Tumoricidal Effects of Macrophage-Activating Immunotherapy in a Murine Model of Relapsed/Refractory Multiple Myeloma

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Abstract

Myeloma remains a virtually incurable malignancy. The inevitable evolution of multidrug-resistant clones and widespread clonal heterogeneity limit the potential of traditional and novel therapies to eliminate minimal residual disease (MRD), a reliable harbinger of relapse. Here, we show potent anti-myeloma activity of macrophage-activating immunotherapy (αCD40+CpG) that resulted in prolongation of progression-free survival (PFS) and overall survival (OS) in an immunocompetent, preclinically validated, transplant-based model of multidrug-resistant, relapsed/refractory myeloma (t-VE-MYC). αCD40+CpG was effective in vivo in the absence of cytolytic natural killer, T, or B cells and resulted in expansion of M1-polarized (cytolytic/tumoricidal) macrophages in the bone marrow. Moreover, we show that concurrent loss/inhibition of Tpl2 kinase (Cot, Map3k8), a MAP3K that is recruited to activated CD40 complex and regulates macrophage activation/cytokine production, potentiated direct, ex vivo anti-myeloma tumoricidal activity of αCD40+CpG-activated macrophages, promoted production of antitumor cytokine IL12 in vitro and in vivo, and synergized with αCD40+CpG to further prolong PFS and OS in vivo. Our results support the combination of αCD40-based macrophage activation and TPL2 inhibition for myeloma immunotherapy. We propose that αCD40-mediated activation of innate antitumor immunity may be a promising approach to control/eradicate MRD following cytoreduction with traditional or novel anti-myeloma therapies.

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Introduction

Despite the advent of novel therapies for multiple myeloma, a cancer of mature lymphocytes that produce antibody, the disease remains incurable. Only one in three patients diagnosed with myeloma will be alive 10 years after the diagnosis (1). Lack of curative approaches is attributable to early and near-universal clonal heterogeneity (2, 3), and the persistence of minimal residual disease (MRD) following traditional and novel therapies, including high-dose therapy with autologous stem cell rescue. Recent evidence has confirmed that detectable MRD constitutes a harbinger for relapse and predicts adverse clinical outcomes (4, 5). Thus, eradication of MRD is a priority in designing curative approaches against myeloma.

We have proposed that therapeutic manipulation of the microenvironment may be an essential component of curative interventions in myeloma (6). Proof-of-principle for this concept is provided by the fact that the only known potentially curative therapy for myeloma, allogeneic transplantation, exerts its effects through modulation of the microenvironment to produce a graft-versus-myeloma effect, albeit at the cost of considerable toxicity (7). Therefore, strategies to render the microenvironment inhospitable or overtly hostile to myeloma cells are urgently needed.

Macrophages are a crucial, and somewhat neglected, component of the myeloma niche. We have previously shown that myeloma-associated monocytes/macrophages (MAM) are key modulators of the inflammatory milieu of the myeloma niche and important producers of cytokines that are known to promote growth of nascent myeloma tumors (8). MAM continue to produce inflammatory cytokines (IL1β, IL6, and TNFα) even as they acquire tumor-promoting (‘M2’) characteristics. This “mixed” or “intermediate” state of macrophage polarization in vivo is similar to previously described “M2b macrophages” or “MSC-educated macrophages” (9–11).

We and others have shown that therapeutic macrophage repolarization (toward an M1-tumoricidal phenotype) using CD40 ligation can be harnessed to exert antitumor activity in vivo (12–21). Therapeutic activation of macrophages typically requires two sequential signals, a “priming signal” delivered
through agonistic CD40 stimulation and a secondary "triggering signal" delivered through Toll-like receptor (TLR) stimulation. The resultant tumoricidal activity has been shown to be independent of T cells and has shown promise even in very difficult cancers: Thus, clinical administration of anti-CD40 agonist monoclonal antibody, without TLR activation, has demonstrated clinical benefit in patients with pancreatic cancer, acting through macrophage activation (21). Macrophages are particularly attractive as anti-myeloma effectors because of their association with myeloma lesions outside the bone marrow (22) where active MRD may localize.

