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Therapeutic effects of CSF1R-blocking antibodies in multiple myeloma

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Abstract

Our previous studies showed that macrophages (MΦs), especially myeloma-associated MΦs (MAMs) induce chemoresistance in human myeloma. Here we explored the potential of targeting MΦs, by using colony-stimulating factor 1 receptor (CSF1R)-blocking mAbs, to treat myeloma. Our results showed that CSF1R blockade specifically inhibited the differentiation, proliferation and survival of murine M2 MΦs and MAMs, and repolarized MAMs towards M1-like MΦs *in vitro*. CSF1R blockade alone inhibited myeloma growth *in vivo*, by partially depleting MAMs, polarizing MAMs to the M1 phenotype, and inducing a tumor-specific cytotoxic CD4⁺ T cell response. Similarly, genetically depleting MΦs in myeloma-bearing MM^{DTR} mice retarded myeloma growth *in vivo*. Furthermore, the combination of CSF1R blockade and chemotherapy such as bortezomib or melphalan displayed an additive therapeutic efficacy against established myeloma. Finally, a fully human CSF1R blocking mAb, similar to its murine counterpart, was able to inhibit the differentiation, proliferation and survival of human MΦs. Thus, this study provides the first direct *in vivo* evidence that MΦs and MAMs are indeed important for myeloma development and progression. Our results also suggest that targeting MAMs by CSF1R blocking

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Author contributions

Q.Y., Q.W., and S.W. initiated the work; S.W., Q.W., and Q.Y. wrote the manuscript; Q.W., Y. L., R. L., and Y. J. performed majority of the experiments; Y. Z., J.Q., and E.B. performed some experiments; J.H. and C.Z. provided critical suggestions to this study.

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mAbs may be promising methods to (re)sensitize myeloma cells to chemotherapy and promote anti-myeloma immune responses in patients.

Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by proliferation of monoclonal plasma cells in the bone marrow (BM) and destructive lytic bone disease.^{1, 2} In recent years, chemotherapy combined with novel agents, such as lenalidomide and bortezomib, has greatly improved the therapeutic efficacy for patients with MM; however, MM still remains largely incurable. Therefore, investigation of novel strategies for MM therapy has important clinical significance.

Macrophages (MΦs) are heterogeneous cell populations originating from blood monocytes.³ In general, MΦs can be divided into two major phenotypes, M1 and M2. M1 MΦs are polarized under inflammatory conditions and have a central role in fighting pathogen infections, whereas M2 MΦs are associated with anti-inflammatory responses.^{4–6} Tumor-associated MΦs are an important component of cancers. M1-like tumor-associated MΦs suppress cancer progression, whereas M2-like tumor-associated MΦs promote tumor growth.^{7, 8} In the tumor microenvironment, MΦs are often educated to develop into M2-like tumor-associated MΦs by tumor-derived cytokines, such as IL-10, IL-6, and TGF-β.^{9, 10} We previously found that MΦs accumulate in the MM BM microenvironment and protect MM cells from chemotherapy drug-induced apoptosis *in vitro*.^{11, 12} However, the role of MΦs in MM development and progression *in vivo* is not fully defined.

Colony-stimulating factor 1 receptor (CSF1R) is mainly expressed by monocytes, MΦs, and monocyte-like myeloid-derived suppressor cells (MDSCs).^{13, 14} The CSF1/CSF1R axis is important in the regulation of recruitment, differentiation, and functions of tissue MΦs.¹⁵ The CSF1/CSF1R axis also has a role in educating MΦs to become M2 MΦs.^{16, 17} Recent studies have shown that CSF1R blockade by inhibitors and antibodies improves therapeutic efficacy in multiple cancers, such as glioma, pancreatic cancer, and diffuse-type giant cell tumor.^{14, 18, 19} Based on these findings, we hypothesized that CSF1R blockade could be an important novel approach to improving MM therapy. Here, we investigated the role of MΦs in MM growth *in vivo* and the effect of targeting MΦs by CSF1R blocking antibodies in MM therapy.

