Clinical Cancer Research

Targeting CD38 Suppresses Induction and Function of T Regulatory Cells to Mitigate Immunosuppression in Multiple Myeloma

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Abstract

Purpose: We study CD38 levels in immunosuppressive $CD4^+CD25^{high}Foxp3^+$ regulatory T cells (Treg) and further define immunomodulating effects of a therapeutic CD38 mAb isatuximab/SAR650984 in multiple myeloma.

Experimental Design: We evaluated percentages of CD38-expressing subsets in Tregs from normal donors and multiple myeloma patients. Peripheral blood mononuclear cells (PBMC) were then treated with isatuximab with or without lenalidomide or pomalidomide to identify their impact on the percentage and immunosuppressive activity of Tregs on $CD4^+CD25^-$ T cells (Tcons). We investigated the mechanism of increased Tregs in multiple myeloma patients in *ex vivo* cocultures of multiple myeloma cells with PBMCs or Tcons.

Results: CD38 expression is higher on Tregs than Tcons from multiple myeloma patients versus normal donors. CD38 levels and the percentages of $CD38^{high}$ Tregs are increased by lenalidomide and pomalidomide. Isatuximab preferentially decreases Treg and increases Tcon frequencies, which is enhanced by pomalidomide/lenalidomide. Isatuximab reduces Foxp3 and IL10 in Tregs and restores proliferation and function of Tcons. It augments multiple myeloma cell lysis by $CD8^+$ T and natural killer cells. Coculture of multiple myeloma cells with Tcons significantly induces Tregs (iTregs), which express even higher CD38, CD25, and FoxP3 than natural Tregs. This is associated with elevated circulating $CD38^+$ Tregs in multiple myeloma patients versus normal donors. Conversely, isatuximab decreases multiple myeloma cell- and bone marrow stromal cell–induced iTreg by inhibiting both cell–cell contact and TGFβ/IL10. Finally, CD38 levels correlate with differential inhibition by isatuximab of Tregs from multiple myeloma versus normal donors.

Conclusions: Targeting CD38 by isatuximab can preferentially block immunosuppressive Tregs and thereby restore immune effector function against multiple myeloma. *Clin Cancer Res; 23(15); 4290–300. ©2017 AACR.*

Introduction

mAbs targeting SLAMF7 and CD38 have become available to treat relapsed/refractory multiple myeloma. Specifically, the first CD38 mAb daratumumab was approved in 2015 to treat relapsed/refractory multiple myeloma (1) and is effective as a monotherapy (2, 3). Isatuximab/SAR650984 (4), another therapeutic CD38 mAb currently under clinical development, also shows significant clinical activity in heavily pretreated patients with relapsed/refractory multiple myeloma, both as a monotherapy and combined with lenalidomide/dexamethasone (5). In addition to Fc-dependent cytotoxicity mediated by IgG_1-based CD38 mAbs, isatuximab induces direct killing of p53-mutated multiple myeloma cells expressing high levels of CD38 in *ex vivo* cultures without effector cells and Fc cross-linking reagents (6). Isatuximab significantly kills $CD38^{high}$-expressing multiple myeloma cells via induction of homotypic aggregation, leakage of lysosome-associated cathepsin B and lysosomal-associated membrane protein-1 (LAMP-1), and generation of reactive oxygen species (6). Furthermore, apoptosis is significantly enhanced when isatuximab is combined with pomalidomide/lenalidomide (6). As CD38 is widely expressed on hematopoietic cells, it is important to study whether isatuximab also has impact on these cells. To date, the effects of isatuximab on CD38-expressing immune cells and modulation of immune function is not defined.

Regulatory T cells (Treg) play a crucial role in immune surveillance by suppressing activation, expansion, and function of target cells including $CD4^+CD25^-$ conventional T cells (Tcons), cytotoxic $CD8^+$ T cells, as well as natural killer (NK) cells (7). They inhibit both cellular and humoral immune responses (8, 9). Two forms of Tregs, "natural" and "induced," have been reported (10, 11). Naturally occurring Tregs (nTreg), which constitute 5%–10% $CD4^+$ lymphocytes, originate in the thymus and disseminate to periphery. Induced Tregs (iTreg) are generated in the periphery by soluble cytokines and cell–cell contact (10–12).

In parallel, tumor cells have the capacity to avoid immune recognition, to induce immune cell dysfunction, and to escape from immune surveillance via Tregs (9, 13). The proportions of

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

CD38 mAb isatuximab, as a monotherapy and in combinational use, have shown significant clinical activities against human multiple myeloma. Here we characterize that CD38 levels are higher in $CD4^+CD25^{high}Foxp3^+$ regulatory T cells (Treg) versus Tcon ($CD4^+CD25^-$), and multiple myeloma patients have increased percentages of $CD38^{high}$ Tregs than normal donors. Importantly, isatuximab inhibits suppressive function of Tregs highly expressing CD38 by decreasing their percentages, reducing immune inhibitory cytokines (TGFβ, IL10), and blocking their trafficking. Isatuximab further enhances NK- and $CD8^+$ T effector cell–mediated antitumor immune responses, which can be further enhanced by immunomodulatory drugs (IMiDs). In *ex vivo* cocultures, multiple myeloma cells significantly induce functional Tregs (iTreg) highly expressing CD38 and suppressing Tcons. Furthermore, increased circulating iTregs in multiple myeloma patients are derived from Tcons in both cell–cell contact-dependent and -independent manners. Importantly, isatuximab blocks these processes. Targeting CD38 with isatuximab may therefore induce immunomodulatory effects, which both relieve immunosuppression and trigger anti-multiple myeloma immunity.

Tregs are elevated in the circulation of patients with solid tumors and hematologic malignancies (14–19). Increasing levels of Tregs often correlate with tumor burden and disease progression (16, 19–22). Accumulation of Treg in the tumor microenvironment is associated with reduced survival (15, 17, 18, 23). Moreover, antitumor immune responses are enhanced in animal models after Treg depletion using mAbs against surface markers highly expressed on Tregs, that is, CD25 (24), CTLA-4 and OX40 (25–27), and in transgenic DEREG mice (28). Thus, Tregs present a promising therapeutic target to restore antitumor immune responses.

CD38 is expressed on several lineages of hematopoietic cells including Tregs, and its levels correlate with the suppressive function of Tregs. CD38-knockout mice present with a loss of $Foxp3^+$ Tregs (29, 30). Conversely, high CD38 expression may define a highly suppressive subset of Tregs (31–33). We here characterize the role of CD38 in Treg inhibitory function both on Tcons and other immune effector cells in multiple myeloma. We then show that isatuximab, alone or with lenalidomide/pomalidomide, modulates the frequency and function of Tregs dependent on CD38, thereby restoring immune effector function in multiple myeloma.

Materials and Methods

Cell lines, medium, and reagents

Human multiple myeloma cell lines (U266, RPMI8226) were cultured in mycoplasma-free condition and maintained in complete culture medium (RPMI1640 medium supplemented by 10% FBS, 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin) in ventilated tissue culture flasks at 37°C in a 5% CO_2 humidified incubator.