We have demonstrated a role for control of macrophage polarization and cytokine production by TPL2, a MAP3K operating at the interface between NFκB and MAPK pathways (8, 23). Moreover, we have attributed roles for Tpl2 in myeloma progression in vivo and have shown thatTpl2 activity promotes macrophage polarization toward pro-tumor (M2) phenotype. Tpl2 is activated by stimuli that activate macrophages (such as TLR ligands and CD40; ref. 24), but its actions limit the production of crucial antitumor effectors (such as nitric oxide; ref. 25) or antitumor immunomodulatory cytokines [such as IL12 (ref. 26) and IFNβ (ref. 27)]. At the same time Tpl2 promotes promyeloma immunomodulatory cytokines IIβ, II6, and II10 (8). Tpl2 signaling could, therefore, be envisaged as an "innate immune checkpoint" that modulates innate anti-myeloma immunity.

In this article, we show that anti-CD40-mediated macrophage repolarization results in potent anti-myeloma activity both ex vivo and in vivo. Host Tpl2 loss promoted production of II12, an M1-polarization agent and a powerful antitumor cytokine (28) and prolonged survival. Our results may open new avenues for controlling myeloma relapse by harnessing the power of innate antitumor immunity.

### Materials and Methods

#### Antibodies and reagents

The FGK45.5 hybridoma–producing antiCD40 was a gift from Dr. F. Melchers (Basel Institute for Immunology, Switzerland). The endotoxin content of our FGK45.5 preparation was directly quantified using the E-Toxate Kit (Sigma) and was found to be below detection limit [0.05–0.1 endotoxin units (EU) per mL]. We have previously reported that FGK45.5–activated macrophages harvested from endotoxin-resistant C3H/HeJ mice retained potent ex vivo antitumor activity, suggesting that macrophage activation was not the result of inadvertent endotoxin contamination of antiCD40 antibody (20). Endotoxin-free CpG1826 [TCCATGACGTTCCTGACGTT] was purchased from Copley Pharmaceuticals Group. Monophosphoryl Lipid A (MPL), L-NG-nitroarginine methyl ester (L-NAME; final concentration, 5 mmol/L), and rat IgG were purchased from Sigma. Pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK; final concentration, 20 μmol/L) was purchased from Promega.

### Ex vivo macrophage cytotoxicity assays

Mice received 0.5 mg antiCD40 FGK45.5 or rat IgG (Sigma, I4131) i.p. and 3 days later peritoneal exudate cells (PEC) were obtained by peritoneal elution. Red blood cell lysis was performed by rapid exposure to deionized water. PECs were seeded onto 96-well plates and allowed to adhere for 90 minutes. MM1.S-mCherry/Luc cells and 5 μg/mL of CpG or MPL were subsequently added to adherent PECs. MM1.S-mCherry/Luc cells were a generous gift from Dr. Constantine Mitsiades (Dana-Farber Cancer Institute, Boston, MA). Tumor cell viability in PEC/tumor cell co-cultures was assessed after 48 hours by adding sterile-filtered luciferin (Promega; P1043) diluted in RPMI complete media to a final concentration of 250 μg/mL, incubating the co-culture at 37°C 5% CO2 for 30 minutes, and then measuring luminescence using a BioTek Synergy4 plate reader. Supernatant nitrite levels were measured using a Griess Reagent-based colorimetric assay (Sigma, G4410). Cytokine levels were measured using the bead-based Bio-Plex System (Bio-Rad). Tranwell experiments were performed using inserts with 3.0-μm diameter pores, a polycarbonate construction, and a separation distance of 0.127 cm between the bottom of the insert and the top of the plate (Corning; 3385).

### In vivo tumor model

Vx12598 tumor cells were described previo usly (29). Tumor cells were injected via intracardiac injection at a dose of 3 × 10⁵ CD138⁺ cells per recipient mouse, without irradiation (day 0). Mice were treated with intraperitoneal, saline-dissolved injections of 0.25 mg antiCD40 or rat IgG on day 5, and 25 μg CpG or PBS on day 8 post-injection, repeated every 2 weeks. Tumor burden was assessed using serum obtained after clotting 20 mL of blood obtained from tail grazes. Sera were analyzed for monoclonal gammapathy using a Sebia Hydrasys/Gelscan system. M-spike band/total protein ratio was multiplied by the total protein content of the serum, as determined using Bradford reagent, yielding absolute quantification of monoclonal gammapathy. Mice were bled and analyzed weekly starting at day 17. Mice that never showed any gammapathy (potential lack of engraftment) were excluded from analyses. Treatment with antiCD40+CpG did not affect the engraftment rate (~85%).

