Priority Brief

Cancer Immunology Research

Targeting Immune Suppression with PDE5 Inhibition in End-Stage Multiple Myeloma

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Abstract

Myeloid-derived suppressor cells (MDSC) play a significant role in tumor-induced immune suppression. Targeting their function could improve antitumor therapies. Previously, we demonstrated that phosphodiesterase 5 (PDE5) inhibition in MDSCs augmented antitumor immunity in murine models. Here, we show how the addition of the PDE5 inhibitor, tadalafil, in a patient with end-stage relapsed/refractory multiple myeloma reduced MDSC function and generated a dramatic and durable antimyeloma immune and clinical response. Strategies targeting MDSC function with PDE5 inhibitors represent a novel approach that can augment the efficacy of tumor-directed therapies. *Cancer Immunol Res; 2(8); 725–31. ©2014 AACR.*

Introduction

A major impediment to effective cancer immunotherapy lies in the host's inability to overcome the intrinsic immunosuppressive mechanisms associated with increasing tumor growth. A complex immunosuppressive network has been described ranging from immune editing of the tumor to the ability of the tumor to delete or anergize tumor-specific T-cell function (1). This negative immune feedback mechanism, which initially evolved to control excessive inflammation, limits the generation of effective tumor-specific immunity. Myeloid-derived suppressor cells (MDSC) play a central role in mediating tumor-induced tolerance (2). Numerous tumor-derived factors induce MDSCs and lead to their accumulation that parallels the increasing tumor burden. MDSC-induced immune suppression is accomplished primarily through upregulation of inducible nitric oxide synthase (iNOS) and overexpression of arginase-1 (Arg-1). As such, therapies aimed at inhibiting iNOS and Arg-1 production could enhance antitumor immunity. Previously, we have demonstrated the ability of phosphodiesterase-5 (PDE5) inhibitors to augment antitumor immunity through the downregulation of MDSC-dependent iNOS and Arg-1 activity in murine tumor models (3). Now, we describe a patient with end-stage multiple myeloma previously refractory to lenalidomide in whom responsiveness to lenalidomide-based therapy was restored upon the addition of the PDE5 inhibitor, tadalafil.

Case Report

A 50-year-old man was diagnosed with immunoglobulin Gκ (IgGκ), Durie Salmon stage IIIb myeloma in 2002. He presented with a hemoglobin level of 6 g/dL and acute renal failure (creatinine level of 4.3 mg/dL). At diagnosis, his serum monoclonal spike (M-spike) was 8 g/dL, and a 24-hour urine sample revealed a urine M-spike of 11.7 g. The bone marrow showed hyperdiploidy with a 13q deletion. He received induction therapy with vincristine, Adriamycin (doxorubicin), and dexamethasone (VAD) followed by autologous stem cell transplant with which he achieved a near-complete remission (CR) but relapsed 1 year later. He was treated with multiple agents including IFN-α, thalidomide, bortezomib–thalidomide–dexamethasone, and high-dose cyclophosphamide. Five years after his initial presentation, he was started on lenalidomide and dexamethasone with a reduction in his monoclonal protein after two cycles. However, drug-related toxicity resulted in lenalidomide dose reductions with subsequent increases in the disease burden. Adding clarithromycin to lenalidomide and dexamethasone resulted in a slight reduction in disease burden but ultimately led to discontinuation of lenalidomide due to drug intolerance. This regimen was followed by a cycle of melphalan, and subsequently bortezomib-pegylated doxorubicin–dexamethasone with progressive disease. His M-spike then increased to 5.35 g/dL with significant marrow suppression requiring 1 to 2 weekly red blood cell (RBC) and platelet transfusions (Fig. 1 and Table 1). Aware of our previous work, the patient initiated himself on treatment with the PDE5 inhibitor, tadalafil, while on bortezomib with no response. He was then switched to lenalidomide–dexamethasone because of lenalidomide's immunomodulatory properties. Despite his prior intolerance to lenalidomide, he was now able to tolerate the lenalidomide–dexamethasone in combination with tadalafil and showed a clinical benefit with a decline in his M-spike to 4.4 g/dL. Clarithromycin was then added because of its antimyeloma efficacy (4), and the four-drug combination resulted in a dramatic clinical response. He had a 90% reduction in his disease burden (very good partial response) and his serum M-spike reached a nadir at 0.58 g/dL after 11 months of treatment with this combination therapy. Importantly, his quality of life improved significantly. He became transfusion independent within 7 months of this combination, reported

