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Antibody dependent cellular phagocytosis by macrophages is a novel mechanism of action of elotuzumab

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Abstract

Elotuzumab, a recently approved antibody for the treatment of multiple myeloma (MM), has been shown to stimulate Fc γ receptor (Fc γ R)-mediated antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells towards myeloma cells. The modulatory effects of elotuzumab on other effector cells in the tumor microenvironment, however, has not been fully explored. Antibody dependent cellular phagocytosis (ADCP) is a mechanism by which macrophages contribute to anti-tumor potency of monoclonal antibodies. Herein, we studied the NK cell independent effect of elotuzumab on tumor associated macrophages (TAMs) using a xenograft tumor model deficient in NK and adaptive immune cells. We demonstrate significant anti-tumor efficacy of single agent elotuzumab in immunocompromised xenograft models of multiple myeloma, which is in part mediated by Fc-Fc γ R interaction of elotuzumab with macrophages. Elotuzumab is shown in this study to induce phenotypic activation of macrophages *in-vivo* and mediates ADCP of myeloma cells through a Fc γ R dependent manner *in-vitro*. Together, these findings propose a novel immune mediated mechanism by which elotuzumab exerts anti-myeloma activity and helps to provide rationale for combination therapies that can enhance macrophage activity.

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Conflict-of-interest disclosure:

I.M.G serves as a consultant on the elotuzumab advisory board of Bristol-Myers Squibb. A.R serves on the advisory board of Amgen. The remaining authors declare no potential competing conflicts of interests.

Keywords

Multiple Myeloma; Elotuzumab; Macrophages; Phagocytosis; Fc γ Receptor

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Introduction

MM is a plasma cell dyscrasia characterized by clonal expansion of malignant plasma cells in the bone marrow leading to lytic bone lesions, cytopenias, and immunodeficiency. [1] In light of recent advances of therapy in MM including proteasome inhibitors and immunomodulatory therapies, MM has transformed into a chronic disease with an approximate median overall survival of 7-10 years. [2] Despite the advances in therapeutic regimens available, MM largely remains incurable with the eventual progression to drug resistant or relapsed disease. [2] Recent MM treatment strategies have focused on the use of novel therapies and combinations that harness the anti-tumor potential of the immune system with the aim of improving prognosis of the disease. [3] The implementation of monoclonal antibodies (mAbs) in the treatment of solid and hematologic malignancies has resulted in considerably improved clinical outcomes for several malignancies. [4,5] Elotuzumab is a novel humanized immunoglobulin G1 (IgG1) mAb that has recently been approved for the treatment of relapsed or refractory MM in combination with lenalidomide and dexamethasone. [6] This mAb recognizes a cell surface glycoprotein signaling lymphocytic activation molecule F7 (SLAMF7) that is primarily expressed on myeloma and other cells of hematopoietic lineage including NK cells. [7,8] While shown to induce NK cellular activation, the binding of elotuzumab to SLAMF7 on myeloma cells has not been shown to induce cellular proliferation or promote tumor cell death. [9] Additionally, the fragment crystallizable (Fc) region of elotuzumab has been shown to activate NK cells by binding to the Fc gamma receptor III (Fc γ RIII) which induces myeloma directed ADCC. [7,8]

The role of the innate immune system has not been thoroughly examined in MM. Effector functions of the innate immune system (such as ADCP and ADCC) have been shown to be dependent on the predominantly stimulatory role of Fc γ R. [10] The effect of elotuzumab on other effector immune cells expressing SLAMF7 as well as Fc γ R remains widely unexplored. However, an understanding of these mechanisms is necessary if we are to fully exploit the potential of this drug in immune-modulating combinations [11] and further define specific subgroups of patients who would benefit the most from this agent. Within the context of MM, TAMs have demonstrated a non-redundant and central role in tumor niche homeostasis. [12] Fc γ R expressing macrophages have been shown to play a considerable role that contributes to efficacy of several widely used mAbs used in anti-tumor therapy. [13,14] Targeting Her2/neu, CD20, and CD38 respectively, trastuzumab, rituximab, and daratumumab are high Fc γ R affinity IgG1 antibodies that induce ADCP by macrophages. [15, 16, 17] In this study, we demonstrate via *in-vivo* xenograft and syngeneic tumor models in addition to *in-vitro* assays the potential role of elotuzumab in inducing Fc γ R-mediated anti-tumor effects of macrophages.

Materials and Methods

Cell lines

The MM.1S GFP⁺Luc⁺ cell line was generated via retroviral transduction using the pGC-GFP/Luc vector (gift of A Kung, DFCI, MA) and cultured in RPMI 1640 medium supplemented with L-glutamine and antibiotics (penicillin and streptomycin at 100 U/mL 100 µg/mL respectively) and 10% FBS. EG7-human SLAMF7 (hSLAMF7) cells [18] were cultured in RPMI with 2 mM L-glutamine, 10% FBS, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 0.4 mg/mL G418 (Life Technologies, California, USA). OPM-2 (DSMZ, Braunschweig, Germany) cells were cultured in RPMI with 10% FBS and 10 mM HEPES.

Mice

Severe combined immunodeficient beige (SCID-beige) and NOD scid gamma (NSG) mice were purchased from Taconic (New York, USA) and used for as myeloma xenograft. C57Bl/6j and SCID mice were purchased from The Jackson Laboratory (Maine, USA) and used for subcutaneous injection models. Female mice used from the indicated strains were 6-7 weeks old. All animal studies were approved by the Dana Farber Cancer Institute IACUC and Bristol-Myers Squibb Animal Care and Use Committee and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Antibodies

Elotuzumab and elotuzumab variants were provided by Bristol-Myers Squibb (BMS; Princeton, NJ). Fc-inert variant of elotuzumab (elotuzumab hIgG1.1): has 5 mutations L234A, L235E, G237A, A330S, P331S in Fc region that abrogate binding to human FcγR. The Fc mutant form of the antibody was expressed by the CHO-S cell line cotransfected with vectors pICOFSCneoK (encoding Elo variable regions) and pODpurIgG1.1f (encoding IgG1 heavy chain constant region with L234A-L235E-G237A-A330S-P331S mutations). To generate a mouse IgG2a variant of elotuzumab (elotuzumab-g2a), the heavy chain variable domain (VH) for the elotuzumab parental mAb was cloned into an expression vector containing the mouse IgG2a constant region. The light chain variable region (Vκ) was cloned into an expression vector containing the mouse light chain constant region. [18] Human IgG1 (hIgG1) and mg2a isotype controls were obtained from R&D systems (Catalog #110-HG, Minneapolis, MN) and BioXCell (clone C1.18.4, West Lebanon, NH) respectively.