Materials and Methods

Rag^{-/-} C57BL/6 mice, OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/J), OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/J), and *Lysm^{Cre} X Csf1r^{LsL}*-DTR C57BL/6 mice (MM^{DTR} mice) were purchased from the Jackson Laboratory. C57BL/KaLwRij (KAL) mice were purchased from Harlan Laboratories. All mice were maintained in specific pathogen-free conditions, and animal study was approved by the Institutional Animal Care and Use Committee of Cleveland Clinic Foundation. The procedures of MΦ generation and differentiation, microarray, mouse experiment are described in Supplemental Methods. The detailed quantitative real-time PCR (RT-PCR), flow cytometry, *in vivo* bioluminescent imaging,

proliferation assay, enzyme-linked immunospot (ELISPOT) assay, and immunohistochemistry (IHC) are also explained in Supplemental Methods.

Results

Blocking CSF1R inhibits the differentiation, survival, and proliferation of murine MΦs and MAMs *in vitro*

MAMs are mainly derived from peripheral blood monocytes;²⁰ and therefore, blocking monocyte differentiation into MΦs may inhibit MΦ infiltration into MM BM. To test this hypothesis, we used a mAb (CS7) against murine CSF1R that is expressed by both murine monocytes and MΦs (Supplementary Figure 1a). Addition of CS7 to monocyte cultures reduced their differentiation to MΦs and induced cell death in a dose-dependent manner (Figure 1a and Supplementary Figure 1b and c). Western blots showed that CS7 treatment downregulated the expression of phosphorylated (p)CSF1R, pERK, pAKT, and mTOR, and cleaved caspases 3, 7, and 8, and cytochrome C in the cells (Supplementary Figure 1d).

Next we examined the effect of CS7 on MAMs. Freshly differentiated normal MΦs were cocultured with 5TGM1 MM cells (to generate MAMs) in the presence of CS7 or control IgG. CS7 but not control IgG treatment inhibited the proliferation of MAMs (Supplementary Figures 1e and 1f). We also determined the effect of CS7 on *in vitro* polarized M1 or M2 MΦs. CS7 treatment significantly and specifically reduced the viability (Figure 1b) and increased apoptosis (Supplementary Figure 1g) of IL-10- or IL-4-polarized M2 MΦs^{21–23} and MAMs, but had minimal effect on GM-CSF- or LPS plus IFN-γ-polarized M1 MΦs^{24, 25}. Interestingly, CS7 treatment significantly downregulated the expression of pAkt and pERK in M2 MΦs and MAMs but not in M1 MΦs (Figure 1c). Thus, these results demonstrated that blocking CSF1R selectively inhibits the development and survival of M2 MΦs and MAMs.

Blocking CSF1R polarizes murine MAMs into M1-like MΦs *in vitro*

Because the above studies showed that CS7 at high doses selectively killed MAMs and M2 MΦs, we wanted to determine whether lower and non-apoptotic doses of CS7 could affect the polarization of MAMs *in vitro*. We cultured BM-derived MΦs (BMDMs) with 5TGM1 cells in the presence of CS7 or control IgG (0.5 nM) for 3 days, and analyzed the global transcriptional profile of the cells by gene array. Compared with control cells, CS7-treated MAMs expressed significantly higher levels of M1 MΦ-related transcriptional factors, cytokines, and other molecules (Figure 2a), and lower levels of M2 MΦ-associated *Il10*, *Fn1*, *Mmp12*, and *Tg*, and transcriptional factors such as *Irf4* and *Myc*^{6, 26–32} (Supplementary Figure 2a). To more accurately assess the effect of CS7 mAb on MAM repolarization, we performed ingenuity pathway analysis (IPA) to identify canonical signaling pathway activation in the cells. CS7-treated MAMs displayed activated signaling pathways of TREM-1, GM-CSF, LPS-stimulated MAPK, and production of nitric oxide and iNOS (Figure 2b) that are highly related to M1 MΦ polarization and function.^{24, 33–35} CS7-treated MAMs also displayed activated pathways of interferon, macropinocytosis, JAK1, JAK2, and TYK2 (Figure 2b), which are also M1 MΦ-specific gene clusters³⁶. Quantitative RT-PCR showed that 5TGM1-cocultured MΦs (MAMs) expressed greater mRNA levels of

M2 MΦ-associated *Irf4*, *Arg1*, and *Il10* and lower levels of M1 MΦ-associated *Il12a* and *Tnfa* than untreated MΦs, whereas *Irf4* and *Il10* mRNA levels were decreased and *Tnfa* and *Nos2* levels were elevated in CS7-treated MAMs (Figure 2c). Moreover, CS7 treatment significantly downregulated the expression of CD206 in MAMs (Supplementary Figures 2b and c), which is a surface marker for M2 MΦs²⁶. Taken together, these results demonstrated that blocking CSF1R repolarizes M2-type MAMs to M1-like MΦs *in vitro*.