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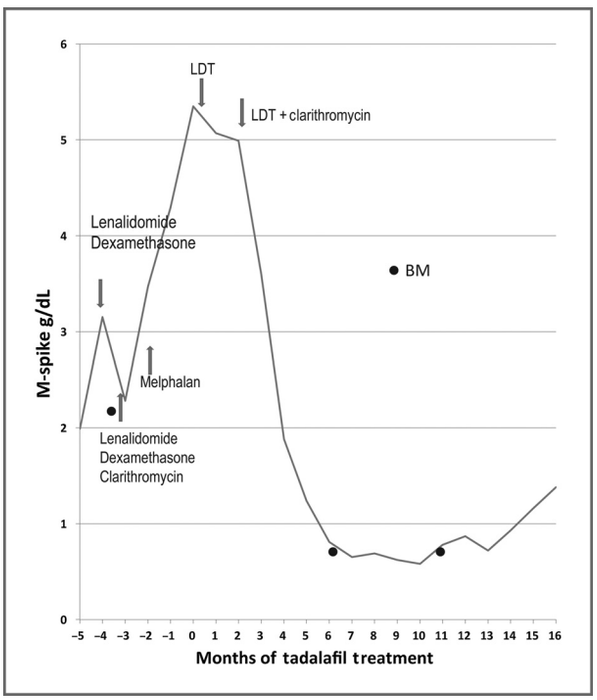

Figure 1. M-spike graph. M-spike (g/dL) of a 56-year-old male patient with IgGκ stage IIIb myeloma. The patient relapsed in month −5 and showed evidence of end-stage multiple myeloma refractory to all prior therapies. Tadalafil was added to the lenalidomide–dexamethasone treatment (LDT) in month 0. The M-spike fell from 5.35 g/dL at the start of tadalafil therapy to 4.4 g/dL. After 2 months, clarithromycin was added and the M-spike dropped further to a low of 0.58 g/dL. Fifteen months after the addition of tadalafil, the patient began showing an increase in his M-spike. The patient eventually died because of complications that arose from an H1N1 infection and not from myeloma. Bone marrow (BM) samples collected are indicated with filled circles, and peripheral blood collections are indicated with filled diamond shapes.

considerable improvement in fatigue, and became a licensed scuba diver shortly thereafter. He enjoyed a progression-free interval of 14 months. He died from complications of an H1N1 infection. After 18 months on treatment, he showed evidence of disease progression with an M-spike of 1.38 g/dL.

Materials and Methods

Patient samples

All samples were procured under an Institutional Review Board (IRB)–approved informed consent at the indicated time points. Bone marrow and peripheral blood lymphocyte (PBL) samples were ficolled and frozen in 90% autologous serum and 10% dimethyl sulfoxide (DMSO).

Flow cytometry

Fluorochrome-labeled CD14, HLA-DR, CD15, IL4Rα, CD4, CD25, FOXP3, and T-cell receptor-ζ (TCR-ζ) antibodies and isotype controls were purchased from BD Pharmingen. Reactive oxygen species (ROS) antibodies and isotype controls (Imagine-IT Live Green) were purchased from Invitrogen. Bone marrow and PBL samples were viably thawed, stained, and analyzed with multicolor flow cytometry on the BD FACSCalibur. Data were acquired and analyzed using CellQuest software (BD).