### Statistical analysis

All in vivo experiments were performed in triplicate and statistical significance between groups in a single experiment was assessed using the two-tailed Student t test. ANOVA methods were used to test differences between groups across multiple independent experiments. For in vivo experiments, progression-free survival (PFS) was defined by a monoclonal gammapathy <0.5 g/dL. Time to progression was determined by linearly interpolating between the two measured gammapathy time points above and below 0.5 g/dL. Kaplan–Meier survival curves were analyzed using the log-rank test and using a Cox proportional hazards model for estimating the interaction between antiCD40+CpG and Tpl2 status.
Macrophages can be activated to exert tumoricidal activity against myeloma cells ex vivo

To confirm that macrophages possess intrinsic anti-myeloma activity, we collected PECs 3 days after in vivo priming with αCD40 or control rat IgG antibody. We confirmed that adherent PECs represent peritoneal-origin macrophages: CD115 expression was used to confirm their monocytic identity—more than 95% of adherent PECs expressed CD115 (data not shown). PECs were seeded in 96-well plates to which luminescent myeloma MM1.S cells (MM1.S-mCherry/Luc) were subsequently added at various ratios of PECs to target cells (effector:target, E:T ratio) (30). TLR agonists or PBS were added to the in vitro co-cultures, as described in Materials and Methods. Emitted bioluminescence exhibited an almost perfectly linear correlation to the absolute number of myeloma tumor cells in each well when myeloma cells were cultured in isolation or in co-cultures with nonluminescent PECs ($r^2 = 0.99$, Fig. 1A).

Addition of TLR agonists CpG (TLR9) or MPL (TLR4) to myeloma cells did not result in appreciable toxicity (Fig. 1B). By contrast, tumoricidal effects of activated PECs were observed in PEC–myeloma co-cultures. TLR stimulation alone, in the absence of prior αCD40 stimulation, was only effective at higher E:T ratios, particularly with the potent TLR4 agonist MPL (Fig. 1B). Prior stimulation of PECs with αCD40 significantly enhanced the tumoricidal activity of PECs, at lower E:T ratios, in the presence of either CpG or MPL. The most potent antitumor cytotoxicity was obtained by αCD40-primed macrophages that were stimulated with MPL. However, CpG stimulation also produced significant antitumor cytotoxic effects in a dose-dependent manner (Fig. 1B). Myeloma cell demise was associated with apoptosis induction. Macrophage-mediated cytotoxicity was abrogated in the presence of the pan-caspase inhibitor Z-VAD-FMK (Supplementary Fig. S1A) and Annexin V/propidium iodide (PI) staining corroborated apoptotic mechanisms (Supplementary Fig. S1B).

The observed cytotoxic effects of different combinations of stimuli correlated with the degree of nitric oxide (NO) production by activated macrophages. Thus, the most potent tumoricidal combination (αCD40 and MPL at 20:1 E:T ratio) also resulted in the most enhanced NO production (Fig. 1B). To determine whether NO was necessary for the observed antitumor activities by PECs, we blocked NO production with L-NAME, a pan-NO synthase inhibitor. Treatment with L-NAME resulted in attenuation of NO production in conjunction with attenuation of antitumor cytotoxic effects (Fig. 1C). We conclude that activated macrophages can exert cytotoxic effects against myeloma cells ex vivo that are partially NO dependent.

Prior reports have suggested a role for cell-to-cell contact in mediating protective effects of macrophages toward myeloma cells (31). Moreover, direct phagocytosis of myeloma cells by PECs, at lower E:T ratios, in the presence of either CpG or MPL. Thus, the most potent antitumor cytotoxicity was obtained by αCD40-primed macrophages that were stimulated with MPL. However, CpG stimulation also produced significant antitumor cytotoxic effects in a dose-dependent manner (Fig. 1B). Myeloma cell demise was associated with apoptosis induction. Macrophage-mediated cytotoxicity was abrogated in the presence of the pan-caspase inhibitor Z-VAD-FMK (Supplementary Fig. S1A) and Annexin V/propidium iodide (PI) staining corroborated apoptotic mechanisms (Supplementary Fig. S1B).