Blocking CSF1R inhibits tumor growth in murine MM models

We reported that MΦs, especially MAMs, protected MM cells from drug-induced apoptosis.¹² To test our hypothesis that depleting MΦs and MAMs *in vivo* could sensitize MM cells to chemotherapy, we determined the effect of CS7 on MM growth *in vivo* using 5TGM1-bearing KAL mice (Supplementary Figure 3a). Treatment with CS7 but not control IgG significantly inhibited tumor growth, as detected by bioluminescent imaging (Figure 3a and Supplementary Figure 3b) and measurement of serum IgG2b secreted by MM cells (Figure 3b). Furthermore, CS7 treatment significantly decreased the number of 5TGM1 cells in hind leg BM and spleen (Supplementary Figure 3c and d). Notably, spleens from CS7-treated, MM-bearing KAL mice were significantly smaller than those treated with control IgG (Supplementary Figure 3e and f).

As monocytes and MΦs in MM^{DTR} mice can be almost completely depleted *in vivo* by diphtheria toxin (DT) injection,³⁷ we used the MM^{DTR} mouse model to confirm the role of MΦs in MM development and progression. As shown in Figure 3c, ablation of monocytes and F4/80⁺ MΦs in MM^{DTR} mice by DT treatment significantly reduced tumor burden detected as the level of circulating mouse IgG2b. These results provides the first direct evidence that MΦs play an important role in MM growth and progression *in vivo*, and suggest targeting these cells by CSF1R inhibitors may be therapeutic in human MM.

Blocking CSF1R induces an anti-MM microenvironment *in vivo*

Mechanistic studies were conducted to determine the therapeutic effects of CSF1R mAbs *in vivo*. CS7 treatment significantly reduced the numbers of CD11b⁺ Ly6C⁺CD115⁺ monocytes in the blood and spleen (Supplementary Figure 4a), and the percentages and total numbers of CD11b⁺ F4/80⁺ MΦs in the peritoneal cavity, BM, and spleen of 5TGM1-bearing KAL mice (Figures 4a and b and Supplementary Figure 4b and c). Moreover, immunohistochemistry confirmed that fewer F4/80⁺ MΦs were present in the BM of mice receiving CS7 than those receiving control IgG (Supplementary Figure 4d). These results indicated that blocking CSF1R depletes MΦs and MAMs and/or inhibits the development of these cells *in vivo*. As expected, fewer MΦs were detected in diphtheria toxin-treated MM^{DTR} mice than controls (Supplementary Figure 4e), confirming that blocking CSF1R efficiently depletes MΦs and MAMs *in vivo*.

We then explored the role of CSF1R blockade in MΦ polarization *in vivo* by examining the percentage and cytokine profile of (CD206⁺) MΦs in CS7-treated MM-bearing mice. The results showed that mice treated with CS7 had fewer CD206⁺ MΦs in spleen than those treated with control IgG (Supplementary Figure 4f). In addition, although CS7 treatment did not significantly change the percentage of CD206⁺ MΦs in the BM, MΦs/MAMs isolated

from BM of CS7-treated MM-bearing mice expressed significantly lower levels of *Il10* and higher levels of *Il12a* and *Tnfa* than those from control IgG-treated MM-bearing mice (Figure 4c). These results indicate that CSF1R blockade skews MAM phenotype to M1-like MΦs *in vivo*.

We also determined whether blocking CSF1R affected the development of MDSCs *in vivo*. As compared to control IgG, CS7 treatment had only minor effects on MDSCs (Supplementary Figure 4g), indicating that CSF1R-blocking mAb does not deplete MDSCs *in vivo*. Taken together, these results demonstrate that blocking CSF1R induces an anti-MM microenvironment by partially depleting MΦs and MAMs and polarizing MAMs to M1-like MΦs *in vivo*.