Peripheral blood mononuclear cells (PBMC) were collected from fresh buffy coat of healthy donors and multiple myeloma patients after informed consent, in accordance with the Declaration of Helsinki and under the auspices of a Dana-Farber Cancer Institute Institutional Review Board–approved protocol. PBMCs were expanded in complete culture medium with 20 IU/mL rIL2 (Miltenyi Biotec). Isatuximab and its F(ab)'2 fragments were obtained from Sanofi (4, 6). Lenalidomide/pomalidomide were purchased from Selectchem, anti-PD1/PD-L1 mAb from Biolegend, and Mitomycin C from Sigma-Aldrich.

Proliferation assay for Tregs and Tcons

PBMCs from normal donors were pretreated with or without 1 μmol/L lenalidomide/pomalidomide for 3 days, stained by 5 μmol/L Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen), and then plated in the presence or absence of indicated doses of isatuximab. After 5-day incubation, proliferating Tregs and Tcons were identified as CFSE-diluted subsets in $CD4^+CD25^{high}Foxp3^+$ Tregs and $CD4^+CD25^-$ Tcons, respectively. Unlabeled cells were used as a control.

Phenotyping and FACS analyses

Antibodies used for flow cytometry were as follows: CD4-Pacific Blue, CD25-PE, CD25-APC, CD127-FITC, Foxp3-PE, CD38-PE-Cy7, AnnexinV-PE, PD1-APC, CD8-FITC, CTLA4-PE-Cy7, CD44-FITC, CD62L-FITC, ICOS-FITC, GITR-PE, OX40-FITC, CD138-FITC, PD-L1-PE, and their isotype-matched mAbs (all from Biolegend). Intracellular stainings of Foxp3, CTLA4, GITR, and OX40 were performed after fixation and permeabilization using cytofix/cytoperm kit (BD Biosciences), according to the manufacturer's protocol. Tregs were gated as $CD4^+CD25^{high}Foxp3^+$ cells in $CD4^+$ population and then sequential markers were assayed on Tregs, whereas $CD4^+CD25^-$ cells were identified as Tcons. The remaining $CD4^+CD25^{low}$/intermediate subset was excluded in the current study because of their limited immunosuppressive activity compared with $CD25^{high}$ population (34). To avoid the effect of permeabilization when apoptosis assay was performed, $CD4^+CD25^{high}CD127^{low/-}$ cells were identified as Tregs (35). All flow cytometry was performed by BD FACSCantoII, and analyzed on FlowJo software version 10 (Treestar).

Cell purification and immunosuppressive function assay

Tregs were purified using $CD4^+CD25^+$ Regulatory T cell Isolation Kit (Miltenyi Biotec). The purity of isolated population was >95%. Tcons were used as target cells in immunosuppressive assays. In brief, Tcons were cultured alone or with autologous Tregs in 96-well tissue culture plates in the presence of isatuximab, alone or with lenalidomide/pomalidomide, and stimulated with anti-CD3/CD28 beads (Miltenyi Biotec), according to the manufacturer's recommendation. Proliferation was measured by [^{3}H]-thymidine incorporation.

Activation of immune effector cells detected by degranulation (CD107a) and intracellular IFNγ production in response to multiple myeloma cells

PBMCs from normal donors or multiple myeloma patients were treated with serial doses of isatuximab and/or 1 μmol/L lenalidomide/pomalidomide for 2–3 days, followed by addition of multiple myeloma cells at effector:target (E:T) ratio of 10:1 together with CD107a antibody (36). After 1-hour incubation, protein transport inhibitors Brefeldin A and Monensin (BD Biosciences) were added for an additional 4 hours. Cells were then harvested and stained with surface markers (CD3-Pacific Blue, CD56-FITC, CD8-APC) and fixed/permeabilized,

followed by staining with anti-IFNγ mAb. All antibodies used were from Biolegend.

Ex vivo coculture in the generation of iTregs

Multiple myeloma cells were pretreated with mitomycin C to block their proliferation, followed by 3 washes. They were next cocultured with PBMCs or Tcons in tissue culture plates. PBMCs or Tcons alone were used as controls. Isatuximab was added into cocultures for 7 days, followed by FACS analysis, to determine the frequency and phenotype of Tregs. Supernatants were also collected for cytokine assessment.

Statistical analyses

Results are shown as means ± SEM or ranges, as appropriate. The Student *t* test was used to compare two groups. One-way ANOVA test was used to compare three or more groups. Two-way ANOVA test was used when there were two variables. Statistical analyses were carried out with Prism software (GraphPad Software, Inc). $P < 0.05$ was determined to be statistically significant.

Results

CD38 expression is higher on Tregs versus Tcons, and CD38^{high} subsets are increased on Tregs of multiple myeloma patients versus normal donors

We first examined CD38 expression on CD4$^+$CD25^{high}Foxp3$^+$ Tregs and CD4$^+$CD25$^-$ Tcons. Three subsets were seen in Tregs and Tcons: CD38 negative ($^-$), low, and high expression populations. In a representative sample, Tregs have higher percentages of CD38-expressing subsets with increased CD38 expression versus Tcons [63.8 % vs. 17.2%; median fluorescence intensity (MFI) of 466 vs. 55.7; Fig. 1A; Supplementary Fig. S1A]. Specifically, percentages of CD38^{high} subsets are increased in Tregs versus Tcons from PBMCs of 8 normal donors (Fig. 1B, left); conversely, frequencies of CD38$^{low/-}$ Tregs are decreased in Tregs compared with Tcons (Supplementary Fig. S1B, bottom). MFIs of CD38 are higher for Tregs versus Tcons (Supplementary Fig. S1C). Significantly, percentages of CD38$^+$ subset (Supplementary Fig. S1D and S1E) and CD38^{high} Tregs are increased in multiple myeloma patients ($n = 11$) versus normal donors ($n = 8$; Fig. 1B, right;