Quantitative RT-PCR

$CD14^+$ cells were selected from unfractionated bone marrow using MACS antibodies and columns from Miltenyi Biotec. The PureLink RNA Micro Kit (Invitrogen) was used to isolate total RNA from 5×10^5 cells as per the manufacturer's instructions. Reverse transcription PCR (RT-PCR) was performed using the Applied Biosystems High Capacity RNA-to-cDNA as per the manufacturer's instructions. Real-time PCR was performed using the following primers: Arg-1 (forward primer: AAG GAA AGA TTC CCG ATG TG; reverse primer: CCA CGT CTC TCA AGC CAA TA) and iNOS (forward primer: TGC GTT ACT CCA CCA ACA AT; reverse primer: ATG AGC TGA GCA TTC CAC AC). The expression of iNOS and Arg-1 was determined using real-time PCR performed on an AB 7500 Real-Time PCR system (Applied Biosystems). β-Actin was used as an internal reference.

Immunohistochemistry

Slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems) as per the manufacturer's protocol. Briefly, slides were deparaffinized with EZ Prep solution (Ventana). Heat-induced antigen retrieval method was used in RiboCC (cat. no. 760-107; Ventana). The slides were incubated with the murine monoclonal nitrotyrosine antibody (cat. no. MAB5404; Millipore) for 32 minutes. The Ventana anti-mouse secondary antibody was used for 16 minutes. The detection system used was the Ventana OmniMap Kit and slides were then counterstained with hematoxylin.

Tumor specificity

Unfractionated bone marrow cells from the indicated time points were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using the CellTrace CFSE Kit from Invitrogen and then pulsed for 72 hours at 37°C with either 50 μg/mL of U266 and H929 myeloma cell line lysates, SW780, a bladder carcinoma cell line, or left unpulsed. The cells were then harvested and stained for CD3 and IFNγ. Tumor-specific T cells were quantified by the $IFN\gamma^+$ production of $CD3^+/CFSE^{low}$ cells.

T-cell expansion

Dynabeads Human T-Activator CD3/CD28 beads (Life Sciences) were added to the unselected and CD14-depleted bone marrow at a ratio of 3:1, beads:T cell, and cultured for 5 days. The beads were magnetically removed, and the overall CD3 expansion was calculated.

Results

Effect of PDE5 inhibition on MDSCs

Although no clear phenotype exists for describing human MDSCs, IL4Rα expression has been associated with the suppressive phenotype of MDSCs (5). In this study, MDSCs were identified as $IL14^+$ or $IL15^+/HLA\text{-}DR^{low}/IL4R\alpha^+$. With tadalafil treatment, the actual number of $CD14^+$ cells decreased from 7.5% before treatment to 1.9% at 11 months. Although the

Table 1. Clinical course and transfusion requirements in response to various regimens

Month	M-spike, mg/dL	RBC transfusion	Platelet transfusion	Regimen	Reason for change in regimen
−6	1.99	0	0	LD	
−5	3.15	0	0	LD	
−4	2.28	0	0	LDC	Disease progression on LD
−3	3.47	4	4	Melphalan	Intolerability to LDC
−2	NM	12	12	None	
−1	4.86	6	6	VDD	Disease progression
0	5.35	6	6	VDDT/LDT	Tadalafil initiated by patient
1	5.07	8	8	LDT	Disease progression on VDD
2	4.4	6	6	LDT	
3	NM	6	6	LDCT	Synergy of clarithromycin and lenalidomide
4	3.6	4	4	LDCT	
5	1.88	2	0	LDCT	
6	1.24	0	0	LDCT	
7	0.81	2	0	LDCT	
8	0.65	0	0	LDCT	
9	0.69	0	0	LDCT	
10	0.62	0	0	LDCT	
11	0.58	0	0	LDCT	
12	0.78	0	0	LDCT	
13	0.87	0	0	LDCT	
14	0.72	0	0	LDCT	
15	0.93	0	0	LDCT	
16	1.16	2	0	LDCT	

NOTE: RBC transfusion measured as units of RBCs transfused per month. Platelet transfusion measured as units of single donor platelets transfused per month.