Myeloma xenograft models

SCID-beige and NSG mice were injected with 5 million MM1S GFP⁺ Luc⁺ cells intravenously (i.v.). For the early tumor model, after 48 hours of tumor injection, mice were treated with intraperitoneal (i.p.) injections of hIgG1, Fc-inert variant of elotuzumab, or elotuzumab at a 10mg/kg dose twice weekly. The late tumor model comprised treatment of mice post tumor development and randomization, using bioluminescence imaging as described below. SCID mice were injected subcutaneously in their hind flanks with 13×10^6

million OPM2 cells suspended in matrigel diluted in PBS (1:1). Antibody treatment with 1mg/kg of hIgG1 or 0.5mg/kg and 1mg/kg doses of elotuzumab i.p. was initiated once tumor volume reached an average of 135mm³ and administered on days 7, 10 and 14.

Syngeneic tumor model

C57Bl/6j mice were subcutaneously injected in their hind flanks with 5×10^6 million EG7-hSLAMF7 cells. After 5–7 days, mice were randomized into treatment groups when tumor volumes reached 80–120 mm³. Mice were then injected intraperitoneally with elotuzumab-g2a or mg2a isotype control (10mg/kg) at the indicated times for a total of 3 doses. NK cells and macrophages were depleted *in-vivo* using 50µg of anti-asialo GM1 [19] (eBioscience; San Diego CA) or 600µg anti -CSF1R i.p. [20] (clone AFS98, BioXCell) respectively. Depletion antibodies were administered to separate groups 3 days before initiation of elotuzumab-g2a or mg2a treatment and every 7 days thereafter. Tumor volume was measured biweekly by digital calipers (Fowler, Newton, MA) and calculated by the formula: length $\times$ width² $\times$ 0.52. [21]

Bioluminescence Imaging

MM1S GFP⁺ Luc⁺ xenograft mice were injected with 75-mg/kg luciferin i.p. (Caliper Life Sciences, Waltham, MA) and imaged with real-time whole-body bioluminescence 5 minutes after the injection. BLI images were obtained via the following settings: Emission Filter Open on IVIS 1327 camera; binning (HR), 4; field of view, 25; flux stop, 1. total flux (photons per second (p/s)) was calculated based on 4 $\times$ 8-cm regions of interest.

In vivo confocal microscopy

In SCID-beige xenograft mice, homing of MM1S GFP⁺ Luc⁺ cells to distant bone marrow niches (skull) was traced in vivo, using in vivo confocal microscopy. After 18 hours of MM1S GFP⁺ Luc⁺ i.v. injection and i.p. treatment with elotuzumab or hIgG1 control, myeloma cell homing to the bone marrow was assessed using a Zeiss 710 confocal system (Carl Zeiss Microimaging, Jena, Germany) with an upright examiner stand and a custom stage. Imaging was done through a skin flap made in the scalp of mice exposing the central and coronary veins on dorsal surface of the skull. GFP was excited using the 488-nm Argon laser while blood vessels were visualized using Evans Blue (100 µL IV) (Sigma-Aldrich) excited via a 633-nm laser. Emission signals were quantified by the Zeiss internal confocal Quasar detectors.

Immunophenotypic analysis

Spleen and subcutaneous tumor from OPM2- or EG7-hSLAMF7-bearing mice were resected and single cell suspensions were prepared by dissociation and passing cells through a 70-µm filter. Cells (5×10^5) were plated in 96-well plates, treated with 2.4G2 (BD Biosciences, San Jose, CA), and stained with fluorochrome-conjugated antibodies against the following surface markers: NK1.1, CD11b, Gr1, F4/80 (Biolegend, San Diego, CA). Stained cells were acquired on the FACS Aria II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Oregon).

Antibody dependent cellular phagocytosis

After 5-6 weeks of injection of MM1S GFP⁺ Luc⁺ in SCID-beige mice, TAMs were isolated as outlined by Zhang et al. [22] from the femurs of mice exhibiting signs of hind limb paralysis. TAMs were cultured in DMEM medium the presence of 40ng/ml of M-CSF for 6 days. Subsequently, TAMs were polarized into an anti-tumor M1 phenotype using 100ng/ml of each IFN- γ and LPS. Concurrently, target MM1S GFP⁺ Luc⁺ cells were co-cultured at an effector to target (E:T) ratio of 3:1. Also, tumor opsonizing antibodies (100 μ g/ml of hIgG1, Fc-inert variant of elotuzumab, or elotuzumab) were added to the co-culture lasting 18-24 hours. Subsequently, cells were analyzed using flow cytometry. Co-localization of the fluorescence of F4/80 (Clone: BM8, Biolegend) stained TAMs and GFP labeled MM1S cells (percentage of double positive events) indicated phagocytosis of the tumor cells.

Confocal Microscopy

TAMs were isolated from MM1S GFP⁺ Luc⁺ xenograft SCID-beige mice and cultured with MM1S GFP⁺ Luc⁺ cells using E:T ration of 1:5 and stained for F4/80 using 5 μ g/ml of Alexa Fluor® 594 anti-mouse F4/80 antibody (Clone: BM8, Biolegend). ADCP was visualized using the Leica Sp8 confocal microscope (HC PL APO CS2 40 $\times$ /1.3 oil objective) at the Harvard NeuroDiscovery Center. Images were saved as .lif files and analyzed in FIJI (ImageJ2).

MTT Cell Proliferation Assay

MM1S GFP⁺ Luc⁺ cells were seeded in a 96 well-plate (10,000 cells/well) and cultured in the presence of hIgG1, Fc-inert variant of elotuzumab, or elotuzumab (100 μ g/ml). Proliferation was assessed at 24, 48, and 72 hours by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International) dye absorbance.