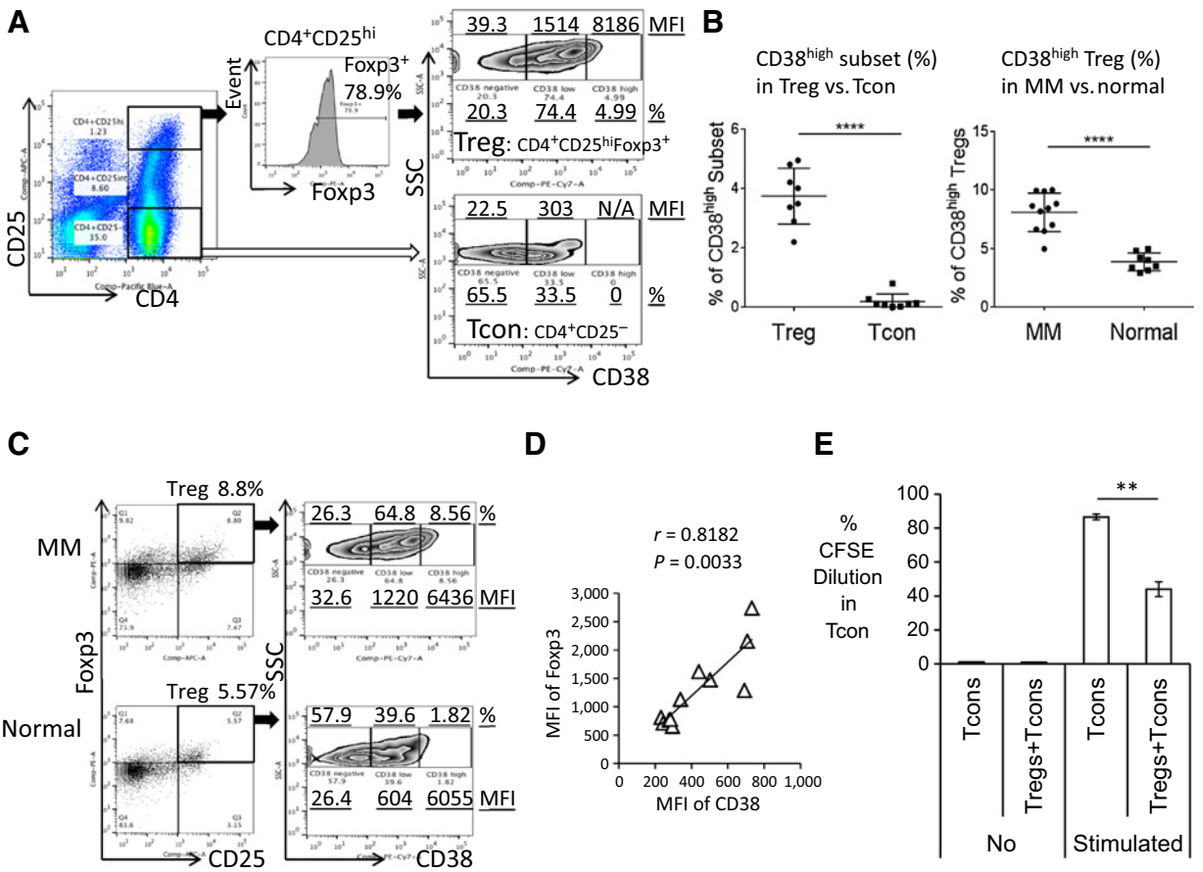

Figure 1.

CD38 level is higher on Treg versus Tcon and percentages of CD38^{high} subset in Tregs are significantly increased in multiple myeloma patients versus normal donors. **A,** The percentage and MFI of CD38^{high}, low, and negative (–) subsets were determined within CD4+CD25^{high}(hi)Foxp3$^+$Tregs (black arrow) and CD4$^+$CD25$^-$ Tcons (open arrow). **B,** Shown are summary (means ± SDs) of percentages of CD38^{high} on Tregs versus Tcons from PBMCs of 8 normal donors (left). Right, percentages of CD38^{high} on Tregs from 11 multiple myeloma patients and 8 normal donors. **C,** Shown are plots of CD38 on Tregs from a representative multiple myeloma patient and normal donor. **D,** Levels of CD38 correlate with Foxp3 in Tregs of 11 multiple myeloma patients. **E,** Treg inhibits proliferation, assessed by CFSE dilution in Tcons, of autologous Tcons from multiple myeloma patients. Stimulation is done using anti-CD3/CD28 beads. **, $P < 0.01$; ****, $P < 0.001$.

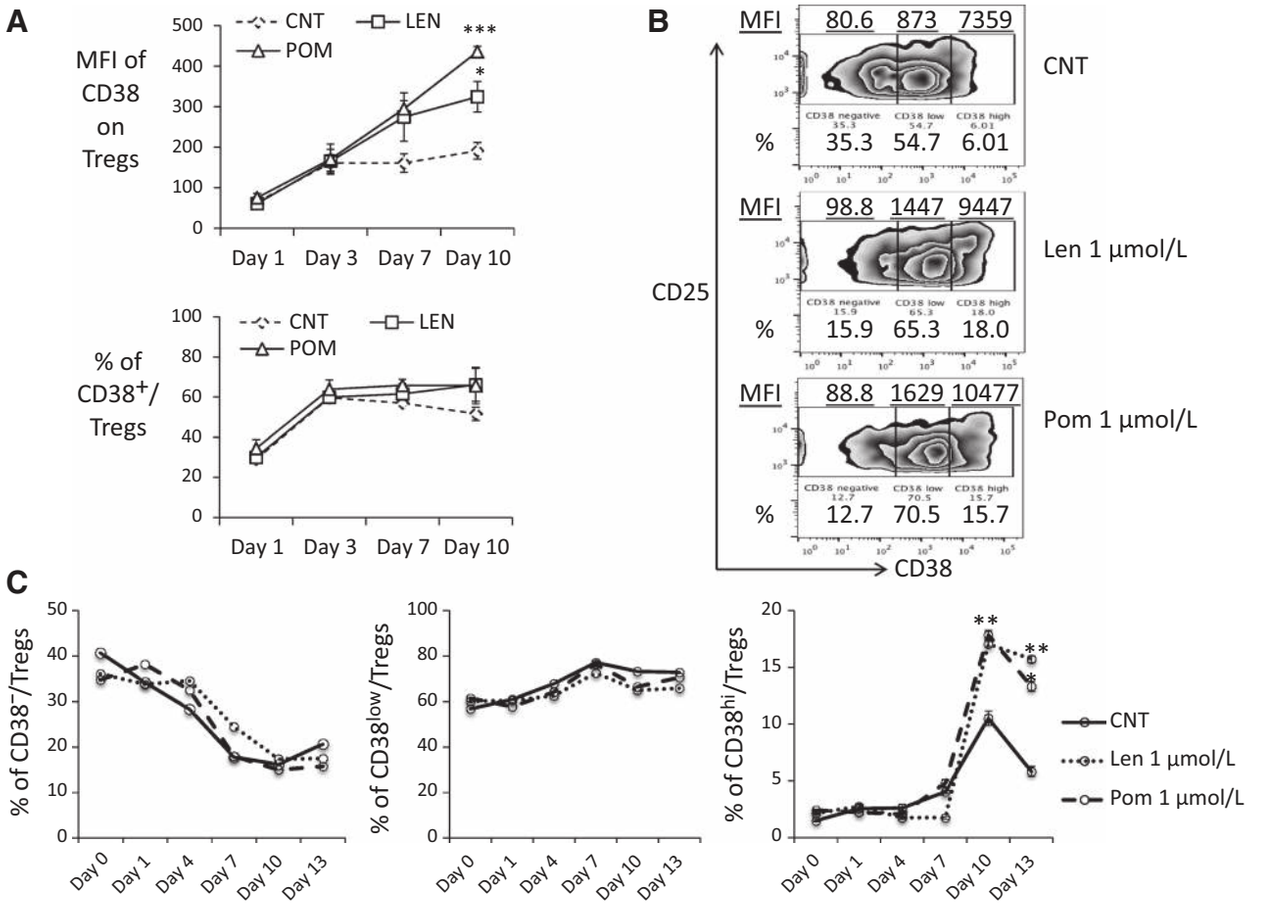

Figure 2.

CD38 expression is upregulated by IMiDs, associated with elevated CD38^{high} Tregs. **A,** PBMCs ($n = 3$) were incubated with lenalidomide (Len) or pomalidomide (Pom) followed by flow cytometry analysis for CD38 levels (MFI) on Tregs and percentage of CD38-expressing Tregs. Shown are means ± SEMs. **B,** Shown are representative plots of CD38-expressing subsets in gated CD4$^+$CD25^{hi}Foxp3$^+$ Tregs from a multiple myeloma patient treated with indicated drugs for 7 days. MFI (underlined) and frequency of CD38$^-$/low/high (hi) subsets are also indicated. **C,** Frequencies of CD38$^-$/low/high Tregs of multiple myeloma PBMCs were followed.