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**Figure 1.**

*Ex vivo* tumoricidal activity of activated macrophages against myeloma cells. A, linear correlation between detectable luminescence and number of viable myeloma MM1.S-mCherry/Luc cells, both in myeloma cell mono-cultures and in co-cultures with nonluminescent peritoneal macrophages (PEC). B, top, potent anti-myeloma tumoricidal effects of activated PECs. PECs were either preactivated following *in vivo* administration of an agonistic αCD40 antibody 3 days before collection or following control IgG administration (IgG). Following collection, PECs were plated with luminescent (MM1.S-mCherry/Luc) myeloma cells at various effector:target (E:T) ratios. TLR agonists CpG or MPL (or PBS, as a control) were added to co-cultures. Control myeloma cell mono-cultures (no M) are shown to the left. Dashed line shows the number of MM1.S-mCherry/Luc cells at start of culture. B, bottom, nitrite, a byproduct of NO processing, was measured in each condition as a surrogate of NO production. C, left, the pan-NO synthase inhibitor, L-NAME, partially reversed macrophage-mediated cytotoxicity at each condition tested. Right, L-NAME effectively reduced NO production at each condition tested. D, top, physical separation between PECs and target (myeloma) cells resulted in partial abrogation of the tumoricidal effect. Left, standard co-cultures between PECs and myeloma cells at E:T = 20:1 (contact occurs between PECs and myeloma cells). Right, physical separation by a Transwell separator, keeping PECs separated from myeloma cells, resulted in partial abrogation of anti-myeloma tumoricidal effects. Dashed line shows the number of MM1.S-mCherry/Luc cells at start of culture. D, bottom, nitrite production by standard (left) and transwell-separated co-cultures were comparable at each condition tested. Statistical significance was determined using the Student’s *t* test and is denoted as follows: ns, not statistically significant; *", *P < 0.05; "*, *P < 0.01; "**, *P < 0.001.
Macrophages has also been shown (32). We tested the hypothesis that cell–cell contact or juxtacrine interactions may be necessary for the observed antitumor effects of activated macrophages. To test this hypothesis we repeated the experiments shown in Fig. 1B with the addition of a Transwell separator that prevents contact between PECs and myeloma cells. As shown in Fig. 1D, separation of PECs from myeloma cells attenuated the antitumor effect. These results demonstrate that activated macrophages exert anti-myeloma activity through direct cell–cell contact or alternatively/additionally, through juxtacrine interactions.

Tpl2 loss enhances tumoricidal activity of activated macrophages ex vivo

Because Tpl2 loss promotes spontaneous macrophage repolarization in the myeloma microenvironment in vivo (8), we hypothesized that Tpl2 loss/inhibition might synergize with therapeutic macrophage repolarization to enhance myeloma cell killing by activated macrophages. Initially, we tested this hypothesis in the ex vivo cytotoxicity assay described above. We compared the relative cytotoxic activity of Tpl2+/+ PECs with Tpl2−/− PECs. As shown in Fig. 2A, Tpl2−/− macrophages exhibited enhanced cytotoxicity compared with Tpl2+/+ macrophages at each condition tested. Both TLR4 stimulation (MPL) and TLR9 stimulation (CpG) enhanced cytotoxicity by Tpl2−/− PECs relative to Tpl2+/+ PECs.

Tpl2 activity has been shown to repress transcription of inducible NO synthase (iNOS) following TLR stimulation in vitro (25). We found that Tpl2 loss also enhanced NO production by CD40+TLR-stimulated PECs (Fig. 2B). Moreover, NO levels directly correlated with enhanced cytotoxicity exhibited by CD40+TLR ligand-treated PECs (Fig. 2A and B).