Statistical Analyses

Statistical analysis of total BLI flux, percentage of macrophages and their associated surface markers, cellular proliferation, phagocytosis assay, and tumor volumes were performed using unpaired student *t*-test or Mann–Whitney *U* test if the data wasn't normally distributed. For survival analyses, Kaplan-Meier survival probability curves were used with log-rank analysis was used to test for statistical significance in survival differences. *P* values < 0.05 were considered statistically significant. Prism 6 (GraphPad Software) was used to calculate and analyze the statistical differences between experimental groups.

Results

Elotuzumab inhibits myeloma progression and prolongs survival in a tumor xenograft model

While elotuzumab has previously been shown to primarily promote myeloma cytotoxicity through the stimulation of NK cells via ADCC, we sought to define the anti-tumor effect of elotuzumab in the absence of functional NK cells by using a severe combined immunodeficiency xenograft mouse model of MM, the SCID-beige mouse model. [23, 24,

25] Macrophages, hence, remain as one of the primary effector immune cells retaining functional anti-tumor activity in this mouse model. [26]

Using the early xenograft model of myeloma (Supplementary Fig. 1a), elotuzumab significantly inhibited tumor growth in treated mice compared to the hIgG1 control group as shown by serial BLI imaging (Fig. 1a, Supplementary Fig. 2a). Moreover, elotuzumab significantly prolonged survival in treated mice compared to the control $P<0.001$ (Fig. 1b). To exclude potential direct effect of elotuzumab on the survival and proliferative ability of injected tumor cells, a methyl tetrazolium (MTT) survival assay was performed in vitro. No notable differences in survival was detected in multiple MM cell lines tested (MM1S, RPMI, U266) upon culture with hIgG1 or elotuzumab for up to 72 hours (Supplementary Fig. 3a). Similarly, the elotuzumab did not affect the migration and homing of MM1S-GFP-Luc+ cells to the bone marrow *in vivo* (Supplementary Fig. 3b). Together, these studies suggest that elotuzumab induces its anti-tumor properties indirectly through immune cell mediators in the xenograft myeloma model.

To further validate the results obtained with the early xenograft myeloma model, the late tumor model was similarly used whereby mice bearing significant tumor burden were treated with elotuzumab (Supplementary Fig. 1a). MM1S GFP+ Luc+ xenograft mice with BLI signal indicating established tumors were randomized into two groups which received either elotuzumab or hIgG1. There was a significant reduction in tumor volume as measured by the BLI signal intensity in the elotuzumab treated group compared to the control group (Fig. 1c, Supplementary Fig. 2b). This translated to improved survival of elotuzumab treated mice in comparison to the control group $P=0.027$ (Fig. 1d).

Beneficial effects of elotuzumab are mediated by activation of macrophages

One of the mainly characterized mechanisms of action of elotuzumab is the activation of NK cells via the Fc γ RIII. Therefore, we aimed to investigate if the anti-tumor effect of elotuzumab in the SCID-beige model is due to the activation of Fc receptors on the main effector cells, macrophages. Since elotuzumab is a humanized antibody, its activity is restricted to hSLAMF7 interactions with human MM cell lines injected into these mice along with the Fc-Fc γ R interaction with murine effector immune cells. The use of the humanized antibody in xenograft animal models therefore, focuses on the effects of engagement of Fc γ R and does not encompass total effects of the antibody comprising SLAMF7 binding on murine immune cells. To delineate the biologic effects mediated via binding of elotuzumab to SLAMF-7 and Fc receptors, an elotuzumab variant incapable of binding to Fc receptors (Fc-inert variant of elotuzumab) was used. Two days after the injection of SCID-beige mice with MM1S GFP+ Luc+ cells, mice were treated with either hIgG1, Fc-inert variant of elotuzumab, or elotuzumab. BLI intensity was significantly reduced in the elotuzumab treated group compared to the hIgG1 and Fc-inert variant of elotuzumab treated groups, indicating a central role of the Fc-Fc γ R interaction in inducing the observed anti-tumor effects. (Fig. 2a, Supplementary Fig. 2c). In addition, elotuzumab resulted in a significantly improved survival benefit in the treatment group compared to both hIgG1 and Fc-inert variant elotuzumab treated groups $P<0.001$ (Fig. 2b). We then used NSG mice that have severe immunodeficiency comprising the function of macrophages [27] to

confirm the requirement of macrophages in the anti-myeloma effect of elotuzumab (Supplementary Fig. 1a). MM1S GFP⁺ Luc⁺ cells were injected into NSG mice and antibody treatment was administered 48 hrs after myeloma cell injection as described previously. Interestingly, the beneficial effect of elotuzumab was abrogated as mice that received the treatment exhibited comparable BLI intensity to mice receiving the Fc-inert variant of the antibody (Fig. 2c). No difference in the survival of mice between groups was observed (Supplementary Fig. 3d). These results demonstrate that elotuzumab enables macrophages to prevent tumor growth and progression through the activation of FcγR.

Elotuzumab exerts its biologic effect comparably through NK cells and macrophages

To further confirm that macrophages are implicated effector cells of anti-myeloma activity, we used a subcutaneous syngeneic tumor model in immunocompetent C57Bl/6j mice (Supplementary Fig. 1a). The use of EG7-hSLAMF7, a mouse cell line expressing hSLAMF7 provided a syngeneic surrogate of human myeloma cells and allowed the *ex-vivo* characterization of effector immune cells mediating the anti-tumor effects of elotuzumab. Since NK cells are well recognized mediators of the anti-tumor properties of elotuzumab, we aimed to compare the anti-tumor potency of macrophages exposed *in-vivo* to elotuzumab to that of NK cells. Consequently, macrophage and NK cells were respectively depleted using anti-CSF1R and anti-asialo GM1 antibodies administered i.p. to separate groups of EG7-hSLAMF7 bearing mice. At day 18 post-treatment, the median tumor growth in control mice receiving mIgG2a (667.17 mm³) was significantly reduced in mice receiving elotuzumab without immune cell depletion (elotuzumab-g2a group) that yielded the lowest tumor burden (median tumor volume= 111.25 mm³) ($P=0.028$). The benefit of elotuzumab treatment in immunocompetent mice (elotuzumab-g2a group) was comparably abrogated upon the depletion of either macrophages (elotuzumab-g2a + CSF1R) or NK cells (elotuzumab-g2a + asialo GM1) given the increase in median tumor volume (669.51 mm³ and 686.56 mm³ respectively, $P=0.037$ for both groups) (Fig. 3a).