Fig. 1C). Levels of CD38 correlate with Foxp3 in Tregs of multiple myeloma patients ($n = 11$, Fig. 1D). Moreover, multiple myeloma patient Tregs inhibit proliferation of autologous Tcons, as demonstrated by significantly decreased percentages of CFSE dilution (Fig. 1E; Supplementary Fig. S1F).

CD38 expression on Tregs is upregulated by IMiDs

We next assessed the impact of lenalidomide/pomalidomide on CD38 expression on viable Tregs. Low-dose (1 µmol/L) lenalidomide and pomalidomide significantly increase MFI of CD38 on Tregs after 3 days persisting until 10 days; moreover, the higher percentage CD38$^+$ Tregs was maintained relative to control medium (Fig. 2A). In addition to higher CD38 on Tregs from multiple myeloma patients versus normal donors, lenalidomide or pomalidomide enhances cell surface CD38 on Tregs of multiple myeloma patients and normal donors (Supplementary Fig. S2). Specifically, lenalidomide or pomalidomide increases 2- to 3-fold the percentage of CD38^{high} subsets and the MFI of CD38 on Tregs in PBMCs from multiple myeloma patients (Fig. 2B and C). These results suggest that IMiDs may enhance the sensitivity of viable Tregs to isatuximab.

Isatuximab decreases Treg frequencies and inhibits Treg suppression of Tcon proliferation, which is enhanced by IMiDs

When PBMCs from normal donors ($n = 10$) were treated with or without isatuximab (1 µg/mL) for 3 days, the frequency of Tregs in CD4$^+$ cells was reduced from 8.88 ± 0.66% at baseline to 4.56 ± 0.77% ($P < 0.01$; Fig. 3A; Supplementary Fig. S3A). In contrast, Tcons are increased from 63.70 ± 7.51% to 75.83 ± 5.87% ($P < 0.05$), associated with decreased ratios of Tregs/Tcons. In multiple myeloma patient samples ($n = 6$), even 0.1 µg/mL of isatuximab decreases Tregs (16.35 ± 1.62% at baseline to 7.34 ± 1.78%, $P < 0.01$) and stimulates Tcons (from 69.03 ± 4.89% to 81.69 ± 2.55%, $P < 0.05$), associated with reduced Tregs/Tcons ratios (Fig. 3B; Supplementary Fig. S3B). Thus, higher CD38 expression on Tregs from multiple myeloma patients versus normal donors correlates with increased isatuximab inhibition of multiple myeloma Tregs. Notably, F(ab)'2 fragments of isatuximab are sufficient to decrease Tregs and stimulate Tcons (Fig. 3C; Supplementary Fig. S3C), confirming that the blockade of Tregs is CD38-specific and Fc-independent.

Pomalidomide alone, more potently than lenalidomide, reduces Tregs and stimulates Tcons (Fig. 3A and B; Supplementary

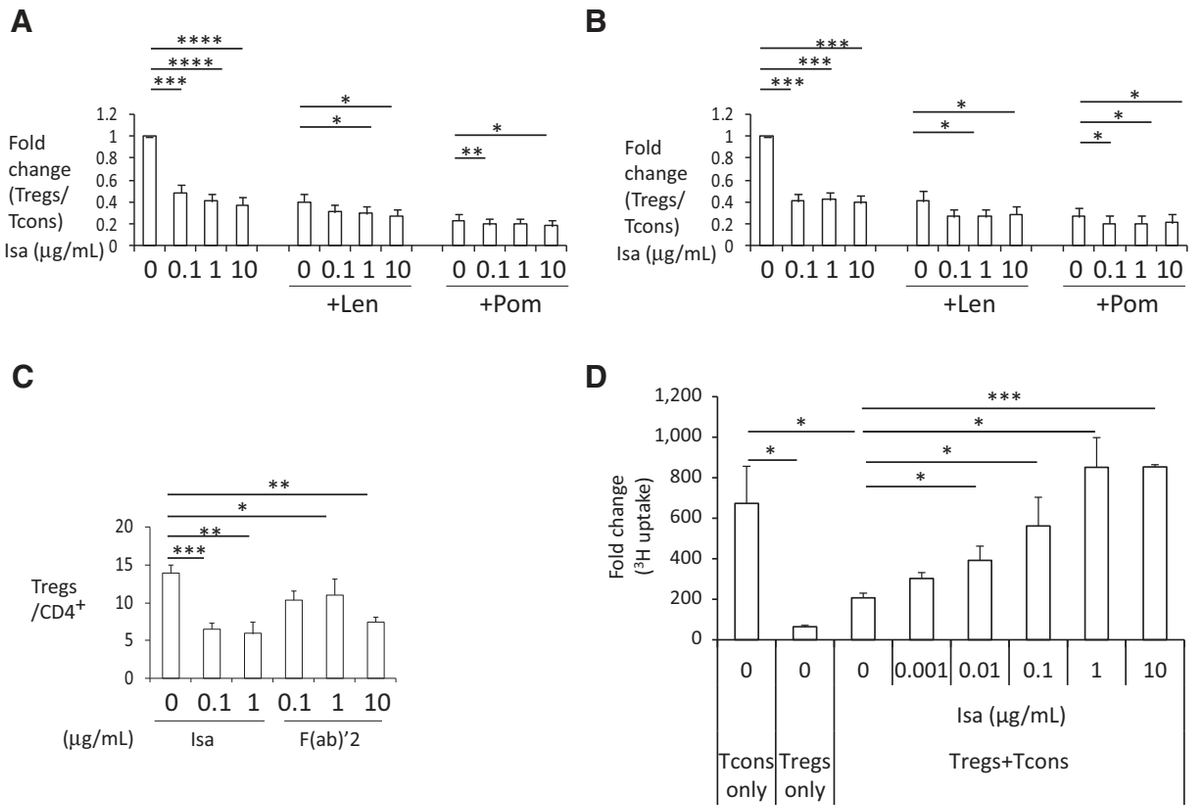

Figure 3.

Isatuximab reduces Treg frequencies and blocks Treg-inhibited proliferation of Tcons, which is enhanced by IMiDs. PBMCs from normal donors (**A**, $n = 10$) or multiple myeloma patients (**B**, $n = 7$) were pretreated with 1 μmol/L lenalidomide (Len) or pomalidomide (Pom) for 3–5 days followed by incubation with isatuximab (Isa), alone or with lenalidomide/pomalidomide, for another 3 days. Flow cytometry was used to determine the percentage of Tregs and Tcons in $CD4^+$ lymphocytes. Tregs/Tcons is then normalized to untreated controls (bottom) and fold changes (means ± SEMs) are shown. Untreated cells are used as controls in all the settings. **C,** PBMCs from 4 multiple myeloma patients were treated with either isatuximab (whole IgG_1) or its F(ab)'2 fragments and the percentage of Tregs is determined by flow cytometry. **D,** Purified Tcons were incubated with or without autologous Tregs isolated from PBMCs at 1:1 ratio for 5 days in the presence of anti-CD3/CD28 activation beads, with indicated concentrations of isatuximab. Proliferation was assessed by [^{3}H] thymidine incorporation added for the last 8 hours. Results are shown as fold changes relative to day 0. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Fig. S3A and S3B). Consistent with upregulation of CD38 on Tregs, pretreatment of PBMCs with lenalidomide/pomalidomide for 3–5 days enhances isatuximab-induced inhibition of Tregs from both normal donors and multiple myeloma patients, evidenced by further decreased Tregs/Tcons ratios (Fig. 3A and B).