Abbreviations: LD, lenalidomide and dexamethasone; LDC, lenalidomide, dexamethasone, and clarithromycin; LDCT, lenalidomide, dexamethasone, clarithromycin, and tadalafil; LDT, lenalidomide, dexamethasone, and tadalafil; NM, not measured; VDD, bortezomib, liposomal doxorubicin, and dexamethasone; VDDT, bortezomib, liposomal doxorubicin, dexamethasone, and tadalafil.

percentage of $CD14^+/HLA\text{-}DR^{low}$ cells increased on treatment (Fig. 2A), IL4Rα expression went from 51.37% of the $CD14^+/HLA\text{-}DR^{low}$ cells pretreatment to 2.13% at 11 months, which corresponded to the same percentage found in the bone marrow of normal donors (Fig. 2B). We then sought to determine whether this reduction in the percentage of "suppressive" MDSCs correlated with changes in their functional characteristics. The expression of both Arg-1 and iNOS was reduced with tadalafil treatment (Fig. 2C). ROS also plays a critical role in suppressing antigen-specific $CD8^+$ T-cell responses (6, 7). A reduction of ROS expression would be critical to both reducing the inhibitory effect of MDSCs and increasing the T cell–mediated antitumor immunity. We thus examined ROS expression on the $CD14^+/HLA\text{-}DR^{low}$ cells (Fig. 2D). ROS levels were reduced to baseline by 6 months. Of note, these changes were significantly more pronounced in the bone marrow (the tumor site) than in the blood.

Effect of PDE5 inhibition on nitrosylation

It has been shown previously that Arg-1 and iNOS together can generate peroxynitrites capable of inducing protein tyrosine nitrosylation (8), which induce CD8 tolerance through the disruption of binding of specific peptide–MHC complex dimers (9) and that inhibiting these enzymes can reverse this process (10). In light of our previous work demonstrating the ability of PDE5 inhibitors to functionally impair both Arg1 and iNOS, we sought to determine whether this pharmacologic inhibition could reverse tyrosine nitrosylation in the bone marrow of our patient. Data supporting the role of this pathway in myeloma show that before initiation of PDE5 inhibition, the vast majority of the bone marrow demonstrated significant tyrosine nitrosylation (Fig. 3A). In parallel with the decrease of MDSCs, as well as both Arg1 and iNOS activity, the late bone marrow biopsy showed minimal amounts of nitrosylation (Fig. 3B), thus demonstrating a direct correlation between reductions in iNOS/Arg-1 levels and tyrosine nitrosylation.

Restoring T-cell function with PDE5 inhibition

Extensive murine data support the notion that abrogating MDSC function can effectively restore tumor-specific immunity (11). As a measure of endogenous T-cell activity, IFNγ

