

Vigorous Premalignancy-specific Effector T Cell Response in the Bone Marrow of Patients with Monoclonal Gammopathy

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Abstract

Most approaches targeting the immune system against tumors have focused on patients with established tumors. However, whether the immune system can recognize preneoplastic stages of human cancer is not known. Here we show that patients with preneoplastic gammopathy mount a vigorous T cell response to autologous premalignant cells. This preneoplasia-specific CD4⁺ and CD8⁺ T cell response is detected in freshly isolated T cells from the BM. T cells from myeloma marrow lack this tumor-specific rapid effector function. These data provide direct evidence for tumor specific immune recognition in human preneoplasia and suggest a possible role for the immune system in influencing the early growth of transformed cells, long before the development of clinical cancer.

Key words: myeloma • vaccine • dendritic cells • prevention • gammopathy

Introduction

Up to 1–3% of elderly humans have clonal expansions of transformed plasma cells called preneoplastic gammopathy (MGUS) (1). Recent studies using interphase cytogenetics and gene expression profiling have shown that transformed plasma cells in MGUS carry most of the known cytogenetic and genomic changes found in its malignant counterpart, multiple myeloma (2–4). Yet, the great majority of MGUS patients remain stable for prolonged periods without ever developing clinical malignancy (1). Whether the immune system can recognize such clonally expanded preneoplastic cells in patients is not known.

In prior studies, we found that freshly isolated T cells from blood and marrow of patients with newly diagnosed multiple myeloma (MM) do not exhibit detectable rapid IFN- γ secretion in response to autologous tumor (5). However, effector cells for viral pathogens were detected, and tumor-reactive killer T cells could be expanded after 2 wk in vitro stimulation with autologous tumor-loaded DCs (5). Here we show that patients with MGUS mount a vigorous immune response to their preneoplastic cells, which is readily detectable in T cells from the BM tumor bed, without the need for prolonged in vitro expansion, as in MM patients.

Materials and Methods

Isolation of Tumor and Immune Cells. Concurrent peripheral blood and BM samples were obtained from 12 patients with a diagnosis of multiple myeloma ($n = 6$) or MGUS ($n = 6$) based on standard criteria (6). All patients signed an informed consent approved by the institutional review board.

Mononuclear cells from blood or BM were obtained by density gradient centrifugation using Ficoll Hypaque. DCs were generated from monocytes by culture of adherent blood mononuclear cells in the presence of GM-CSF (Immunex) and IL-4 (R&D Systems) as described (7). Nonadherent cells were used as a source of blood T cells. BM mononuclear cells were separated into tumor/preneoplastic (CD138 positive) and nontumor (CD138 negative) fractions using CD138 magnetic beads using the manufacturer's protocol (Miltenyi Biotec) (5). Tumor/preneoplastic cells were cryopreserved in aliquots for use as sources of antigen and targets. Nontumor cell fractions were also cryopreserved for use as a source of T cells from the marrow as described previously (5). In some patients with IgG myeloma or MGUS, circulating Igs were purified from plasma by affinity chromatography on a protein G sepharose column (Amersham Biosciences) as described (5).

Generation of Antigen-loaded DCs. Autologous tumor/preneoplastic cells were opsonized using anti-syndecan-1 antibody, γ irradiated, and then fed to immature DCs as described (5, 7). DCs were then induced to mature by overnight culture in the presence of a cytokine cocktail consisting of 10 ng/ml TNF- α 1, 10 ng/ml IL-1 β , IL-6 1,000 U/ml (all from R&D Systems) and PGE₂ (1 μ g/ml; Amersham Biosciences). For some experiments, autologous DCs were pulsed overnight with purified Ig as described (5).

ELISPOT Assay for Quantitation of Cytokine-producing T Cells. The presence of tumor/preneoplasia-reactive, IFN- γ -producing T cells in both blood and marrow T cells was quantified both directly ex vivo and after in vitro stimulation with antigen-

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pulsed DCs using a 16 h ELISPOT assay as described (7). Briefly, autologous T cells from blood or marrow were cocultured with unpulsed DCs, or DCs loaded with autologous or allogeneic myeloma cells, at a T:APC ratio of 10:1. For some experiments, autologous tumor/preneoplasia (CD138⁺) or nontumor cells (CD138⁻) fraction from the BM were used as APCs. After 16 h, the presence of antigen-specific IFN- γ spot-forming cells were quantified as described (5, 7).