We next determined whether isatuximab modulates the suppressive activity of Tregs on Tcons. Cocultures of Tregs with autologous Tcons diminished proliferation of Tcons from 100% to 27.35% ± 2.99%; conversely, isatuximab suppresses the inhibition of Tregs on Tcon proliferation in a dose-dependent manner (Fig. 3D; Supplementary Fig. S3D).

Isatuximab induces apoptosis and inhibits proliferation of Tregs

We next evaluated mechanisms of isatuximab-induced cytotoxicities against Tregs. Isatuximab (0.1 μg/mL) induces approximately 2-fold higher percentage annexin V^+ Tregs than control media (Fig. 4A and B, A, normal donors; B, multiple myeloma patients) or isotype IgG_1 (data not shown), which is further enhanced by lenalidomide or pomalidomide (Fig. 4A). The impact of isatuximab on the proliferation of Tregs was examined by staining PBMCs with CFSE for 5 days, followed by flow cytometry analysis gated on Tregs. Isatuximab decreases proliferation of Tregs in a dose-dependent manner, which is enhanced by pomalidomide more potently than lenalidomide (Fig. 4C). In contrast, lenalidomide or pomalidomide alone slightly induce apoptosis and significantly decrease proliferation of Tregs in *ex vivo* culture.

Using real-time qRT-PCR, isatuximab decreased Foxp3, a key immunosuppressive transcriptional factor, in Tregs from normal donors and multiple myeloma patients (Fig. 4D; Supplementary Fig. S4). Inhibitory cytokine IL10 is also reduced in isatuximab–treated Tregs (Fig. 4D).

Isatuximab enhances $CD8^+$ T and NK-mediated lysis of multiple myeloma cells, which is further enhanced by lenalidomide/pomalidomide

As Tregs also influence NK and $CD8^+$ T effector cells, we next assessed the effects of isatuximab on their function. PBMCs from 3 normal donors were treated for 3 days with isatuximab, with or without lenalidomide/pomalidomide (1 μmol/L), prior the addition of RPMI8226 multiple myeloma cells and flow cytometry

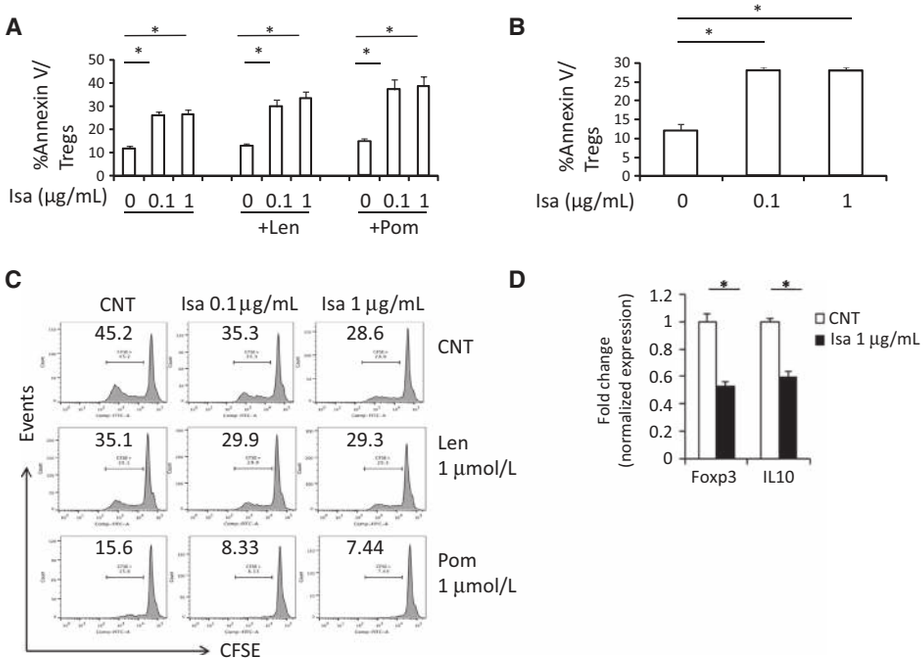

Figure 4.

Isatuximab (Isa) induces cytotoxicity and inhibits immunosuppressive molecules of Tregs. Isatuximab-induced cytotoxicity in Tregs from normal donors (**A**) and multiple myeloma patients (**B**) is shown as percentage of Annexin V^+ cells in Tregs. Shown are means ± SEMs of three independent experiments. **C,** PBMCs were stained with 5 μmol/L CFSE and incubated with indicated drugs for 5 days followed by flow cytometry analysis to determine the percentage of diluted CFSE in Tregs. **D,** Tregs were purified from PBMCs of normal donors treated with or without 1 μg/mL isatuximab for 1 day followed by real-time qRT-PCR assay for Foxp3 and IL10. Shown are fold changes relative to control groups after normalization with GAPDH control; *, $P < 0.05$. Len, lenalidomide; Pom, pomalidomide.

analysis. Upregulation of cell surface CD107a and IFNγ production is associated with cytotoxicity induced in these effector cells (36). Isatuximab increases percentages of CD107a and IFNγ in immune effector cells of normal donors (Supplementary Fig. S5A and S5B), whereas an isotype control IgG_1 mAb has no effects (data not shown). Isatuximab (0.1 μg/mL) significantly increases the ability of these two immune effector cells to lyse target multiple myeloma cells, confirmed by depletion of $CD138^+$ multiple myeloma cells in these cultures (data not shown). Although low-dose lenalidomide or pomalidomide (1 μmol/L) alone for 3 days minimally increased both CD107a and IFNγ, combined treatment of isatuximab with pomalidomide, more potently than lenalidomide, further augments activation of these effector cells (Supplementary Fig. S5A and S5B). Importantly, isatuximab, in a dose-dependent manner, upregulates effector function of $CD8^+$ T and NK cells from multiple myeloma patients ($n = 3$; Fig. 5A and B), which is further augmented by pomalidomide, more potently than lenalidomide.