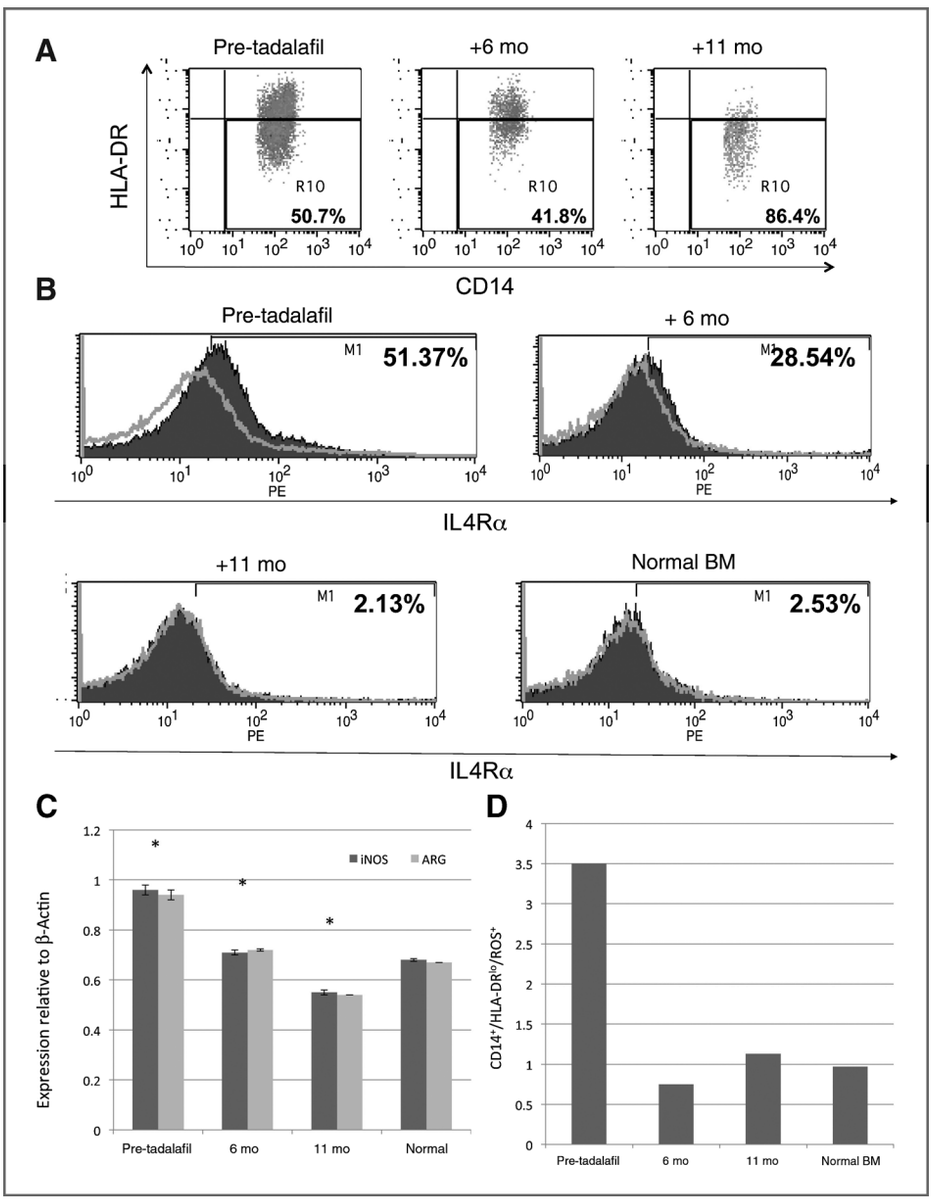

Figure 2. Characterization of bone marrow (BM) MDSC. A, total number of bone marrow $CD14^+$ cells decreased over time when staining the pre-tadalafil, +6 months, and +11 months time points (B) IL4Rα phycoerythrin (PE; fill) and isotype control (line) staining of $CD14^+$/$HLA\text{-}DR^{lo}$ cells over time. IL4Rα expression showed a decrease from pre-tadalafil at +6 months, to near normal expression at +11 months of therapy. C, iNOS and Arg-1 expression levels relative to β-actin in pre-tadalafil, +6 months, +11 months and a normal bone marrow sample. D, ROS staining of $CD14^+$/$HLA\text{-}DR^{lo}$ cells at the pre-tadalafil, +6 months, and +11 months time points as well as a normal bone marrow for comparison. *, $P < 0.005$ of pretreatment vs. normal or pretreatment vs. 6 months or 11 months.

expression increased during treatment although never to the level seen in normal donors (Fig. 4A). We had shown previously that elimination of the MDSC population either physically or through PDE5 blockade resulted in enhanced T-cell proliferation to anti-CD3/CD28 beads (3). We thus repeated this assay in our patient. Although the later time points showed increased proliferation in both groups, CD14 depletion further increased T-cell proliferation at 6 and 11 months (Fig. 4B). Interestingly, despite CD14 depletion, no proliferation was observed at baseline. One mechanism mediating T-cell dysfunction is the extracellular depletion of arginine resulting from the upregulation of Arg-1 by MDSCs. This downregulates the TCR-ζ chain resulting in decreased T-cell proliferation and function (12). TCR-ζ chain expression was initially downregulated and significantly increased upon tadalafil treatment (Fig. 4C). Finally, we sought to determine whether our clinical findings and data demonstrating enhanced immune function correlated with increased tumor-specific immunity. As shown in Fig. 4D, the reduction in tumor burden was associated with a dramatic increase in tumor-specific immunity of the marrow-infiltrating T cells after 11 months of treatment with 51.9% of the divided T cells showing antimyeloma specificity. Taken together, these data demonstrate the ability of PDE5 inhibition to restore T-cell function and augment antitumor immunity.