The anti-MM effects of CSF1R blockade rely on tumor-specific CD4⁺ T cell responses

To determine whether the immune system was required for the observed CS7-induced anti-MM effect *in vivo*, immunodeficient Rag^{-/-} mice were used to establish MM (5TGM1 cells), followed by injection of CS7 or control IgG. MM growth and tumor cell infiltration of BM and spleen were similar between mice treated with CS7 or IgG control (Figure 5a and Supplementary Figure 5a and b), indicating that the immune system or, specifically, lymphocytes were required for CS7 mAb-induced antitumor effect *in vivo*.

To determine the role of T cells in the antitumor effects induced by CSF1R blockade, 5TGM1-bearing immunocompetent KAL mice were again used. Although the total number of CD4⁺ or CD8⁺ T cells infiltrating BM and lymph nodes (LNs) did not differ between MM-bearing mice treated with CS7 or control IgG (Supplementary Figure 5c), the percentage and number of granzyme-B-expressing CD4⁺ and CD8⁺ T cells in BM and LNs were significantly higher in mice treated with CS7 (Supplementary Figure 5d and e). Similarly, DT-treated MM-bearing MM^{DTR} mice had higher percentages of granzyme-B-expressing CD4⁺ and CD8⁺ T cells in the BM and LNs than PBS-treated mice (Supplementary Figure 5f and g). More importantly, *in vivo* depletion of CD4⁺, but not CD8⁺, T cells completely abrogated CS7-induced inhibition of tumor growth and tumor cell infiltration of the BM (Figure 5b and Supplementary Figure 5h). ELISPOT analysis revealed an increased number of granzyme-B-secreting CD4⁺, but not CD8⁺, T cells after *ex vivo* restimulation with mature BM-derived dendritic cells (BMDCs) pulsed with irradiated 5TGM1, in the spleens and LNs of MM-bearing mice treated with CS7 compared to mice treated with control IgG (Figure 5c and Supplementary Figure 5i). These results indicate that CS7 treatment induces or enhances a tumor (5TGM1)-specific CD4⁺ T cell response that may have contributed to the observed anti-MM effects *in vivo*.

CS7-treated MAMs are immunogenic to tumor-specific CD4⁺ T cells

To elucidate the mechanism underlying CS7-mediated induction of tumor-specific CD4⁺ T cell response *in vivo*, we determined the effects of CS7 on the immunogenicity of MAMs *in vitro*. First, we examined the capacity of tumor (OVA)-pulsed MAMs, generated by coculturing MΦs with irradiated 5TGM1-OVA cells overnight and then treating them with CS7 or control IgG for another 24 hours to induce proliferation of OVA-specific CD4⁺ (OT-II) T cells *in vitro*. Irradiated 5TGM1 cells were used as antigen-negative control cells. The

results showed that CS7-treated 5TGM1-OVA-cocultured MAMs induced proliferation of OVA-specific CD4⁺ T cells (Figure 6a and Supplementary Figure 6a) and upregulated mRNA expression of *Gzmb* and *Eomes* in OVA-specific CD4⁺ T cells (Figure 6b). No T cell proliferation was induced by control IgG-treated, 5TGM1-OVA-cocultured or CS7-treated, 5TGM1-cocultured MAMs. These results indicated that CS7 treatment enhances the antigen-presenting capacity of MAMs to promote tumor-specific cytotoxic CD4⁺ T cell response.

To further analyze the antigen presentation capacity of CS7-treated MAMs, IPA was used to identify canonical signaling pathways of immune activation in the cells. As shown in Supplementary Figure 6b, CS7-treated MAMs were highly functional immune cells as compared with IgG-treated cells. Indeed, we also observed higher expression of genes involved in MHC class II-associated antigen processing and presentation, such as *Cd86*, *H2-Dma*, *H2-Ab1*, *H2-Aa*, *Cd40*, *Cd80*, *Ciita*, and *H2-Dmb2*^{38–40}, in CS7-treated MAMs as compared with IgG-treated cells (Supplementary Figure 6c). Flow and western blot analyses confirmed upregulated expression of CD40, CD80, CD86, and MHC II molecules (Figure 6c and Supplementary Figure 6d) and CIITA (Supplementary Figure 6e) on CS7-treated MAMs. Hence, these results indicate that CS7 treatment enhances the antigen presentation capacity of MAMs to promote a cytotoxic tumor-specific CD4⁺ T cell response.