We next tested the hypothesis that Tpl2 loss might enhance production of antitumor cytokine IL12 by CD40+TLR-stimulated macrophages. IL12 and IL10 have been considered markers of M1 and M2 macrophage polarization, respectively. Tpl2 signaling has been shown to exert repressive effects on IL12 transcription following TLR9 stimulation in vitro (26), but the impact of this observation on antitumor immunity has been unclear. We found that TLR9 (CpG) but not TLR4 (MPL) stimulation led to increased IL12p40 production by CD40-activated macrophages.
that exert anti-myeloma activity ex vivo (Fig. 2C). Whereas Tpl2 loss enhanced IL12p40 in both macrophage mono-cultures and macrophage–myeloma cell co-cultures, no IL12p40 production was found in myeloma cell mono-cultures. IL10 production was not affected by Tpl2 genotype (Fig. 2C).

Tumoricidal effects of αCD40+ CpG-activated macrophages require intact Nfkβ1 activity

Tpl2 associates with Nfkβ1 (p105), a core component of the NFκB canonical signaling pathway (33). p105 is the precursor of the active NFκB subunit p50. Tpl2 kinase activity is repressed when Tpl2 is bound to p105. Signals that activate the canonical NFκB pathway lead to p105 degradation, and thus to Tpl2 release and activation. Because Tpl2 is only stable in association with p105, Nfkβ1-null animals are also functionally Tpl2-null (Fig. 3A).

We asked whether both components of the p105/Tpl2 complex modulate therapeutic macrophage activation. To this end, we used Nfkβ1-null (p105/p50-null) macrophages in our in vitro anti-myeloma assay. We found that macrophage-mediated tumoricidal activity was impaired by the loss/deletion of Nfkβ1, particularly when TLR9 (CpG) costimulation was used (Fig. 3B). Concurrently, with attenuation of tumoricidal activity, NO production (Fig. 3C) as well as IL12 and IL10 production (Fig. 3D) were reduced in αCD40+ CpG–stimulated Nfkβ1−/− PECs.

Our results show that Nfkβ1 (p105/p50) is involved in the activation of macrophages in response to αCD40+ CpG stimulation. Because Tpl2 loss enhances antitumor potential only in the presence of functional Nfkβ1, we conclude that p105/p50 acts upstream of Tpl2 in macrophages that respond to αCD40+ CpG. In other words, Tpl2 modulates the effects of p105/p50-mediated signaling in αCD40+ CpG-activated macrophages.

Therapeutic macrophage activation results in prolonged survival in a model of drug-resistant, relapsed/refractory myeloma

We hypothesized that therapeutic macrophage activation may exert antitumor effects in "minimal disease" states in myeloma. To model the growth and progression of MRD in an immunocompetent host, we used a transplant-based model in which a threshold inoculum of tumor cells was delivered 5 days before beginning of treatment. Engraftment and progression of t-VeMYC (Vc12598), a model for relapsed/refractory myeloma (29), recapitulates high-risk, drug-resistant...
myeloma growth. Injection of Vk12598 cells through the intracardiac route into immunocompetent syngeneic C57BL/6J recipients, without pre-conditioning irradiation, resulted in universal death in engrafted animals (median, 32 days, 95% confidence interval, 30–37) from progressive myeloma (Fig. 4A). The rapid clinical course allowed overall survival (OS) to be determined as a study endpoint. Moreover, the multidrug-resistent profile of Vk12598 (29) offers an ideal vehicle to test the concept that therapeutic macrophage activation can inhibit drug-resistant, proliferative myeloma.

Treatment of animals with αCD40+CpG was initiated 5 days after intracardiac injection to avoid any confounding effects of treatment on tumor-cell engraftment rate. As shown in Fig. 4A, treatment of animals with αCD40+CpG resulted in significant prolongation of OS that was further enhanced inTpl2-null recipients. Figure 4B shows a significant positive effect of treatment on prolongation of PFS (progression defined as M-spike >0.5 g/dL), which was further enhanced by recipientTpl2 loss. Log-rank statistical significance values are given in Table 1. Cox proportional hazard analysis (as detailed in the Supplementary Data) estimated that, among αCD40+CpG-treated animals, wild-type genotype conferred a relative risk of 2.21 for death (P = 0.02) and 2.22 for progression (P = 0.02), compared with that of the Tpl2-null genotype (Supplementary Tables S1 and S2).