Tumor cells induce generation of iTregs

We found that circulating Tregs in multiple myeloma patients are significantly higher compared with normal donors (Fig. 6A), as in previous reports (20, 22, 37). This could be due to generation of Tregs in the periphery, designated as tumor-induced Tregs (iTregs) derived from naïve $CD4^+$ cells or $CD4^+CD62L^+$ central memory cells (38–41). To study whether such a mechanism leads to elevated percentage of Tregs in multiple myeloma patients, PBMCs were cocultured with multiple myeloma cells for 7 days, followed by flow cytometry analysis. Using the same $CD4^+CD25^{high}Foxp3^+$ gating strategy, the frequency of Tregs in $CD4^+$ populations significantly increases when cocultured with either RPMI8226 or U266 multiple myeloma cells (Supplementary Fig. S6A, left). To further identify the origin of these iTregs, purified Tcons were cocultured with multiple myeloma cells. Multiple myeloma cells induce even more iTregs from Tcons than from whole PBMC populations (Supplementary Fig. S6A, right): fold increases in iTregs induced by multiple myeloma cells were significantly higher from Tcons ($n = 11$) than PBMCs ($n = 5$; Supplementary Fig. S6B). We found that iTregs in cocultures of PBMCs with multiple myeloma cells increase expression of CD38 with time (Supplementary Fig. S6C). Fold induction in CD38 levels is significantly higher in iTregs versus nTregs in *ex vivo* cocultures containing low-dose IL2 (Fig. 6B). Importantly, proliferation of autologous Tcons is inhibited by iTregs induced by multiple myeloma cells in these *ex vivo* cocultures (Fig. 6C). Whether the source of iTreg is PBMCs or Tcons, their phenotype is characterized by higher CD38, CD25, Foxp3, CD44, ICOS, and PD1, as well as lower CD127 expression, compared with nTregs (Supplementary Fig. S6C–S6E). Changes in these cell surface proteins are even more significant on iTregs derived from Tcons versus PBMCs, following culture with multiple $CD38^{low/-}$ multiple myeloma cell lines (Supplementary Fig. S6C–S6E).

We next studied mechanisms of iTreg generation. PD-L1 is concurrently increased on multiple myeloma cells in these cocultures (Supplementary Fig. S7A). Conditioned media from multiple myeloma cells increase frequency of iTregs (Supplementary Fig. S7B), indicating the importance of cytokines secreted from multiple myeloma cells in the induction of iTregs. Specifically, both TGFβ and IL10 are significantly increased in supernatants from cocultures of multiple myeloma cells with either PBMCs or Tcons (Supplementary Fig. S7C; Fig. 6E). Stronger induction of both cytokines from Tcons versus PBMCs correlates with greater extent of iTreg generation from Tcons versus PBMCs (Supplementary Figs. S6B and S7C). Neutralizing anti-TGFβ, -PD1, and -PD-L1 mAb partially blocked iTregs induction (Supplementary Fig. S7D and S7E), supporting their roles in generation of iTregs. Combined with decreased induction of iTregs when using transwell plates to separate Tcons from multiple myeloma cells, these data indicate that cell-to-cell contact also contributes to the generation of iTregs (data not shown).

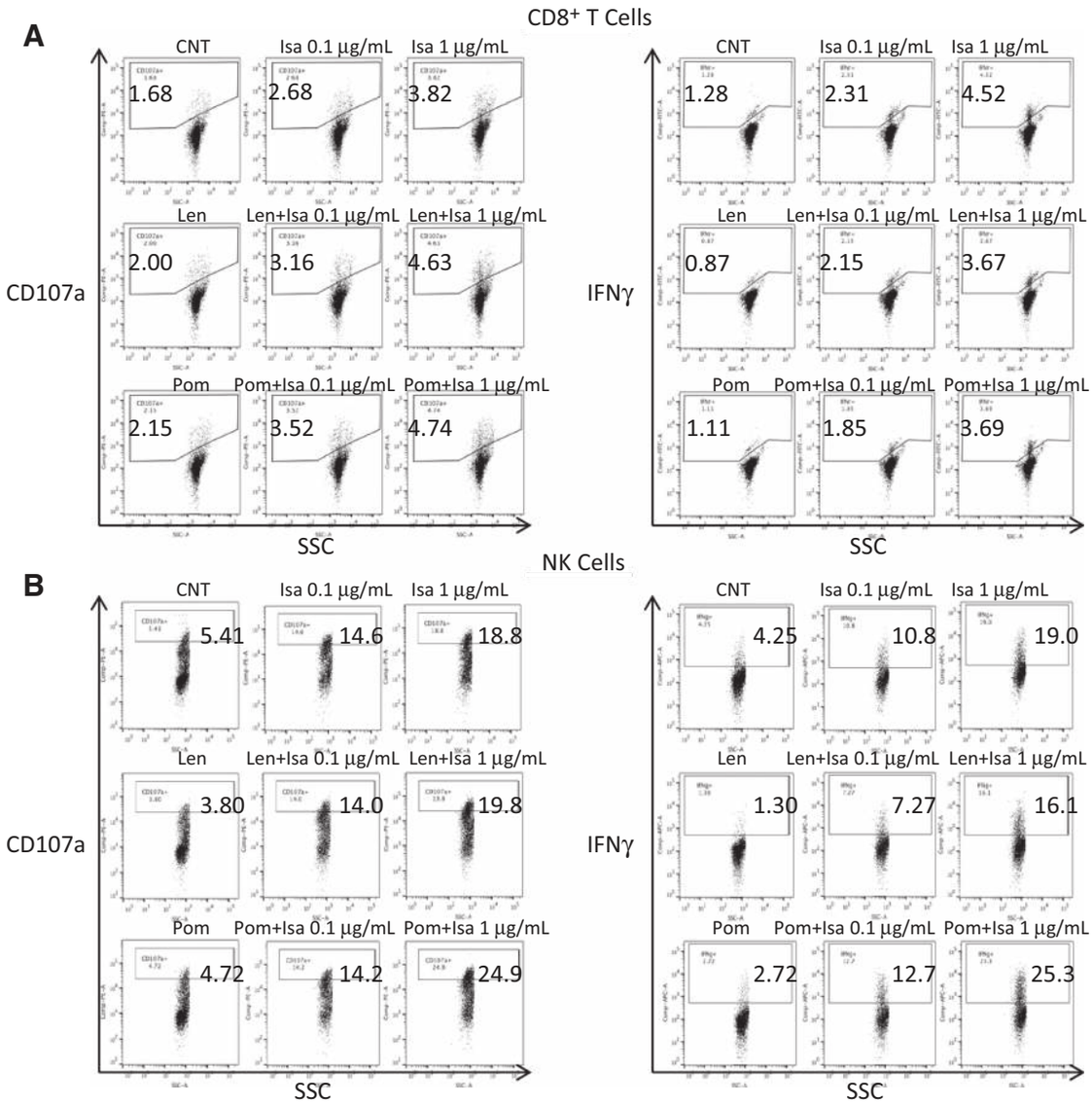

Figure 5.

Isatuximab (Isa) augments $CD8^+$ T and NK effector cell–mediated cytotoxicity against multiple myeloma cells, which is further enhanced by lenalidomide (Len)/pomalidomide (Pom). PBMCs from multiple myeloma patients were pretreated with isatuximab, alone or with lenalidomide/pomalidomide for 2–3 days, and then RPMI8226 multiple myeloma cells were added at E:T ratio of 10:1 for 5 hours, followed by flow cytometry analysis for cell membrane CD107a and intracellular IFNγ. Dot plots are shown for CD107a (left) and IFNγ (right) in $CD3^+CD8^+$ T cells (**A**) and NK cells (**B**) of multiple myeloma patient PBMCs treated as indicated.