Flow Cytometric Assay for the Detection of Intracellular Cytokines. Tumor antigen-reactive cells in freshly isolated blood or marrow T cells were also analyzed by flow cytometry-based assay for the detection of intracellular cytokines as described previously (8). Briefly, blood or BM T cells were cultured for 12 h with autologous unpulsed mature DCs, or DCs loaded with autologous tumor, or cag tumor cell line in the presence of 0.7 μ g/ml of Golgistop (from Cytofix/CytoPerm Plus kit; BD Biosciences). Cells were then fixed and permeabilized in 100 μ l cytofix/cytoperm solution using manufacturer's directions and stained for intracellular cytokines (IFN- γ). Samples were analyzed on FACSsort or FACS Calibur (Becton Dickinson) instrument using CellQuest software. Lymphocyte events collected were typically 1–3 $\times 10^5$ events per sample.

Expansion of Tumor-reactive T Cells in Culture. Antigen-loaded (or unpulsed) mature DCs were cocultured with autologous T cells (2×10^5 cells/well) from either the marrow or blood as described previously (5). After 12–16 d of culture, T cells from several microcultures were pooled, and tumor antigen-reactive T cells were assayed by ELISPOT assays for reactivity against autologous tumor/preneoplasia-loaded DCs as described earlier (5).

Statistical Analysis. Data for immune response between two groups were compared using the Mann Whitney test, and significance set at $P < 0.05$.

Results

To study the interactions of the immune system with preneoplastic or neoplastic cells, we obtained paired blood and BM specimens from 12 consecutively referred patients with a diagnosis of MGUS ($n = 6$) or MM ($n = 6$) based on standard diagnostic criteria (6). MM patients included those with both early (Durie-Salmon stage I/II; $n = 2$) or advanced (stage III; $n = 4$) stage disease and were studied before the initiation of any chemotherapy. Freshly isolated T cells from either blood or BM of myeloma patients did not react detectably to autologous tumor-loaded DCs in an effector assay measuring IFN- γ secretion, consistent with findings in prior studies (5) (Fig. 1). In contrast, when MGUS patients were studied IFN- γ -producing T cells in response to preneoplasia-pulsed DCs were readily detected in freshly isolated T cells from all patients, without the need for prolonged ex vivo expansion. Therefore, we refer to these cells as rapid effectors to distinguish them from those that require 1–2 wk in vitro culture. Preneoplasia/tumor-specific IFN- γ producers in the BM, as detected by the ELISPOT assay, were significantly higher in MGUS relative to myeloma (mean 55 versus 1 IFN- γ producers/ 10^5 cells in MGUS versus MM, respectively; $P = 0.002$). This effector T cell response in MGUS was significantly enriched in the marrow relative to the blood (mean 55 versus 7 IFN- γ pro-

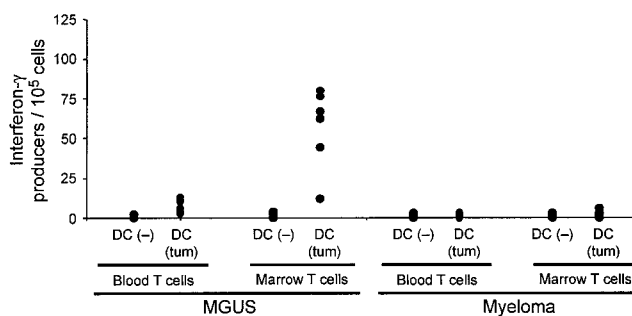


Figure 1. Enrichment of preneoplasia-specific effectors in the BM of MGUS patients. Freshly isolated blood or BM T cells from patients with MGUS ($n = 6$) or myeloma ($n = 6$) were tested for the recognition of autologous tumor/preneoplasia-loaded DCs (DC-Tum) or unpulsed DCs (DC) in a 16-h ELISPOT assay for IFN- γ -producing cells.

ducers/ 10^5 cells in the marrow versus blood, respectively; $P = 0.008$). Therefore, the marrow but not the blood of MGUS patients contains significant numbers of rapid effector T cells that are reacting to antigens from these preneoplastic cells.