Discussion

This is the first demonstration in humans suggesting that PDE5 inhibitors can effectively block MDSC function and

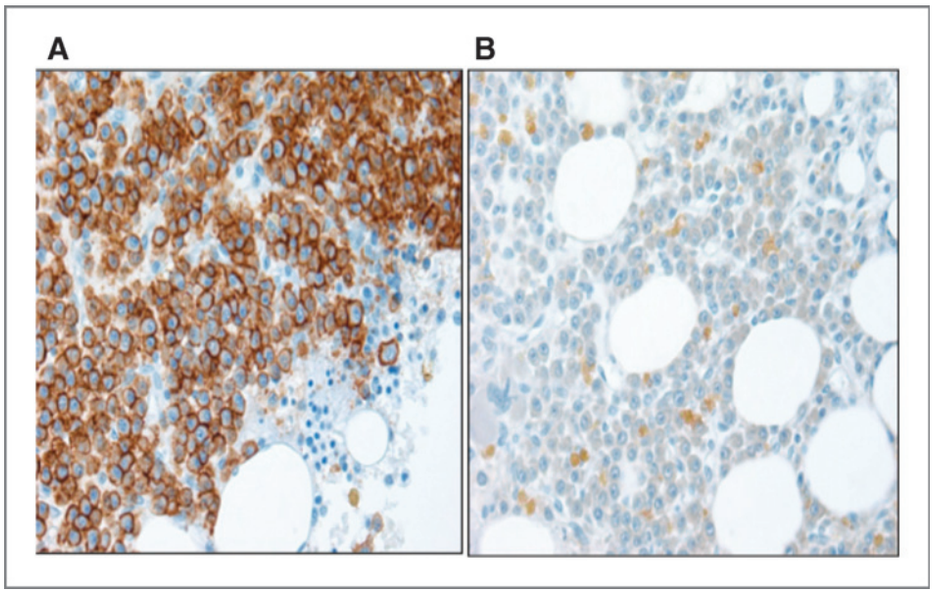

Figure 3. Bone marrow nitrosylation. Bone marrow obtained (A) before initiation of tadalafil treatment and (B) at +11 months was stained with nitrotyrosine.

restore immune responsiveness. Specifically, we observed a reduction in the expression of IL4Rα—a protein shown to be responsible for the MDSC-mediated immune suppression (5) and reductions in iNOS, Arg-1, and ROS expression. These findings further translated into increased expression of IFNγ and TCR-ζ upregulation (13). Finally, enhanced T-cell immunity correlated with the development of a clinically measurable antitumor response in a patient with multiply relapsed/refractory myeloma.

The exact phenotype of MDSCs has become increasingly complex with the recent identification of monocytic ($CD14^+$) or granulocytic ($CD15^+$) subpopulations (14). The dominant population in myeloma remains to be clearly elucidated. We had demonstrated previously that depletion of $CD14^+$ monocytes in myeloma effectively restored T-cell responsiveness to anti-CD3/CD28 stimulation (3). More recently, others have shown that $CD15^+$ MDSCs may play a more prominent role in other malignancies (11). The presence of both populations could possibly explain why CD14 depletion only partially restored T-cell function in this patient.