Isatuximab also significantly inhibits tumor- and bone marrow stromal cell–induced Tregs (iTregs), which highly express CD38

We next determined whether isatuximab inhibits multiple myeloma cell–induced iTregs, which express high levels of CD38 (Fig. 6B; Supplementary Fig. S6C–S6E). Isatuximab blocks induction of iTregs from PBMCs (Fig. 6D) and from Tcons (Supplementary Fig. S8A) in *ex vivo* cocultures with U266 multiple myeloma cells. Similar levels of apoptosis were observed in both isatuximab-treated and untreated CD38-negative U266 multiple myeloma cells, evidenced by Annexin V/PI–based flow cytometry analysis (Supplementary Fig. S8B), excluding the possibility that failure of U266 cells to induce iTregs was due to tumor cell killing by isatuximab. TGFβ and IL10 were significantly elevated in supernatants from cocultures of PBMCs with U266 multiple myeloma cells and significantly reduced by isatuximab (Fig. 6E). As these cytokines are critical in iTreg generation, we also examined whether bone marrow stromal cells (BMSC) induce generation of iTregs. Cocultures of PBMCs with adherent BMSCs from multiple myeloma patients increased the percentage of Tregs, which was also inhibited by isatuximab (Supplementary Fig. S8C). These results indicate that CD38 mAb is capable of blocking iTregs in the bone marrow microenvironment. Finally, isatuximab decreases the percentage of Tregs from multiple

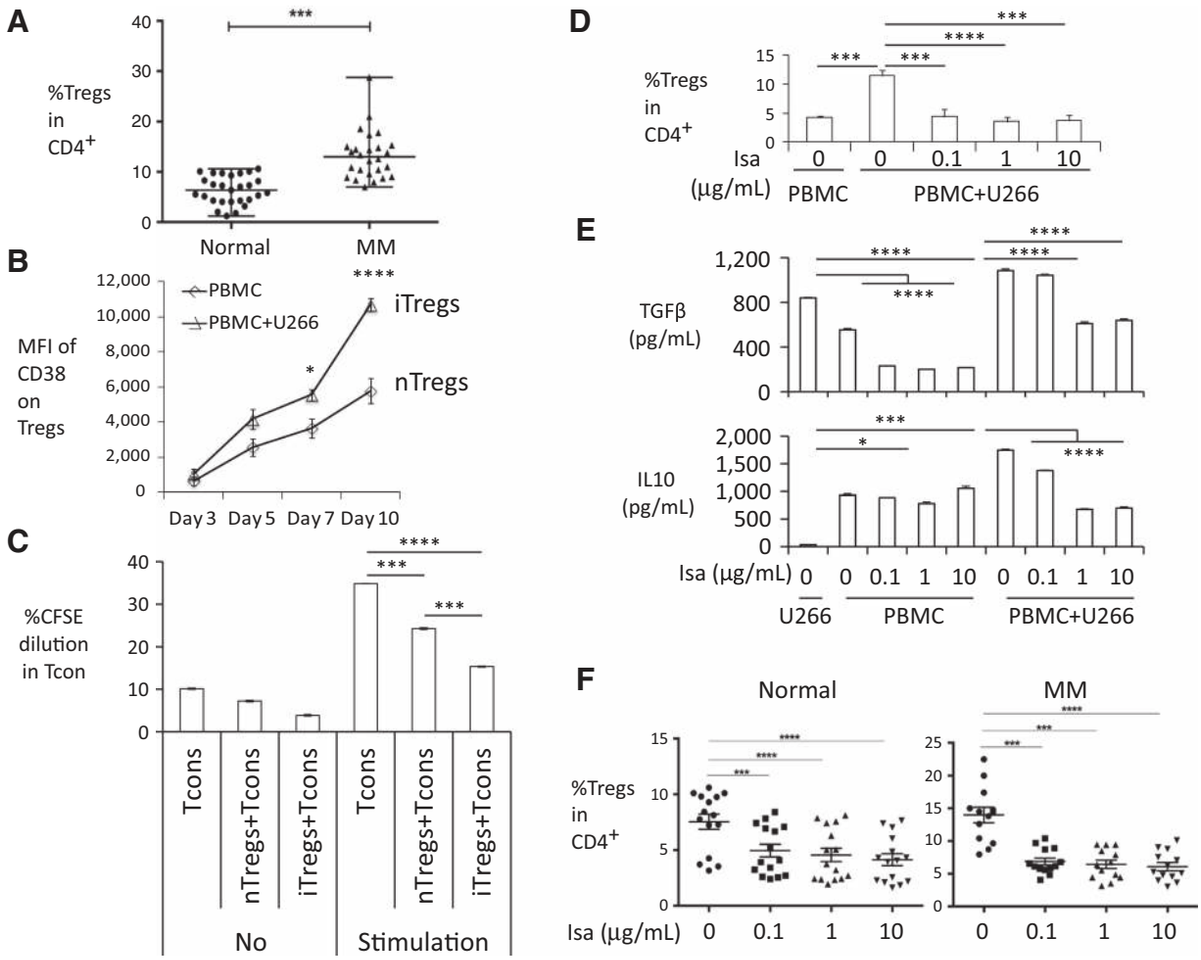

Figure 6.

Multiple myeloma (MM) cell–induced Tregs (iTregs) highly express CD38 and are blocked by isatuximab. **A,** Percentages of Tregs in $CD4^+$ lymphocytes were determined in normal donors ($n = 27$) and multiple myeloma patients ($n = 26$). Shown are median ± ranges. **B,** CD38 levels were examined at indicated time periods in iTregs (triangle) versus nTreg (square) in PBMCs cocultured with or without $CD38^{low/-}$ U266 cells in low-dose IL2-containing culture media. **C,** CFSE-labeled Tcons cocultured with either nTregs isolated from normal donors or iTregs isolated from multiple myeloma cell induction in ex *vivo* coculture system were stimulated with (stimulation) or without (no) anti-CD3/CD28 beads for 6 days. Proliferation was determined by percentage of CFSE dilution in Tcons. **D,** Percentages of iTregs were determined in cocultures of multiple myeloma cells with PBMCs ($n = 4$) treated with indicated doses of isatuximab for 7 days. Shown are means ± SEMs. **E,** Supernatants of *ex vivo* cocultures (**D**) were assayed for TGFβ (top) and IL10 (bottom) by ELISA. **F,** PBMCs from healthy donors ($n = 15$) and multiple myeloma patients ($n = 13$) were treated with isatuximab, and percentage Tregs in $CD4^+$ lymphocytes was measured by flow cytometry analysis. Shown are means ± SEMs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****$P < 0.0001$.

myeloma patients more efficiently than those from normal donors: multiple myeloma patients baseline 15.98% ± 1.19% reduced to 5.87% ± 0.54% at 0.1 μg/mL isatuximab, $n = 13$; normal donors baseline 7.54% ± 0.68% decreased to 4.96% ± 0.57% at 0.1 μg/mL isatuximab, $n = 15$ (Fig. 6F).