We hypothesized that the improvement in PFS and OS conferred by recipientTpl2 loss may be related to enhanced IL12 production in vivo. To test this hypothesis, we measured serum levels of IL12p40 at 4 hours and 24 hours following αCD40+CpG treatment in vivo. Serum was collected at either 4 hours (left) or 24 hours (right) after CpG injection. For C and D, statistical significance was determined using ANOVA analysis on logarithmically transformed values obtained from multiple independent experiments and is denoted as follows: ns, not statistically significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

Figure 4.

In vivo tumoricidal effects of αCD40+CpG in a model of relapsed/refractory myeloma. A and B, t-VkrMyc (Vk12598) cells were injected at day 0 into Tpl2+/− or Tpl2−/− recipients. Treatment with αCD40+CpG or vehicle was initiated at day 5 according to the treatment schedule delineated in Materials and Methods. In the absence of treatment, animals succumbed rapidly to progressive myeloma, irrespective of Tpl2 status. αCD40+CpG treatment significantly prolonged PFS (B) and OS (A). Recipient Tpl2 loss led to further prolongation in PFS and OS. PFS is defined as time to M-spike greater than 0.5 g/dL. The log-rank test P values for comparisons of survival curves are listed in Table I. C, recipient Tpl2 loss leads to sustained high levels of serum IL12p40 following αCD40+CpG in vivo. Serum was collected at either 4 hours (left) or 24 hours (right) after CpG injection. D, recipient Tpl2 loss has no effects on serum concentrations of IL10 following αCD40+CpG treatment in vivo. Serum was collected at either 4 hours (left) or 24 hours (right) after CpG injection. For C and D, statistical significance was determined using ANOVA analysis on logarithmically transformed values obtained from multiple independent experiments and is denoted as follows: ns, not statistically significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
In vivo activity of αCD40+ CpG is independent of cytolytic NK or T-cell activity

Although we have shown that macrophages can exert anti-myeloma activity ex vivo following αCD40+TLR stimulation, we asked whether αCD40+CpG acts primarily through macrophages in vivo. To this end, we transplanted Vk12598 cells into SCID/Beige animals, characterized by defective NK-cell cytolytic activity and absence of mature T or B cells, the latter due to the SCID mutation (35). Vk12598 cells were injected on day 0 and treatment was administered as per the schedule delineated in Materials and Methods. Tumor burden was assessed by serum protein quantitation and by absolute T-cell counts on days 24 and 31 and compared with wild-type C57BL/6J immunocompetent recipients.

The results are shown in Fig. 5. αCD40+CpG treatment was at least partially effective in the absence of cytolytic NK, T, or B cells in SCID/Beige animals. The degree of delay in monoclonal gammopathy (myeloma tumor burden) between αCD40+CpG-treated SCID/Beige and C57BL/6J recipients was comparable at each timepoint tested. These data are consistent with the hypothesis that the anti-myeloma effects of αCD40+CpG treatment in vivo are mediated, in a large part, through macrophages.

αCD40+CpG leads to expansion of M1-polarized bone marrow macrophages in vivo

αCD40+CpG and αCD40+MPL mediate macrophage repolarization and macrophage-mediated anti-myeloma cell toxicity ex vivo (Fig. 1). Moreover, in vivo anti-myeloma effects of αCD40+CpG are detected in the absence of cytolytic NK, T, or B cells (Fig. 5), strongly supporting a central role for macrophages in mediating the in vivo anti-myeloma effects of αCD40-based immunotherapy. We sought to directly determine whether αCD40+CpG treatment actively leads to myeloma-associated macrophage repolarization in vivo. To this end, we compared the polarization status of myeloma-associated macrophages obtained from bone marrows of myeloma tumor-bearing mice following administration of control IgG+PBS or αCD40+CpG.

We have previously demonstrated that macrophages in de novo Vk′MYC tumor-bearing animals partition into a CD68int/Ly6Cint population that expresses iNOS and is consistent with M1-like macrophages and a CD68int/Ly6Gint population that does not express iNOS and is consistent with M2-like macrophages (8). A similar immunophenotyping strategy was used for analysis of myeloid cell subpopulations in the present study with some modifications. First, we eliminated F4/80 staining for the initial gating on the basis of a recent report favoring Ly6C and Ly6G in lieu of F4/80 for analysis of mouse splenic myeloid cell populations (36). Second, we introduced IL4Rx (CD124) to better characterize the M2-like subpopulation (37).