To further characterize the nature of IFN- γ producers in response to autologous tumor cell-loaded DCs, we used intracellular cytokine flow cytometry (Fig. 2). With this assay, the antitumor effector response in MGUS was detected in both CD4⁺ and CD8⁺ T cells from the BM of three out of three patients tested (Fig. 2 A). Consistent with this, the ELISPOT reactivity could not be depleted by removing either CD4⁺ or CD8⁺ population alone (not depicted). Therefore, the preneoplasia-specific effector T cell response in MGUS marrow consists of both CD4⁺ and CD8⁺ T cells.

We next addressed the antigenic specificity of the T cells in MGUS by testing myeloma cell lines and preneoplastic cells from other individuals with MGUS. However, there was no recognition of autologous DCs loaded similarly with allogeneic MGUS tumor cells or cag myeloma cell line (shown previously to highly express several shared cancer-testis antigens) (7), suggesting that the antitumor immune response is largely directed against a pattern of antigens specific to each individual patient's tumor (Fig. 2, A and B). Myeloma and MGUS cells secrete a monoclonal Ig that can serve as a patient-specific tumor antigen (9). However, in contrast to lymphomas it is largely secreted as a soluble antigen, which can lead to the deletion of Ig-reactive T cells (10). Preneoplasia-specific effectors in the marrow did not recognize autologous monoclonal Ig-loaded DCs, indicating that the effector T cells detected in these assays were not directed against Ig-derived determinants (Fig. 2 B). This is remarkably similar to the findings on reactivity of tumor-specific T cells expanded in culture from MM patients using tumor cell-loaded DCs (5). Importantly, the immune T cells also recognized isolated fresh MGUS plasma cells but not nonplasma cells from the BM in the two patients tested (Fig. 2 C). We also tested the ability of autologous preneoplastic cell-loaded DCs to expand preneoplasia-specific T