Discussion

Tregs, as regulatory elements, actively suppress immune responses and represent a predominant tolerance-inducing modality. Conversely, blockade of Tregs may reverse the suppressive immune environment via promoting T-cell activation and cytotoxicity, thereby allowing the immune system to efficiently attack the tumor. We here demonstrate that targeting CD38 by isatuximab, as recently reported by daratumumab (32), preferentially blocks Tregs greater than Tcons due to increased CD38 levels on Tregs. As $CD38^{high}$ Tregs exhibit even higher immunosuppressive ability (32, 42), targeting CD38 can abrogate this subset more effectively than $CD38^{low}$ or negative subsets, thereby relieving the immunosuppressive bone marrow microenvironment. We show that blockade of Tregs by isatuximab restores Tcons and upregulates cytolysis of multiple myeloma cells mediated by cytotoxic T and NK cells, which is further enhanced by IMiDs.

Our studies show that isatuximab reduces the frequency of Tregs and blocks their suppressive function on Tcons from both normal donors and multiple myeloma patients. The increased proportion of Tcons after isatuximab treatment is due to

significantly increased proliferation of Tcons. Correspondingly, the ratio of Tregs to Tcons (Tregs/Tcons) significantly decreased following isatuximab treatment. Fc-independent mechanisms including apoptosis and decreased proliferation could account for Treg inhibition, in addition to antibody-dependent cytotoxicity (ADCC) and antibody-dependent phagocytosis mediated by Fc-expressing NK and macrophages. Our studies show that isatuximab preferentially induces apoptosis of Tregs greater than Tcons due to increased percentages of $CD38^+$ Tregs with higher CD38 expression than Tcons. $CD38^{high}$ Tregs, which have even greater immunosuppressive capacity than $CD38^{-/low}$ Tregs (32), are most sensitive to CD38 targeting. In addition, isatuximab decreases Foxp3 and IL10 in viable Tregs, further targeting the immunosuppressive function of Tregs. It remains to be determined whether such differential effects of isatuximab on Tregs versus Tcons can increase its therapeutic window.

Besides blocking the suppressor cell component, isatuximab spares Tcons, $CD8^+$ T cells, and NK cells, consistent with the limited toxicity observed in clinical trials (5, 43). Isatuximab significantly increases $CD8^+$ T- and NK-cell–mediated anti-multiple myeloma immune response, with enhanced induction of CD107a and IFNγ. Inhibition of immune effector cells by suppressor Tregs is blocked following treatment of PBMCs with isatuximab for at least 2 days. Considering the underlying immune deficiency of multiple myeloma patients, targeting Tregs to restore effective antitumor response represents a promising treatment strategy. Importantly, isatuximab targets $CD38^{high}$ Tregs in an Fc-independent manner, even in multiple myeloma patients with a highly impaired immune system.

IMiDs inhibit proliferation and function of Tregs *in vitro* (44, 45). However, reports of Treg frequency in patients treated with lenalidomide are variable. In CLL patients, lenalidomide treatment reduced proportion of Tregs (46). Conversely, a delayed increase of Tregs after treatment with lenalidomide has been reported in multiple myeloma in the setting of induction, maintenance, or salvage treatment with lenalidomide or pomalidomide (22, 35, 47, 48). Elevated Tregs *in vivo* after immune stimulation by lenalidomide may represent a negative feedback loop to maintain immune homeostasis. Indeed, our data shows that IMiDs reduce Tregs and stimulate Tcons *in vitro*. Importantly, low dose lenalidomide/pomalidomide with isatuximab enhances suppression of Tregs and induces immune effector cell–mediated multiple myeloma cell lysis *in vitro*. Mechanistically, lenalidomide and pomalidomide upregulate CD38 levels on viable Tregs and increase the percentage of $CD38^{high}$ Tregs, thereby conferring further sensitivity to isatuximab treatment.

Increasing evidence indicates that patients with cancer may have higher proportions of Tregs, which may serve as a predictor for survival. Importantly, increased circulating functional Tregs have been noted in multiple myeloma patients compared with normal donors (22, 37). Increased Tregs in patients with cancer can be derived from naïve $CD4^+$ T cells by stimulation with tumor cells and tumor bystander cells. Using an *ex vivo* coculture system to mimic the *in vivo* bone marrow microenvironment, we showed that multiple myeloma cells are able to induce generation of iTregs from both PBMC and Tcons. Specifically, iTregs are induced when coculturing multiple myeloma cells with purified Tcons in the absence of antigen-presenting cells. Furthermore, when compared with nTregs, iTregs express even higher cell surface CD38, Foxp3, CD25, CD44, ICOS and PD1, as well as lower levels of CD127. This suggests an even greater suppressive function of iTregs, further supporting targeting CD38 to block these highly immunosuppressive Tregs derived from Tcons. In addition, PD-L1 is increased on multiple myeloma cells in these cocultures, which may further promote differentiation of Tcons into Tregs via ligation with PD1 (49). PD-L1 upregulation on multiple myeloma cells mediates adherence to bone marrow stromal cells and induction of multiple myeloma—related cytokines, which further increases immunosuppression in the bone marrow microenvironment (42). These results suggest the utility of combining isatuximab with PD-L1 mAb to enhance anti-multiple myeloma immunity.

Both soluble cytokines and cell-to-cell contact are critical in the generation of iTregs. On one hand, inhibitory cytokines IL10 and TGFβ in the culture supernatant are significantly increased when tumor cells are present. Indeed, a blocking anti-TGFβ mAb partially inhibits generation of iTregs. Conditioned media from multiple myeloma cells also induces Tregs from PBMCs, supporting a soluble cytokine mechanism. On the other hand, separating Tcons and multiple myeloma cells by transwell plates attenuates induction of iTregs. Moreover, membrane PD-L1 expression is upregulated on multiple myeloma cells in parallel with increased PD1 receptor on Tregs. Either anti-PD1 mAb or anti-PD-L1 mAb can inhibit induction of iTregs, indicating a role of surface receptor–ligand interaction in this process. Our current findings are consistent with the notion that induction of $CD25^+$ Tregs from $CD25^-$ Tcons was partially abrogated when Tcons were separated from tumor cells (40), indicating a role for cell–cell contact.

Our data show that isatuximab prevents induction of iTregs by multiple myeloma cells and bone marrow microenvironment cells, associated with reduced TGFβ and IL10 in the coculture supernatants. Potential mechanisms whereby isatuximab attenuates iTregs generation include: (i) iTregs express higher levels of CD38 than nTregs, making them even more sensitive to isatuximab; and (ii) soluble cytokines contribute to generation of iTregs, and isatuximab may reduce IL10 production through blocking CXCL12/CXCR4 signaling. For example, CXCL12 costimulates IL10 secretion by a diverse population of $CD45RA^-$ T cells, including Tregs (50). Blocking CD38 impairs CXCL12/CXCR4 signaling pathway in CLL cells (51). Whether this occurs in Tregs remains to be determined.

In summary, isatuximab suppresses the inhibitory function of Tregs, which highly express CD38 by decreasing their cell number, inhibiting immunosuppressive cytokines, and blocking their trafficking. Isatuximab enhances NK- and $CD8^+$ T effector cell–mediated antitumor immune responses, which can be further enhanced by IMiDs. Furthermore, increased circulating iTregs in multiple myeloma patients are derived from Tcons in both cell–cell contact-dependent and -independent manners. Importantly, isatuximab blocks these processes. Targeting CD38 with isatuximab may therefore induce immunomodulatory effects, which both relieve immunosuppression and trigger anti-multiple myeloma immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Feng, L. Zhang, C. Acharya, Y.T. Tai, K.C. Anderson
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