In addition, the difference in the tumor volume among NK cell and macrophage depleted groups not treated with elotuzumab (groups asialo GM1 and CSF1R) to similar groups that were treated further suggests the dependency of anti-tumor effector functions of these cells on elotuzumab. Upon depletion of macrophages, the median tumor volume for mice concurrently receiving elotuzumab treatment (elotuzumab-g2a + CSF1R group: 669.51 mm³) was decreased compared to those subjected only to macrophage depletion (CSF1R group: 956.54 mm³, not statistically significant) and is mainly attributed to the anti-tumor effector role of NK cells (Fig. 3a). However, compared to the tumor volume of NK cell-depleted mice not treated with elotuzumab (asialo GM1 group: 1,264.07 mm³), mice that were subjected to NK cell depletion and received elotuzumab treatment (elotuzumab-g2a + asialo GM1 group) demonstrated a significant reduction in median tumor volume (686.54 mm³) ($P=0.005$) (Fig. 3a). The benefit in tumor volume reduction obtained upon elotuzumab treatment despite the depletion of NK cells strongly suggests the involvement of other effector cells, primarily macrophages, in mediating the anti-tumor effects of elotuzumab in immunocompetent syngeneic tumor bearing mice.

The effect of elotuzumab treatment of EG7-hSLAMF7 bearing mice on the accumulation of macrophages at the tumor site was assessed 11 days after the initiation of elotuzumab treatment (total of 3 administered doses). F4/80 expressing macrophages showed a 1.59-fold increase in percentage upon elotuzumab treatment compared to groups subjected to isotype control ($P=0.042$). The efficacy of macrophage depletion is also illustrated among groups that received anti-CSF1R antibody compared to isotype control (Fig. 3b).

Elotuzumab induces tumor associated macrophage activation

We aimed to investigate how the function and phenotype of macrophages is modulated by elotuzumab. A subcutaneous tumor model was used where OPM2 myeloma cells were injected in SCID mice (Supplementary Fig. 1a). Mice received i.p injections of 1 mg/kg hIgG1 or either 0.5mg/kg or 1mg/kg of elotuzumab. As observed in the other mouse models, a significant reduction in tumor progression was noted among mice treated with elotuzumab 0.5 mg/kg (tumor volume at day 21: $643.7 \pm 703.3 \text{ mm}^3$) compared to the control group ($1,952 \pm 773.3 \text{ mm}^3$, $P<0.001$). Similarly, the tumor volume of mice treated with 1mg/kg elotuzumab ($488 \pm 524 \text{ mm}^3$) demonstrated a significant decrease in tumor volume compared to the hIgG1 treated group ($1,952 \pm 773.3 \text{ mm}^3$, $P<0.001$). In addition, among the elotuzumab treated groups, the lower tumor volume obtained upon treatment with 1mg/kg elotuzumab suggests a dose response (Fig. 4a).

Phenotypically, we investigated if tumor associated macrophages from elotuzumab treated mice had an activated phenotype. Classically activated macrophages through lipopolysaccharide (LPS) and interferon-gamma (IFN- γ) are characterized by an increase in expression of CD86 as well as PD-L1. [28, 29] Tumors and spleens from these mice were isolated on days 1 and 8 post treatment and were analyzed by flow cytometry. A significantly higher percentage of F4/80⁺ TAMs was detected in the 1mg/kg elotuzumab treated group as early as day 1 post treatment compared to the control group ($P=0.004$) (Fig. 4b). Also, the higher dose of elotuzumab resulted in a higher percentage of TAMs as compared to group receiving the lower dose of 0.5mg/kg ($P=0.024$). On day 8, however, the percentage of recruited macrophages among elotuzumab treated mice was comparable to the control group both in the tumor and the spleen. Notably, among the 1mg/kg elotuzumab treated group, TAMs exhibited an early and significantly higher expression of activation markers CD86 ($P=0.0063$) and PD-L1 ($P=0.0107$) compared to the hIgG1 control. Higher TAM expression levels of CD86 and PD-L1 were also noted in the group receiving a lower dose of elotuzumab indicating a dose dependent response. Therefore, while the recruitment of TAMs was decreased by day 8, elotuzumab treatment at both dosages showed sustained phenotypic changes among the macrophages with CD86 and PD-L1 maintaining a higher expression compared to the control group (Fig. 4b). Conversely, macrophages analyzed from spleens did not demonstrate similar phenotypic changes to those noted among TAMs (Fig. 4c). Collectively, these results demonstrate the role of elotuzumab in promoting the accumulation of macrophages at the tumor site and inducing phenotypic changes that likely reflect effector cell activation.

Elotuzumab mediated phagocytosis of myeloma cells is induced via Fc γ R activation of macrophages

Given the role of elotuzumab in inducing macrophage activation in mouse tumor models, we investigated the effect of the mAb on the anti-tumor effector function of macrophages. TAMs isolated from the femurs of SCID-beige mice that were injected with MM1S GFP⁺ Luc⁺ cells were polarized *in-vitro* into the anti-tumor M1 phenotype using IFN γ and LPS. Consequently, the effector M1 macrophages were co-cultured with MM1S GFP⁺ Luc⁺ target cells opsonized with hIgG1, Fc-inert variant of elotuzumab, or elotuzumab. ADCP was analyzed using flow cytometry and assessed through the F4/80 and GFP double positive cells. Elotuzumab opsonized tumor cells resulted in a significantly higher ADCP activity compared to hIgG1 ($P=0.019$). In addition, the use of Fc-inert variant of elotuzumab resulted in a two-fold reduction in ADCP compared to elotuzumab treated cells ($P=0.0077$). Also, the classical activation and polarization of TAMs into the M1 phenotype was crucial in inducing ADCP as evidenced by the loss of in elotuzumab induced ADCP efficacy among myeloma cells treated with the mAb and co-cultured with undifferentiated TAMs ($P=0.0059$) (Fig. 5a and b). These results show the necessity of an intact Fc region for enhanced ADCP activity along with the specific Fc-Fc γ R interactions of elotuzumab with classically activated macrophages that promote myeloma cell phagocytosis.