The combination of CSF-1R blockade and chemotherapy displays an additive therapeutic efficacy against MM *in vivo*

We reported that MΦs conferred MM cell chemoresistance.¹² However, we had not determined whether CSF1R blockade could improve the chemotherapeutic efficacy *in vivo*. To address this issue, the protease inhibitor bortezomib, CS7, and their combination were used to treat 5TGM1-bearing KAL mice. Although mice treated with bortezomib or CS7 alone exhibited smaller tumor burden and longer survival than control IgG-treated mice, the combination of bortezomib and CS7 significantly reduced tumor burden and improved mouse survival as compared with single agent-treated mice (Figure 7a). Similarly, 5TGM1-bearing KAL mice treated with another MM chemotherapeutic drug, melphalan, or CS7 alone exhibited smaller tumor burden and longer survival than mice treated with control IgG (Figure 7b). Moreover, the combination of melphalan and CS7 also significantly reduced tumor burden compared with melphalan therapy alone and improved mouse survival. These results demonstrated that CSF1R blocking mAbs can be used to treat MM, especially in combination with chemotherapy.

Blocking CSF1R inhibits differentiation, survival, and proliferation of human MΦs *in vitro*

Finally, to determine whether our findings can be translated into clinic, we examined the effects of a fully human mAb (CS4) that blocks human CSF1R on human monocytes and MΦs. Similar to its murine counterpart, CS4 was also able to retard monocyte differentiation to MΦs and induced cell death in a dose-dependent manner (Figure 8a and Supplementary 7a and b). Furthermore, CS4 treatment decreased the viability and proliferation, and increased the apoptosis of human MAMs in coculture with human MM (ARP-1) cells (Figures 8b and c and Supplementary Figure 7c and d). We also examined the effect of CS4 on monocyte differentiation to DCs *in vitro*. CS4 did not impair monocyte survival and

differentiation to DCs (Supplementary Figure 7e and f). Finally, we determined the effect of the mAb on human MM cells and found that CS4 did not affect the survival of human MM ARP-1, MM.1S, RPMI 8226, and U266 cells (Supplementary Figure 7g). Taken together, these results demonstrate that blocking CSF1R signaling inhibits human monocyte differentiation into MM-supporting MΦs without affecting DCs or MM cells.

Discussion

We previously reported that CD68⁺ MΦs heavily infiltrate the BM of patients with MM.¹¹ However, the role of MΦs in MM development and progression is not fully defined. In this study, we demonstrated that depleting MΦs in myeloma-bearing MM^{DTR} mice retarded myeloma growth. In addition, targeting MΦs by CSF1R blocking antibodies also inhibited tumor growth *in vivo*. Mechanistic studies showed that MΦs in the BM of patients with MM were mainly of the M2-like phenotype, which promotes tumor progression; and blocking CSF1R partially depleted MΦs and MAMs and effectively skewed the MΦ phenotype from M2 to M1-like phenotype, and inhibited tumor progression. Consistent with the therapeutic effects of CSF1R blockade in solid tumors,^{17,21,22} we found that CSF1R-blocking mAbs inhibited MM growth *in vivo*, and the combination of CSF1R-blocking mAbs and chemotherapy further improved the therapeutic efficacy against established MM. Thus, this study provides the first direct evidence that MΦs and MAMs are important for MM development and progression, and suggests that targeting MΦs will be important for improving the efficacy of chemotherapy in MM.

Tumor-associated MΦs mediate immunosuppression in many cancer types, including MM.^{5, 9, 14} Thus, we speculated that the anti-MM effects induced by CSF1R blockade might be through ablation of MAMs, and subsequent activation of anti-MM immunity *in vivo*. Indeed, in this study, we found that CSF1R blockade failed to inhibit MM growth in immunodeficient mice, which lack B- and T cells. In addition, depletion of CD4⁺ but not CD8⁺ T cells abolished the anti-MM effects induced by CSF1R blockade. Notably, CSF1R blockade increased the number of cytotoxic CD4⁺ (granzyme-B-producing CD4⁺) T cells in both BM and LNs. Our results are consistent with previous investigations showing that circulating CD4⁺ T-cell numbers decrease in MM and its precursor disease MGUS,^{41, 42} and the ratio of CD4⁺ to CD8⁺ T cells was negatively related to tumor burden and MM progression.⁴³ Thus, our data established the important role of cytotoxic CD4⁺ T cells in mediating the anti-MM activity induced by CSF1R blockade.