Treatment with αCD40+CpG led to expansion of the CD68int/Ly6Cint population expressing iNOS (M1-like) (Fig. 6). Figure 6 also shows the data from analysis of all animals in the untreated and αCD40+CpG-treated cohorts in two independent experiments. These results demonstrate that αCD40+CpG leads to expansion of the M1-like macrophage population in myeloma-infiltrated bone marrow in vivo.

Discussion

The incidence of multiple myeloma continues to rise, with an estimated 24,050 new cases in 2014 in the United States (1). Its precursor form, monoclonal gammopathy of undetermined significance (MGUS), is the most common hematologic disorder with a prevalence of 4% in the general population over the age of 40 years. Despite the advent of novel therapies that have revolutionized the treatment landscape, most patients diagnosed with myeloma will die of their disease. Novel therapies and stem-cell transplantation confer excellent cytoreduction and may prolong survival. Whereas current clinical research aims to define the role of autologous transplant in the era of novel therapies, it is clear that none of these approaches are routinely curative (38).

The lack of curative approaches in this disease reflects the persistence of residual myeloma acting as a nidus for regrowth and clinical relapse. In recent years, quantification and characterization of this MRD have received more attention (39). It is now generally accepted that the detection of measurable MRD portends an ominous prognosis (4, 5).

Although there is growing appreciation for the correlation between MRD and clinical outcome, biologic characterization is
still incomplete. Characterization of MRD is likely to be complicated by significant inter- and intrapatient heterogeneity. We have envisaged distinct types of MRD that may be further complicated by overlapping mechanisms: First, MRD may reflect the persistence of cells that have acquired genetic or epigenetic attributes of drug resistance through a process of classical, linear clonal evolution. Second, MRD may represent "tides" involving subclones that have arisen through a process of "branching" evolution (40). Third, MRD may reflect the specific biologic attributes of clonogenic precursors of the disease. Although the nature of these "myeloma stem cells" is hotly debated, they are likely to be relatively resistant to the effects of therapy (41, 42). Alternatively, MRD may reflect the stochastic persistence of residual myeloma cells with tumor-protective niches.

Activation of selected components of the tumor's microenvironment to maximize efficacy and minimize toxicity may be an essential component of approaches aiming at eradication of MRD and achievement of cures. Strategies to mobilize the microenvironment against residual myeloma should work with little regard to the clonal composition of MRD. Among various components of innate immunity in the myeloma niche, our work has focused on monocytes/macrophages (6). Whereas the concepts governing polarization and plasticity of macrophages in solid tumors have been well formulated (43, 44), the mechanisms governing macrophage behavior in hematologic malignancies are not as well understood. Multiple myeloma, a malignancy in which tumor cells are critically dependent on cross-talk with diverse components of the bone marrow microenvironment, provides an excellent model to study interactions between macrophages and tumor cells and evaluate the potential of their therapeutic exploitation.

We have previously shown that monocytes/macrophages provide essential support to tumor cells in the nascent myeloma lesion through the elaboration of critical promyeloma cytokines such as II1β and IL6 (8). We have proposed that approaches to limit production of these cytokines may be useful in controlling indolent myeloma. The Weissman laboratory has previously shown that macrophages may exist in a state of precarious equilibrium with myeloma cells: Simple blockade on protective "don't-eat-me" signals on the surface of the myeloma tumor cells suffice to elicit potent anti-myeloma, macrophage-mediated cytotoxicity (32). Whereas blocking "don't-eat-me" signals constitutes a "passive" approach in turning macrophages into anti-myeloma effectors, "active" methods to repolarize macrophages may hold even better therapeutic promise. We have previously demonstrated that active repolarization of macrophages to elicit tumoricidal activity can be achieved through the administration of sequential CD40-mediated activation and TLR ligation (12, 19). CD40-induced macrophage activation has been shown to result in meaningful clinical tumor regressions in recalcitrant solid tumors (e.g., pancreatic cancer; ref. 21) and in a model of chronic lymphocytic leukemia, a tumor of mature B lymphocytes (15).