Furthermore, confocal microscopy confirmed cytometry findings as a more robust phagocytic activity was found among elotuzumab opsonized tumor cells compared to hIgG1 or the mutant Fc-inert variant of elotuzumab (Fig. 5c). Notably, macrophages that were activated with elotuzumab demonstrated the ability to phagocytose multiple cells under confocal microscopy.

Discussion

Among the most abundant innate immune cells in the tumor microenvironment, TAMs are well recognized as key regulators of tumor progression and metastasis. [30] Clinically, TAMs and their associated markers have been correlated with MM disease progression and proposed as prognostic biomarkers. [31, 32] However, regarded as dysregulated immunosuppressive cells, several immunomodulatory therapies focus on the reprogramming of TAMs to harness their anti-tumor potential. [33, 34, 35] Within the realm of MM, immunotherapy has demonstrated its ability to re-educate TAMs and stimulate anti-tumor activity of macrophages in a refractory/relapsed mouse model of myeloma. [36]

Monoclonal antibody immunotherapy has been shown capable of not only targeting specific surface cellular antigens but also engaging effector immune cells through the Fc-Fc γ R interaction. Engaging the Fc γ R results in the induction of effector immune functions such as ADCC and ADCP among NK cells and macrophages. [10, 37] Previously underestimated, this mechanism of action is gaining wide interest given the increased dependence on immune modulating mAbs in the therapeutic armamentarium against cancer.

Elotuzumab is among the first monoclonal therapies approved for the treatment of multiple myeloma. Aside from NK cell activation via SLAMF7, the other aspect of elotuzumab's dual effect on NK cells comprises inducing Fc γ RIII mediated ADCC against myeloma cells.

[6] Although well described for NK cells, the Fc γ R mediated mechanisms of action of elotuzumab remain to be explored among other effector cells in the tumor microenvironment. Our work presented herein proposes a new mechanism of action of elotuzumab whereby Fc γ R activation of tumor associated macrophages promotes tumor clearance by ADCP. Using the myeloma xenograft model in SCID-beige mice, we showed the central role of intact Fc region of elotuzumab in inhibiting tumor progression and promoting mouse survival. The use of similar model among NSG mice that lack functional macrophages abrogated these beneficial effects. Furthermore, using syngeneic tumor models expressing hSLAMF7 in immunocompetent mice where macrophages and NK cells were selectively depleted, we show that elotuzumab exerts its anti-tumor effect comparably through NK cells and macrophages. Since elotuzumab specifically binds to human SLAMF-7, the effects seen in the *in-vivo* mouse models were isolated to Fc-Fc γ R interactions of the mAb with mouse effector cells. Therefore, contributory effects of SLAMF7 stimulation of effector cells were not feasible to assess in the current study.

Macrophage accumulation at the tumor sites was also augmented through elotuzumab treatment in a dose dependent manner. This can be due to the involvement of macrophage chemoattractants such as MIP-1- α , MCP-1, and MIP-2. [38, 39] Phenotypically, these macrophages exhibited an activated phenotype evidenced by increase in CD86 expression, a hallmark of classically activated M1 macrophages with LPS and IFN- γ . [28] Known to be increased in the presence of IFN- γ [40], PD-L1 expression was also upregulated upon treatment with elotuzumab. Hence, given the co-expression of CD86 and PD-L1, elotuzumab potentially primes the tumor microenvironment with an IFN- γ predominant inflammatory milieu. Although PD-L1 has been associated with an immunosuppressive function of TAMs, its expression corresponds primarily to a negative feedback mechanism aiming to decrease the inflammatory response. [41] Notably, the upregulation of PD-L1 expression on TAMs has been shown to be correspond to higher B and T lymphocyte tumor infiltration. [42, 43, 44, 45, 46] Therefore, this points to potential future benefit of elotuzumab and adjunctive PD1/PD-L1 axis blockade in the treatment of myeloma.

The engagement of human SLAMF7 by elotuzumab holds promise in augmenting the potential Fc-Fc γ R interactions among human effector cells. Transcriptome analysis of the human macrophage surfaceome by Beyer et al has shown differentially higher expression of SLAMF7 among anti-tumor M1 macrophages as compared to M2 macrophages. [47] Also, the expression of SLAMF7 among human M1 macrophages has also been demonstrated in intestinal allografts undergoing rejection. [48] Recently, upon SIRP α -CD47 blockade, SLAMF7 has been shown to play a critical role in the phagocytosis of hematologic tumor cells by macrophages. [49] Given the overexpression of CD47 in several tumor types including myeloma, synergistic blockade of the SIRP α -CD47 axis along with a tumor-directed mAb treatment is a potentially promising strategy to promote phagocytosis effector functions. [13, 16] In the current study, the biologic effects of elotuzumab treatment in the mouse models were due to Fc-Fc γ R interaction given the binding specificity of the mAb to human SLAMF7. Therefore, engaging the SLAMF7 signaling pathway is anticipated to potentially augment the Fc γ R pro-phagocytic properties of elotuzumab. The dual phagocytosis promoting mechanisms of elotuzumab through engagement of SLAMF7 and the Fc γ R might also be further improved by blockade of the SIRP α -CD47 blockade and

warrant future studies. Given that elotuzumab is most clinically efficacious in combination with lenalidomide and dexamethasone, further work is needed to characterize the role of the Fc γ R pro-phagocytic properties of elotuzumab in combination with immunomodulatory therapies.