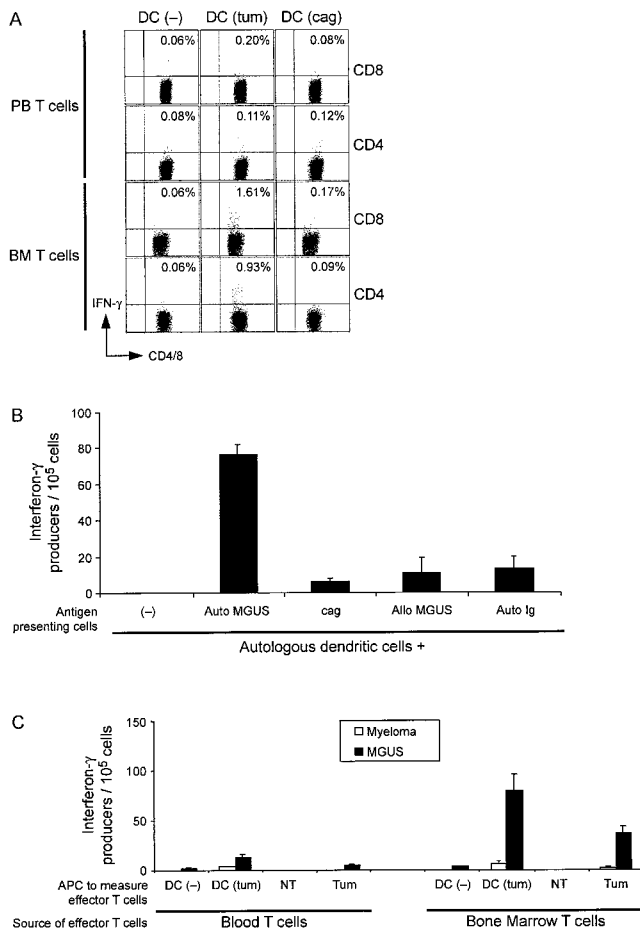


Figure 2. Characterization of preneoplasia-specific immune effectors in the MGUS BM. (A) Preneoplasia-specific IFN- γ -producing T cells consist of both CD4⁺ and CD8⁺ T cells. Freshly isolated blood or BM T cells from a patient with MGUS were cultured for 12 h with autologous control DCs, DCs loaded with autologous preneoplastic cells, and DCs loaded with the cag myeloma cell line. IFN- γ -producing CD4⁺ and CD8⁺ T cells were examined by intracellular cytokine flow cytometry. Percentages refer to the proportion of CD4⁺ or CD8⁺ T cells producing IFN- γ . Data are representative of similar findings on three patients. (B) Preneoplasia-reactive effectors are mostly specific for antigens on autologous preneoplastic cells. BM T cells from a patient with MGUS were tested for the recognition of autologous control DCs, DCs loaded with autologous preneoplastic cells, cag myeloma cell line, preneoplastic cells from a different MGUS patient (allo), or exposed to 10 μ g/ml tumor-derived Ig. IFN- γ -producing cells were quantified by a 16-h ELISPOT assay. Data are representative of similar experiments on two patients. (C) Recognition of fresh autologous MGUS tumor cells by immune cells in the BM. Freshly isolated blood or BM T cells from patients with MGUS or myeloma were tested for the recognition of autologous tumor/preneoplasia-loaded DCs (DC-Tum) or unpulsed DCs (DC), or tumor/preneoplasia (Tum; CD138⁺) or nontumor (NT; CD138⁻) fractions from the BM in a 16-h ELISPOT assay for IFN- γ -producing cells. Data on the recognition of MGUS tumor cells are representative of findings from two patients.

cells in culture in two patients with MGUS. Preneoplasia-specific T cells could be expanded in both patients, although the degree of expansion was greater with BM T cells (Fig. 3). Therefore, the BM of MGUS patients contains effector T cells that are specific to a patient's preneoplastic clonal expansion.

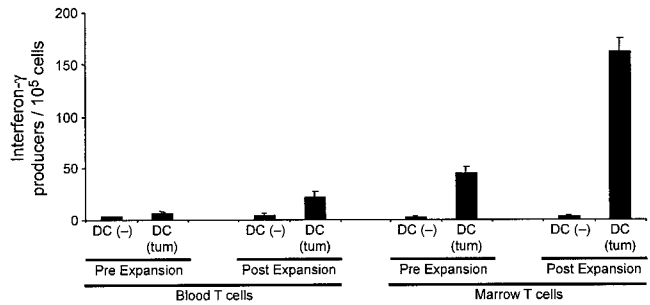


Figure 3. Antipreneoplasia T cells from MGUS patients can be expanded in vitro using autologous preneoplastic cell-loaded DCs. Blood or marrow T cells from a patient with MGUS were cocultured with DCs loaded with autologous MGUS tumor cells. Tumor antigen-specific T cells were quantified by testing reactivity to autologous control DCs or tumor loaded DCs using a 16-h IFN- γ ELISPOT assay. Data shown are representative of similar findings from two patients.

Discussion

To our knowledge, these data provide the first direct evidence for the enrichment of active preneoplasia-specific immune effectors at the site of the transformed cells in patients with a human nonviral preneoplastic state. Importantly, these effector T cells can be detected without the need for prolonged (1–2 wk) in vitro expansion. Such rapid effector function is not observed in T cells from the tumor bed in the clinical cancer, multiple myeloma. Most prior studies studying immune response to autologous tumor in humans have focused on patients with clinical cancer and not preneoplastic states. Prior studies have shown that tumor-specific T cells capable of reacting with autologous tumor cells or cell lines can be expanded from blood, LNs, tumor bed, or BM of tumor-bearing patients (5, 11–14). In certain situations, such as some of the patients with paraneoplastic neuronal disorders, tumors may be somewhat indolent. However, whether T cells from these patients recognize autologous tumor is not known (15). Detection of tumor-specific effector function in cancer patients is not common and generally requires in vitro stimulation using cytokines and antigen-presenting cells (16). However, whether the human immune system mounts a specific response to early “pre-malignant” proliferations of transformed cells in patients, which can persist for many years before the development of clinical malignancy, is not known (16). This is particularly relevant, as recent studies have shown that many of the genes conferring invasive growth in human cancer are already active in the early preinvasive stages (17, 18). Prior studies have documented lymphocytic infiltration in preneoplastic lesions such as dysplastic nevi (19). However the evaluation of specific antitumor immune recognition of these lesions has been difficult, due to the lack of access to adequate amounts of fresh tumor and immune cells from the tumor bed. Patients with MGUS represent a useful model in this regard. In MGUS, both the preneoplastic and the surrounding immune cells can be isolated without the need for prolonged in vitro culture or the generation of cell lines. These considerations

along with recent advances in DC biology (7) allowed us to measure effector T cell function against preneoplastic cells in MGUS.