PDE5 inhibitors have been described as exerting a direct effect on CLL through caspase-3–induced apoptosis likely through the inhibition of PDE4 (15). However, we have been unable to detect a direct antitumor effect of PDE5 inhibitors on myeloma cell lines or primary samples. In contrast, our preclinical studies demonstrated a therapeutic effect of PDE5 inhibition on the $CD11^+/Gr\text{-}1^+$ MDSCs in mice and a $CD14^+$ population in patients with both head and neck cancer and myeloma (3). The data presented here demonstrate a role of MDSCs in myeloma and underscore how inhibiting MDSC function with a noncytotoxic agent can generate clinically meaningful antitumor immunity. This therapeutic effect was achieved through the downregulation of both iNOS and Arg-1. Interestingly, while not considered to have a direct cytotoxic effect on the MDSCs, we observed a significant reduction in MDSC numbers in addition to the expected reduction in IL4Rα expression. This is likely explained by the presence of a positive feedback loop between the tumor and the MDSCs. Abrogation of MDSC function altered the tumor microenvironment, which augmented the tumor-specific T-cell response. This reduced the tumor burden and the secretion of tumor-derived factors, such as granulocyte macrophage colony-stimulating factor (GM-CSF), IL6, and VEGF (2), which, in turn, generated fewer MDSCs, thereby reducing MDSC numbers.

Tadalafil increased IFNγ production and TCR-ζ chain expression on marrow-infiltrating T cells obtained from the tumor microenvironment. Although only these two parameters of T-cell function were examined, other MDSC-induced factors leading to T-cell anergy include depletion of extracellular cystine and cysteine (16), nitration of the TCR and CD8 molecules (9), and induction of regulatory T cells (Treg; ref. 17). We also observed a decrease in Tregs in the blood over time (data not shown). The major effect of PDE5 blockade in this patient was the ability to increase the tumor-specific immune response and to generate a meaningful and durable antimyeloma response using a regimen to which he was previously refractory. It is also worth noting that tumor-specific T-cell responses were increased with PDE5 inhibition despite the presence of chronic corticosteroid therapy given to treat the myeloma. Taken together, these results underscore the critical role of MDSCs within the complex immunosuppressive pathway found in the tumor microenvironment but also suggest the presence of additional inhibitory mechanisms.

These data would suggest that a noncytotoxic, nontumoricidal agent may be capable of targeting MDSC function and generating a potent antitumor immune response with an associated clinical benefit. Despite being refractory and intolerant to lenalidomide in the past, the addition of tadalafil enabled the patient to tolerate lenalidomide-based therapy and led to a significant clinical response with associated transfusion independence and improvement in quality of life. Tadalafil alone is unlikely to generate a measurable clinical