In summary, our study demonstrates the ability of elotuzumab to promote macrophage mediated anti-myeloma phagocytic activity through engaging the Fc γ R. In addition to increasing macrophage accumulation at the tumor site, we show that elotuzumab increases macrophage activation. This novel mechanism of immune mediated anti-myeloma activity may help to further define useful combination strategies for this immunotherapy in MM as well as precursor MM states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kyle RA, Rajkumar SV. Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma. *Leukemia*. 2009; 23:3–9. [PubMed: 18971951]
2. Rajkumar SV. Treatment of multiple myeloma. *Nat Rev Clin Oncol*. 2011; 8:479–491. [PubMed: 21522124]
3. Yang Y. Cancer immunotherapy: harnessing the immune system to battle cancer. *J Clin Invest*. 2015; 125:3335–3337. [PubMed: 26325031]
4. Weiner LM, Dhodapkar MV, Ferrone S. Monoclonal antibodies for cancer immunotherapy. *Lancet*. 2009; 373:1033–1040. [PubMed: 19304016]
5. Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. *Nat Rev Cancer*. 2012; 12:278–287. [PubMed: 22437872]
6. Lonial S, Dimopoulos M, Palumbo A, White D, Grosicki S, Spicka I, et al. Elotuzumab Therapy for Relapsed or Refractory Multiple Myeloma. *N Engl J Med*. 2015; 373:621–631. [PubMed: 26035255]
7. Tai Y-T, Dillon M, Song W, Leiba M, Li XF, Burger P, et al. Anti-CS1 humanized monoclonal antibody HuLuc63 inhibits myeloma cell adhesion and induces antibody-dependent cellular cytotoxicity in the bone marrow milieu. *Blood*. 2008; 112:1329–1337. [PubMed: 17906076]
8. Hsi ED, Steinle R, Balasa B, Szmania S, Draksharapu A, Shum BP, et al. CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma. *Clin Cancer Res*. 2008; 14:2775–2784. [PubMed: 18451245]
9. Guo H, Cruz-Munoz ME, Wu N, Robbins M, Veillette A. Immune cell inhibition by SLAMF7 is mediated by a mechanism requiring src kinases, CD45, and SHIP-1 that is defective in multiple myeloma cells. *Mol Cell Biol*. 2015; 35:41–51. [PubMed: 25312647]

10. Nimmerjahn F, Ravetch JV. Fc gamma receptors as regulators of immune responses. *Nat Rev Immunol.* 2008; 8:34–47. [PubMed: 18064051]
11. Veillette A, Guo H. CS1, a SLAM family receptor involved in immune regulation, is a therapeutic target in multiple myeloma. *Crit Rev Oncol Hematol.* 2013; 88:168–177. [PubMed: 23731618]
12. Asimakopoulos F, Kim J, Denu RA, Hope C, Jensen JL, Ollaret SJ, et al. Macrophages in multiple myeloma: emerging concepts and therapeutic implications. *Leuk Lymphoma.* 2013; 54:2112–2121. [PubMed: 23432691]
13. Weiskopf K, Weissman IL. Macrophages are critical effectors of antibody therapies for cancer. *MAbs.* 2015; 7:303–310. [PubMed: 25667985]
14. Gül N, van Egmond M. Antibody-dependent phagocytosis of tumor cells by macrophages: a potent effector mechanism of monoclonal antibody therapy of cancer. *Cancer Res.* 2015; 75:5008–5013. [PubMed: 26573795]
15. Shi Y, Fan X, Deng H, Brezski RJ, Ryczyn M, Jordan RE, et al. Trastuzumab triggers phagocytic killing of high HER2 cancer cells *in vitro* and *in vivo* by interaction with Fcγ receptors on macrophages. *J Immunol.* 2015; 194:4379–4386. [PubMed: 25795760]
16. Chao MP, Alizadeh AA, Tang C, Myklebust JH, Varghese B, Gill S, et al. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell.* 2010; 142:699–713. [PubMed: 20813259]
17. Overdijk M, Verploegen S, Bögels M, van Egmond M, Lammerts van Bueren J, Mutis T, et al. Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma. *MAbs.* 2015; 7:311–321. [PubMed: 25760767]
18. Bezman NA, Jhatakia A, Kearney AY, Brender T, Maurer M, Henning K, et al. PD-1 blockade enhances elotuzumab efficacy in mouse tumor models. *Blood Advances.* 2017; 1:753–765. [PubMed: 29296719]
19. Kasai M, Yoneda T, Habu S, Maruyama Y, Okumura K, Tokunaga T. In vivo effect of anti-asialo GM1 antibody on natural killer activity. *Nature.* 1981; 291:334–335. [PubMed: 7231554]
20. MacDonald KP, Palmer JS, Cronau S, Seppanen E, Olver S, Raffelt NC, et al. An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation. *Blood.* 2010; 116:3955–3963. [PubMed: 20682855]
21. Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol.* 1989; 24:148–154. [PubMed: 2544306]
22. Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. *Curr Protoc Immunol.* 2008 Chapter 14:Unit 14.1.
23. Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. *Nature.* 1983; 301:527–530. [PubMed: 6823332]
24. Roder J, Duwe A. The beige mutation in the mouse selectively impairs natural killer cell function. *Nature.* 1979; 278:451–453. [PubMed: 313007]
25. Joliat MJ, Shultz LD. The molecular bases of spontaneous immunological mutations in the mouse and their homologous human diseases. *Clin Immunol.* 2001; 101:113–129. [PubMed: 11683570]
26. Grugan KD, McCabe FL, Kinder M, Greenplate AR, Harman BC, Ekert JE, et al. Tumor-associated macrophages promote invasion while retaining Fc-dependent anti-tumor function. *J Immunol.* 2012; 189:5457–5466. [PubMed: 23105143]
27. Shultz LD, Schweitzer PA, Christianson SW, et al. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol.* 1995; 154:180–191. [PubMed: 7995938]
28. Wang C, Yu X, Cao Q, Wang Y, Zheng G, Tan TK, et al. Characterization of murine macrophages from bone marrow, spleen and peritoneum. *BMC Immunol.* 2013; 14:6. [PubMed: 23384230]
29. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of Programmed Death 1 Ligands by Murine T Cells and APC. *J Immunol.* 2002; 169:5538–5545. [PubMed: 12421930]
30. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell.* 2010; 141:39–51. [PubMed: 20371344]