The degree to which the antitumor immune response in humans is directed against tumor-derived unique or shared antigens is of central importance to tumor immunotherapy (20). Our data suggest that in the early stages of preneoplastic growth, when an active immune effector function in T cells removed from the tumor bed is detectable, most of the reactivity is against the pattern of antigens unique to each patient's preneoplastic cells, since the T cells did not recognize DCs loaded with allogeneic MGUS cells, or cag myeloma cells (7) that express high levels of several shared cancer-testis antigens. Recent studies by our group and others have shown that T cells from myeloma patients expanded in vitro using autologous tumor-loaded DCs have a similar reactivity, mostly against antigens from uncultured autologous tumors (5, 21). Limitations in cell numbers prevented us from testing multiple allogeneic tumor targets or cell lines. Therefore, more rigorous testing is required to fully assess the degree to which target antigens are shared between these patients. Clinical progression to myeloma is associated with the loss of rapid tumor-specific effector function in the tumor bed. This may be due to the lack of optimal antigen presentation by resident DCs, induction of suppressor T cells, or the presence of immune inhibitory factors in the tumor microenvironment (16, 22).

Our finding that there is a vigorous preneoplasia-specific effector T cell response in the MGUS marrow raises important questions about the potential impact of this immune response on the preneoplastic cells, and perhaps more importantly, their microenvironment (1). In principle, the immune system can mediate both tumor suppressive and promoting effects in vivo (16). In addition to the direct antitumor effects of T cells, IFN- γ produced by tumor-specific effectors can also mediate the inhibition of myeloma tumor growth by modulating IL-6 and STAT-3 signaling in tumor cells in vivo (23, 24). IFN- γ may also mediate antimyeloma effects by inhibiting angiogenesis (25) and osteoclastogenesis (26), both of which are observed in MM but not MGUS. Recent studies from our laboratory suggest that tumor progression in clinical myeloma is also associated with a functional defect in glycolipid-reactive NK T cells, suggesting a possible role for these innate lymphocytes in the immune control of myeloma as well (8). Interestingly, tumor-reactive effector T cell function is lost even in patients with early myeloma wherein NK T cell function is well preserved (8), suggesting that the loss of tumor-specific effector T cell function is an earlier event during tumor progression.

However, interactions of tumor and immune cells is complex, and under certain circumstances immune activation, particularly of CD4⁺ T cells in tumor bearing mice, may be deleterious (27–29). Requirement for such “reverse immune surveillance” has been well documented for the growth of some germinal center-derived lymphomas in mice (30). T cells have also been suggested to play an important role in both oncogene-driven and pristane oil-

induced plasmacytomagenesis in mice (31, 32). The observed host response may therefore play a critical role in the maintenance of MGUS clone in vivo. Transformation to multiple myeloma may then depend on the acquired ability of transformed cells to overcome dependence on paracrine signals derived from this microenvironment modulated by the host immune response.

Further studies are needed to more fully characterize the clinical and biologic significance of the observed host response to MGUS cells in patients. Therefore, we have recently initiated a US national cooperative group effort under the auspices of South West Oncology Group to prospectively study these patients. Preliminary data from this ongoing trial confirm the observations in this paper and may allow correlations with clinical, cytogenetic, and genomic changes in the preneoplastic cells (unpublished data). Most of the attention to date for harnessing the immune system against human cancer has been for the therapy as opposed to prevention of cancer. Our finding that human immune system can mediate vigorous immune response to antigens from preneoplastic cells suggests the need to similarly study host response in other human preneoplastic states. Harnessing the immune system for immune prevention or delay of cancer may have a major impact on reducing net cancer burden in humans as with infectious diseases (33).

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