Of interest is our finding that cytotoxic CD4⁺ T cells are critical for the eradication of MM during CSF1R blockade treatment, but most MM cells express a low level of MHC class II antigens on their surface.⁴⁴ Therefore, whether and how cytotoxic CD4⁺ T cells recognize MHC class II^{low} MM cells remains unclear. Tveita et al⁴⁵ addressed the question whether MHC II-deficient MM cells could be eradicated by tumor-specific CD4⁺ T cells and demonstrated that CD4⁺ T cells specific for MM antigens could eliminate MM cells in the absence of endogenous MHC II expression on the tumor cells through antigen uptake and indirect presentation on tumor-infiltrating MΦs. Our data showed that CSF1R blockade enhanced MHC II antigen presentation of MAMs, which stimulated cytotoxic CD4⁺ T cell proliferation and cytolytic function *in vitro*. Thus, these findings provide a plausible

explanation as to why CD4⁺ T cells are crucial for CSF1R-mediated anti-MM immune responses *in vivo*.

In conclusion, our study provides the first direct *in vivo* evidence that MΦs and MAMs are indeed important for myeloma development and progression and may have important clinical implications for the treatment of MM by targeting its microenvironment components.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Blocking CSF1R inhibits differentiation, survival, and proliferation of murine M2 and MAMs *in vitro*

(a) Viability by MTS assay in 3-day culture of CD14⁺ monocytes in the presence of M-CSF (CSF1) and CS7 mAb or control IgG. (b) Viability detected by MTS of murine MΦs in 3-day coculture with IL-10, IL-4, GM-CSF, LPS plus IFN- γ , or MM cells (5TGM1) in the presence of CS7 or control IgG. (c) Western blot showing the expression of phosphorylated (p) Akt and ERK in murine BMDMs cultured with M-CSF, IL-4, 5TGM1, GM-CSF, or LPS plus IFN- γ in the presence of 5 nM IgG or CS7 for 24 hours, shown is representative of at least three independent experiments. Summarized data from at least three independent experiments are shown. NS: not significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2. Blocking CSF1R redirects the polarization of murine MAMs *in vitro*

BMDMs were cocultured with 5TGM1 in the presence of 0.5 nM CS7 (CS7) or control IgG (MAM) for 3 days. RNA (biological samples from three mice) was extracted for gene microarray and the array data were analyzed. (a) Heat maps illustrating the log₂-fold change of M1 MΦ-related gene sets as indicated. (b) IPA identifying activation of specific canonical pathways as indicated, in MAMs treated with CS7 or IgG. The circle surface area is proportional to -log (p-value) and the color intensity of circles indicates the Z score. (c) Ratio in mRNA expression of indicated M1 or M2 signature genes in murine MΦs in 3-day coculture with or without 5TGM1 cells in the presence of CS4 or IgG by RT-PCR. Summarized data from at least three independent experiments are shown. NS: not significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 3. Blocking CSF1R inhibits murine MM growth *in vivo*

On day -16, KAL mice were injected with 2×10^6 5TGM1-luc cells via the tail vein. Starting on day 0, mice were injected intraperitoneally with 10 mg/kg CS7 (n=6 per group) or control IgG (n=6 per group) twice a week. Mouse serum was collected once a week. On day 21, mice were sacrificed for analysis. **(a)** Bioluminescent imaging of 5TGM1 tumor burden on day 21 (n=6 mice per group). **(b)** Tumor burden measured as the serum concentration of IgG2b secreted by MM cells in mice (n=6) treated with CS7 or control IgG. **(c)** Level of serum IgG2b on day 21 in 5TGM1-bearing MM^{DTR} mice (n=4 per group) treated with PBS or diphtheria toxin (DT). Data are presented as mean $\pm$ SD, * $P < 0.05$.