In this study, we demonstrate that macrophages can be induced to elicit potent anti-myeloma tumoricidal activity both in vivo and in vitro. Activation of murine macrophages through administration of CD40 with sequential TLR activation in vitro led to dose-dependent cytotoxic activity against myeloma cells. Gene loss of the MAP3K, Tpl2, promoted the tumoricidal activity of activated macrophages. The increased cytotoxic potential of Tpl2-null macrophages correlated with higher production of tumoricidal effectors, such as NO.

Figure 6. 
αCD40 + CpG treatment promotes expansion of M1-like macrophages in t-VEx MYC animals. A, representative flow cytometric plots of CD11b(+)Ly6G(-)–gated bone marrow cells are shown from both control-treated and αCD40 + CpG-treated, myeloma tumor-bearing animals. We have previously demonstrated that the CD68(int)/Ly6C(int) population expresses iNOS (consistent with M1-like macrophages; ref. 10). By contrast, CD68(+)Ly6C(int) cells express low levels of iNOS and high levels of CD24 (L44R), consistent with an M2-like phenotype. αCD40 + CpG treatment results in expansion of the CD68(int)/Ly6C(int) population in myeloma-bearing mice. B, proportion of M1-like CD68(int)/Ly6C(int) cells over total CD11b(+)Ly6G(-) cells in individual animals, treated with αCD40 + CpG or with IgG + PBS control. For B, statistical significance was determined using ANOVA analysis across two independent experiments.
Macrophage-Activating Immunotherapy in Myeloma

TPL2 loss led to enhanced production of IL12, both in vitro and in vivo. IL12 is a powerful antitumor cytokine with pleiotropic antitumor effects, encompassing both innate and adaptive antitumor immunity whose clinical utility has not yet been fully harnessed (28). Therefore, TPL2 loss not only promoted macrophage-mediated tumor cell killing, but may have also led to amplification of anti-myeloma response through additional effects on NK and T-cell–mediated antitumor immunity.

Therapeutic macrophage activation led to prolongation of disease PFS and OS in a model of relapsed/refractory myeloma, t-Vk+MYC (29, 45). We have demonstrated that the high prolif-erative rate, aggressive clinical course, and drug resistance of t-Vk+MYC accurately model end-stage, relapsed/refractory myeloma (29). The use of a drug-resistant t-Vk+MYC model allowed us to test the hypothesis that therapeutic macrophage activation may be effective in controlling drug-resistant myeloma. This model may have important clinical implications given the paucity of clinical approaches to treat drug-resistant, end-stage myeloma.

Weakly agonistic or antagonistic cCD40 antibodies have shown modest clinical activity and an acceptable safety profile in multiple myeloma (46, 47). In this article, we provide a rationale for strongly agonistic cCD40 immunotherapy in myeloma. TPL2 inhibition could provide an ideal adjunct to strongly agonistic cCD40 immuno-therapy when the tumor cells express CD40. CD40-mediated MAPK pathway activation is dependent upon TPL2 activity: B cells from Tpl2−/− mice fail to activate ERK in response to CD40 stimulation (48). Therefore, TPL2 inhibition may prevent a cell-autonomous growth-promoting effect of cCD40 on CD40-expressing myeloma cells while also further activating cCD40-bearing macrophages to elicit tumoricidal activity.

In summary, we present that therapeutic macrophage repolarization coupled with TPL2 inhibition may be a promising approach to control drug-resistant residual disease in myeloma. TPL2 inhibitors are in advanced pharmaceutical development (49). We have previously proposed single-agent TPL2 inhibition as monotherapy to delay progression of indolent myeloma through interference with elaboration of pro-myeloma cyto-kines by MAM (8) and potentially other microenvironmental cell types. Advanced myeloma may be less responsive to TPL2 mono-therapy because of acquisition of autocrine cytokine support and/or mutations in crucial signaling pathways, for example, NF-κB mutations, considered to be progression events (50). Therapeutic activation of macrophages may be an effective means to control progression of relapsed/refractory myeloma relapse. Moreover, TPL2 inhibition offers an attractive adjunct to therapeutic macrophage activation in multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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