31. Suyan E, Sucak GT, Akyürek N, Sahin S, Baysal NA, Yaıcı M, et al. Tumor-associated macrophages as a prognostic parameter in multiple myeloma. *Ann Hematol.* 2013; 92:669–677. [PubMed: 23334187]
32. Andersen MN, Abildgaard N, Maniecki MB, Møller HJ, Andersen NF. Monocyte/macrophage-derived soluble CD163: A novel biomarker in multiple myeloma. *Eur J Haematol.* 2014; 93:41–47. 16. [PubMed: 24612259]
33. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity.* 2014; 41:49–61. [PubMed: 25035953]
34. Gutiérrez-González A, Martínez-Moreno M, Samaniego R, Arellano-Sánchez N, Salinas-Muñoz L, Relloso M, et al. Evaluation of the potential therapeutic benefits of macrophage reprogramming in multiple myeloma. *Blood.* 2016; 128:2241–2252. [PubMed: 27625360]
35. Georgoudaki AM, Prokopec KE, Boura VF, Hellqvist E, Sohn S, Östling J, et al. Reprogramming tumor-associated macrophages by antibody targeting inhibits cancer progression and metastasis. *Cell Rep* 2016; 15:2000–2011.
36. Jensen JL, Rakhmilevich A, Heninger E, Broman AT, Hope C, Phan F, et al. Tumoricidal Effects of Macrophage-Activating Immunotherapy in a Murine Model of Relapsed/Refractory Multiple Myeloma. *Cancer Immunol Res.* 2015; 3:881–90. [PubMed: 25941352]
37. Barnhart BC, Quigley M. Role of Fc–FcγR interactions in the antitumor activity of therapeutic antibodies. *Immunol Cell Biol.* 2017; 95:340–346. [PubMed: 27974746]
38. Roussou M, Tasidou A, Dimopoulos MA, Kastritis E, Migkou M, Christoulas D, et al. Increased expression of macrophage inflammatory protein-1α on trephine biopsies correlates with extensive bone disease, increased angiogenesis and advanced stage in newly diagnosed patients with multiple myeloma. *Leukemia.* 2009; 23:2177–2181. [PubMed: 19587705]
39. Zhu EF, Gai SA, Opel CF, Kwan BH, Surana R, Mihm MC, et al. Synergistic innate and adaptive immune response to combination immunotherapy with anti-tumor antigen antibodies and extended serum half-life IL-2. *Cancer Cell.* 2015; 27:489–501. [PubMed: 25873172]
40. Kondo A, Yamashita T, Tamura H, Zhao W, Tsuji T, Shimizu M, et al. Interferon-γ and tumor necrosis factor-α induce an immunoinhibitory molecule, B7-H1, via nuclear factor-κB activation in blasts in myelodysplastic syndromes. *Blood.* 2010; 116:1124–1131. [PubMed: 20472834]
41. Chen L, Han X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J Clin Invest.* 2015; 125:3384–3391. [PubMed: 26325035]
42. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. *Nat Med.* 2002; 8:793–800. [PubMed: 12091876]
43. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of Inflammatory Response with B7-H1 Expression in Human Melanocytic Lesions Supports an Adaptive Resistance Mechanism of Immune Escape. *Sci Transl Med.* 2012; 4:127ra37.
44. Schalper KA, Carvajal-Hausdorf D, McLaughlin J, Velcheti V, Chen L, Sanmamed M, et al. Clinical significance of PD-L1 protein expression on tumor-associated macrophages in lung cancer. *J Immunother Cancer.* 2015; 3:P415.
45. Webb JR, Milne K, Kroeger DR, Nelson BH. PD-L1 expression is associated with tumor-infiltrating T cells and favorable prognosis in high-grade serous ovarian cancer. *Gynecol Oncol.* 2016; 141:293–302. [PubMed: 26972336]
46. Kim HR, Ha SJ, Hong MH, Heo SJ, Koh YW, Choi EC, et al. PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients. *Sci Rep.* 2016; 6:36956. [PubMed: 27841362]
47. Beyer M, Mallmann MR, Xue J, Staratschek-Jox A, Vorholt D, Krebs W, et al. High-Resolution Transcriptome of Human Macrophages. *PLoS ONE.* 2012; 7:e45466. [PubMed: 23029029]
48. Ashokkumar C, Ningappa M, Ranganathan S, Higgs BW, Sun Q, Schmitt L, et al. Increased expression of peripheral blood leukocyte genes implicate CD14+ tissue macrophages in cellular intestine allograft rejection. *Am J Pathol.* 2011; 179:1929–1938. [PubMed: 21854741]
49. Chen J, Zhong MC, Guo H, Davidson D, Mishel S, Lu Y, et al. SLAMF7 is critical for phagocytosis of haematopoietic tumour cells via Mac-1 integrin. *Nature.* 2017; 544:493–497. [PubMed: 28424516]

Figure 1. Elotuzumab inhibits tumor progression and promotes mouse survival in early and late myeloma xenograft models

(a) Weekly ventral BLI acquired over the course of 6 weeks of SCID-beige mice ($n=8$ per group) injected i.v. with 5 million MM1S GFP⁺ Luc⁺ cells. Treatment in the early xenograft model was initiated 48 hours later and comprised the administration of 10mg/kg of hIgG1 or elotuzumab i.p. twice weekly. (b) Early xenograft model Kaplan-Meier survival analysis showing significantly prolonged survival among elotuzumab treated mice (mean survival= 87 days) compared to hIgG1 treated group (mean survival=38 days) $P<0.001$. (c) Ventral BLI imaging at week 5 of late xenograft model where mice ($n=6-7$ per group) were randomized into the two treatment groups upon showing first positive BLI signal at week 2. A total of 5 doses of 10mg/kg of elotuzumab or hIgG1 were administered resulting in significant decrease in BLI intensity. (d) Late xenograft model Kaplan-Meier survival analysis showing significantly prolonged survival among elotuzumab treated mice (mean survival= 58.5 days) compared to hIgG1 treated group (mean survival=38 days) $P= 0.027$.

Figure 2. Elotuzumab induces $Fc\gamma R$ mediated immunostimulation of macrophages

(a) Weekly ventral BLI acquired over the course of 6 weeks of SCID-beige mice (n=5 per group) injected i.v. with 5 million MM1S GFP⁺ Luc⁺ cells. Treatment in the early xenograft model was initiated 48 hours later and comprised the administration of 10mg/kg of hIgG1, Fc-inert variant of elotuzumab, or elotuzumab i.p. twice weekly. (b) Early xenograft model Kaplan-Meier survival analysis showing significantly prolonged survival among elotuzumab treated mice (mean survival= 50 days) compared to hIgG1 and Fc-inert variant of elotuzumab treated groups (mean survival=38 and 36 days respectively) $P<0.001$. (c) Dorsal BLI imaging of early tumor xenograft model among NSG mice (n=3 per group) treated with elotuzumab or its Fc-inert variant at dose of 10mg/kg administered i.p. twice per week.

