7-specific T cells were detected following alloTCD-HSCT and DLI and were present at significantly higher levels than those detected before autoSCT and DLI (data not shown).

Autologous and allogeneic transplantation may enhance adaptive cellular and humoral immune responses via numerous mechanisms. First, chemotherapy applied before transplant and/or DLI likely promotes the release of tumor antigen upon tumor necrosis, making antigen available for uptake and processing by antigen-presenting cells, and subsequent presentation to tumor-specific T cells (32). The posttransplant lymphopenic host immune environment may further augment both types of immune responses via homeostatic proliferation of T cells and antibody-producing B cells. The elimination of suppressor cell subsets, removal of cytokine sinks, and greater availability and activation of antigen-presenting cells may also promote antitumor immune responses in the lymphopenic setting (33). It has also been postulated that the allogeneic immune environment itself may promote the persistence and efficacy of antitumor immune responses by providing "danger signals" that attenuate self-limiting immune mechanisms (14). In addition to boosting "host" immunity, these mechanisms also enhance the efficacy of adoptively transferred antigen-specific CD8+ T cells (34). Conceivably, the presence of these mechanisms in the posttransplantation lymphopenic host environment promoted the expansion of CT7-specific precursors contained within the DLI products administered to some of our patients described herein, thereby generating or enhancing the graft-versus-myeloma effect.

To eliminate MRD, CT7-specific T cells would need to target the putative multiple myeloma stem cell (MMSC), as well as the malignant plasma cells making up the bulk of the disease. Cancer stem cells are valid targets for immunotherapy due to their expression of CTAs, and their lysis by both T cells and antibody-producing B cells. The elimination of both types of immune responses via homeostatic proliferation of T cells and antibody-producing B cells. The elimination of suppressor cell subsets, removal of cytokine sinks, and greater availability and activation of antigen-presenting cells may also promote antitumor immune responses in the lymphopenic setting (33). It has also been postulated that the allogeneic immune environment itself may promote the persistence and efficacy of antitumor immune responses by providing "danger signals" that attenuate self-limiting immune mechanisms (14). In addition to boosting "host" immunity, these mechanisms also enhance the efficacy of adoptively transferred antigen-specific CD8+ T cells (34). Conceivably, the presence of these mechanisms in the posttransplantation lymphopenic host environment promoted the expansion of CT7-specific precursors contained within the DLI products administered to some of our patients described herein, thereby generating or enhancing the graft-versus-myeloma effect.

This study indicates that CT7 protein expression in the bone marrow of patients with multiple myeloma is associated with the disease course, and may serve as a prognostic marker. The identification of a novel epitope of CT7 that is restricted by the HLA-A’0201 allele facilitated the development of a tetramer for serial monitoring of CT7-specific T-cell frequencies in patients with multiple myeloma. Longitudinal analyses of CT7-specific T-cell frequencies by IFN-γ and tetramer analyses revealed an association between T-cell responses and disease outcome, with patients who developed marked populations of CT7-specific T cells entering CR, and those failing to expand CT7-specific T cells exhibiting progressive disease courses. These findings suggest that CT7-specific immune responses play a role in modulating myeloma disease, and support the development of immunotherapeutic approaches targeting CT7 for the treatment of multiple myeloma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** E.M. Tyler, A.A. Jungbluth, S. Gnjatic, R.J. O'Reilly, G. Koehne  
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**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** E.M. Tyler, R.J. O'Reilly, G. Koehne  
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**References**


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