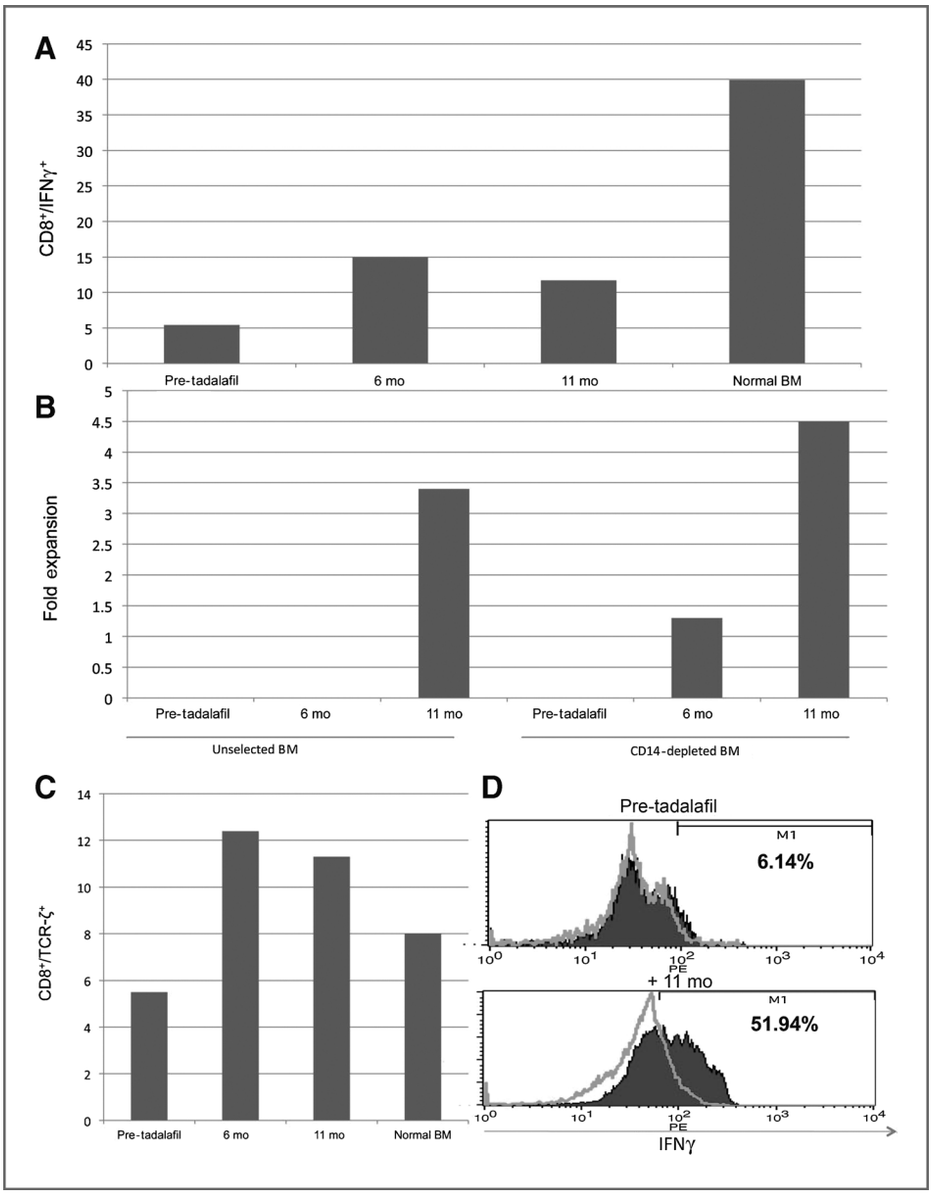

Figure 4. Characterization of T-cell response over time. A, bone marrow (BM) was stimulated with phorbol 12 myristate 13 acetate (PMA)/ionomycin for 4 hours and the overall intracellular expression of IFNγ on $CD8^+$ cells was determined for the pre-tadalafil, +6 months, +11 months, and normal bone marrow samples. B, PBL from two post-tadalafil time points (+5 and +8 months) as well as a normal PBL were stained for $CD4^+/CD25^+$ and intracellular expression of FOXP3 was determined. C, TCR-ζ expression was determined on bone marrow $CD8^+$ cells at the pre-tadalafil, +6 months, +11 months, and normal bone marrow samples. D, bone marrow samples from the pre-tadalafil and +11 months tadalafil time points were CFSE-labeled and then pulsed with either H929/U266 (fill) or SW780 lysates (data not shown/no increase from unpulsed) or left unpulsed (line). After a 5-day stimulation, the cells were harvested and stained with CD3 and intracellularly for IFNγ. $CD3^+/CFSE^{lo}/IFN\gamma^+$ cells are shown in this graph. E, CD3/CD28 T-cell stimulations of either unselected bone marrow or $CD14^+$ depleted bone marrow. CD3/CD28 beads were added at a 3:1 bead to T-cell ratio in both groups for a 5-day stimulation. On day 5, the cells were harvested, beads were removed, and the overall expansion of $CD3^+$ cells was determined.

response. One possible explanation for this synergy is that the immune-mediated efficacy of lenalidomide was augmented by tadalafil inhibition of MDSC function, which would justify combination of PDE5 inhibitors with other immunotherapeutic approaches. A clinical trial in myeloma is under way examining the therapeutic efficacy of PDE5 inhibitors in conjunction with a lenalidomide-based regimen.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K.A. Noonan, N. Ghosh, I. Borrello
Development of methodology: K.A. Noonan, N. Ghosh, L. Rudraraju, M. Bui, I. Borrello
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.A. Noonan, N. Ghosh, L. Rudraraju, M. Bui
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.A. Noonan, N. Ghosh, L. Rudraraju, M. Bui, I. Borrello
Writing, review, and/or revision of the manuscript: K.A. Noonan, N. Ghosh, M. Bui, I. Borrello
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.A. Noonan

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