Figure 4. Blocking CSF1R induces an anti-tumor microenvironment in MM-bearing mice
 Shown are numbers of MΦs in different tissues of 5TGM1-bearing KAL mice 21 days after treatment with CS7 or control IgG. **(a)** Dot plots showing the percentage and total number of CD11b⁺F4/80⁺ MΦs in peritoneal leukocytes, results from 1 representative mouse of total 6 mice are shown. **(b)** Summarized data of dot plots showing the percentage and total number of CD11b⁺Ly6C⁺F4/80⁺ MΦs in mouse BM and spleen. **(c)** Fold change in the expression of M1 or M2 cytokine genes in MΦs isolated from BM of 5TGM1-bearing KAL mice (n=4) treated with CS7 or control IgG (right). Data are presented as mean ± SD. * *P* < 0.05; ** *P* < 0.01.

Figure 5. Blocking CSF1R stimulates a tumor-specific CD4⁺ T cell response *in vivo*

(a) Tumor burden of Rag^{-/-} mice (n=9 per group) injected with 2×10^6 5TGM1 cells and treated with CS7 or control IgG. Tumor burden was assessed as the serum IgG2b concentration. (b) Tumor burden detected as the circulating IgG2b concentration from 5TGM1-bearing KAL mice (n=5) treated with control IgG, CS7, CS7 plus CD8-neutralizing antibody (CS7+ α CD8), CS7 plus CD4-neutralizing antibody (CS7+ α CD4), or CS7 plus CD4 and CD8 neutralizing antibodies (CS7+ α CD8+ α CD4). (c) Representative ELISPOT images showing the number of granzyme B-secreting MM-specific CD4⁺ or CD8⁺ T cells from spleens and draining LNs of 5TGM1-bearing KAL mice treated with control IgG, CS7, CS7+ α CD4, or CS7+ α CD8. T cells from spleens and LNs were cocultured with mature murine DCs pulsed with or without irradiated 5TGM1 cells for 2 days before assay. Shown are results from 1 representative experiment of 4 performed (n=4 mice). Data are presented as mean $\pm$ SD of at least three independent experiments. NS: not significant.

Figure 6. CS7-treated MAMs promote a cytotoxic CD4⁺ T cell response by enhancing antigen presentation

(a) BMDMs were cocultured with irradiated 5TGM1 or 5TGM1-OVA cells in the presence of IgG or CS7 antibody for 24hs, washed four times with PBS to remove the MM cells, and then cocultured with CFSE-labeled OT-II CD4⁺ T cells *in vitro* for 2 days. Summarized results showing the percentage of CFSE^{low} proliferative cells determined by FACS. (b) RT-PCR determination of relative *Gzmb* and *Eomes* mRNA expression in MAM-cocultured OT-II CD4⁺ T cells. (c) Summarized data showing the expression of CD40, CD80, CD86, and MHC II by MAMs treated with 1nM IgG or CS7 for 3 days. Data are presented as mean $\pm$ SD of at least three independent experiments. NS: not significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 7. The combination of CSF1R blocking mAbs and chemotherapy induces potent therapeutic efficacy of MM *in vivo*

(a) Tumor burden (left) and survival curves (right) of 5TGM1-bearing KAL mice (n=7 per group) treated with CS7, bortezomib (BTZ), or a combination of both. (b) Tumor burden (left) and survival curves (right) of 5TGM1-bearing KAL mice (n=7 per group) treated with CS7, melphalan (Mel), or a combination of both. Isotype IgG was used as a control for CS7. Serum samples were collected once a week. Mice were sacrificed when moribund. Tumor burden was assessed as the level of serum IgG2b. * $P < 0.05$; ** $P < 0.01$.

Figure 8. Blocking CSF1R inhibits differentiation, survival, and proliferation of human MΦs and MAM *in vitro*

(a) Viability in 3-day culture of human monocytes in the presence of human M-CSF (CSF1) and CS4 mAb or control IgG. (b) Viability in 3-day coculture of human MΦs with MM cells (ARP-1) in the presence of CS4 or control IgG. (c) Summarized data showing the percentage of Ki67⁺CD11b⁺ proliferating MΦs in 24-hour coculture with MM cells (ARP-1) in the presence of CS4 or control IgG. Summarized data from three independent experiments are shown. NS: not significant. * $P < 0.05$; ** $P < 0.01$.