Figure 3. Elotuzumab promotes recruitment of macrophages capable of mediating a potent anti-tumor effect comparable to that of NK cells

(a) Tumor growth curves of EG7-hSLAMF7 injected subcutaneously to C57Bl/6j mice ($n=8$ per group). Where indicated, mice were either depleted of macrophages (anti-CSF1R antibody) or NK cells (asialo GM1 antibody) and concurrently received elotuzumab-g2a or mIgG2a isotype control treatment. Analysis of the median tumor volume among the different treatment groups was conducted on day 18 post treatment. Mice receiving elotuzumab treatment (group: elotuzumab-g2a) had the significant reduction in median tumor volume (111.25 mm³) compared to that of isotype control treated group (mIgG2a) (667.17 mm³, $P=0.028$). NK cell depleted mice that concurrently received elotuzumab (group: elotuzumab-g2a + asialo GM1) demonstrated a significant reduction in tumor volume (686.56 mm³) compared to mice that were only depleted of NK cells (group: asialo

GM1, median tumor volume= 1,264.07 mm³, $P=0.005$) signifying the involvement of other effector cells in mediating anti-tumor effects of elotuzumab. A similar reduction of tumor volume among macrophage depleted mice treated with elotuzumab (group: elotuzumab + CSF-1R, 669.51 mm³) compared to macrophage depleted mice (group: CSF1R, 954.56 mm³) that was not statistically significant. The depletion of macrophages in elotuzumab treated mice (group: elotuzumab-g2a + CSF1R, median tumor volume = 669.51 mm³) abrogated the elotuzumab induced reduction in median tumor volume in immunocompetent mice (group: elotuzumab-g2a, 111.25 mm³, $P=0.037$). NK cell depletion also resulted in a comparable loss of elotuzumab efficacy (686.56 mm³, $P=0.037$). **(b)** Among tumor infiltrating leukocytes (TIL), the percentage of F4/80⁺ macrophages was analyzed 11 days after treatment initiation among mice treated with elotuzumab or isotype control (n= 7-8 mice per group) with and without antibody based depletion of CSF1R bearing macrophages (n= 3 mice per group). There is a 1.59-fold increase in the percentage of macrophages accumulating at the tumor site among mice treated with elotuzumab compared to isotype control ($P=0.042$). Efficacious depletion of macrophages using CSF1R depleting antibody among mice that received treatment with elotuzumab or isotype control is demonstrated with the significant reduction in F4/80⁺ populations ($P=0.026$ and $P=0.009$ respectively). Statistical significance was determined by * $P<0.05$, ** $P<0.01$ by two-tailed nonparametric Mann Whitney U test (A) and one-tailed unpaired t-test (B). Bars on day 18 represent median $\pm$ deviation

Figure 4. Dose dependent accumulation and phenotypic activation of TAMs by elotuzumab
(a) Individual tumor growth curves of subcutaneously injected OPM2 cells in SCID mice (n= 10 per group) randomized for hIgG1 or elotuzumab (0.5mg/kg or 1mg/kg) daily treatment for a total of 5 doses. **(b, c)** Flow cytometry analysis of TAMs and spleen macrophages at days 1 and 8 post treatment (n=3-5 per group per timepoint) for the expression of F4/80⁺ macrophages and CD86 and PD-L1 surface markers. **(b)** TAMs from elotuzumab (1mg/kg) treated mice showed statistically significant increase in the percentage of F4/80⁺ macrophages ($P=0.007$) expressing CD86 ($P=0.003$) and PD-L1 ($P=0.018$) 1 day after treatment compared to the hIgG1 control. A dose dependent response was also noted with the 1mg/kg elotuzumab dosed group showing a statistically significant increase in the percentage of F4/80⁺TAMs expressing CD86 ($P=0.009$) and PD-L1. While the increase in F4/80⁺ TAM percentage was not sustained till day 8 after treatment, phenotypic changes were preserved. Also, the percentage of CD86 and PD-L1 expressing macrophages became comparable among groups receiving elotuzumab at both dosages. **(c)** Among macrophages isolated from the spleen, elotuzumab treated mice did not demonstrate an increase in the percentage of F4/80⁺ macrophages or the percentage of CD86⁺ or PD-L1⁺ macrophages compared to the hIgG1 control. The analysis of marker expression on days 1 and day 8 was separately normalized to the hIgG1 control of the same day experiment. The data is

representative of two independent experiments and statistical significance was determined by $*P<0.05$, $**P<0.001$ by two tailed unpaired t-test.

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Figure 5. Flow cytometry and confocal microscopy reveal Fc γ R mediated ADCP of myeloma cells

(a) ADCP flow cytometry analysis of undifferentiated TAMs and M1 polarized TAMs that were co-cultured with MM1S GFP⁺ Luc⁺ opsonized with hIgG1, Fc-inert variant of elotuzumab, or elotuzumab. The double positive quadrant denotes the percentage of phagocytosis after 18-24 hrs of co-culture. (b) Bar graph representation of ADCP from 3 independent experiments. M1 polarized macrophages with LPS and IFN γ show significant increase in Fc γ R dependent phagocytosis of myeloma cells opsonized with elotuzumab as compared to hIgG1 and Fc-inert variant of elotuzumab. Despite treatment with elotuzumab, ADCP was significantly reduced among unpolarized TAMs. (c) Confocal microscopy of M1 polarized TAMs stained for F4/80 (Alexa Fluor 594) co-cultured with MM1S GFP⁺ Luc⁺ opsonized with hIgG1, Fc-inert variant of elotuzumab, or elotuzumab. Robust internalization of multiple elotuzumab opsonized myeloma cells by M1 polarized macrophages is shown compared to hIgG1 and Fc-inert elotuzumab treated cells. Indicated calibration bars corresponding to 30 μ m. Data represents three independent experiments and the bars represent the mean $\pm$ S.E.M. * P <0.05, ** P <0.01 by two-tailed unpaired t-test.