Pre-treatment CD38-positive regulatory T cells affect the durable response to daratumumab in relapsed/refractory multiple myeloma patients

CD38 is highly expressed on plasma cells and considered a good target for the treatment of multiple myeloma. Daratumumab, a humanized monoclonal antibody against CD38, has emerged as a promising drug for relapsed/refractory multiple myeloma. It has shown substantial clinical activity in several clinical trials, particularly in combination with immunomodulatory drugs (IMiD).[1-3] Considering the high activity and favorable toxicity profile of daratumumab, other CD38 antibodies, e.g. isatuximab, are currently under development.[4] Daratumumab has classic Fc-dependent immune effector mechanisms, including antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity.[5] In fact, pre-treatment CD38 expression levels in myeloma cells have been shown to significantly correlate with the response to daratumumab.[6] However, daratumumab rapidly reduced CD38 expression levels through trogocytosis even after the first infusion.[7] Moreover, such a reduction occurred in all patients, including those with deep and durable responses,[7] suggesting the presence of mechanisms that target myeloma cells indirectly. Daratumumab also targets CD38-expressing non-plasma cells, including CD38-positive ($CD38^+$) regulatory T cells (Tregs).[8] Indeed, it has been shown that cytotoxic T-cell number, activation, and clonality increase following daratumumab treatment.[8] Thus, although various mechanisms of daratumumab action have been suggested, they have not been examined in detail in the real-world clinical setting. Moreover, it remains unclear whether immunomodulating or direct actions are important or both in these mechanisms. In addition, response markers other than CD38 expression on myeloma cells have not been established. Here we show that in addition to CD38 expression levels in myeloma cells, the frequency of circulating $CD38^+$ Tregs present before the treatment is also associated with the extent of response to daratumumab, particularly in the case of the durable response.

This study comprised 44 patients with relapsed/refractory multiple myeloma (median age: 77 years, range: 50-92 years) who were treated with daratumumab at the Kameda Medical Center. The patient population included all patients who had an evaluable response and were followed up for ≥1 cycle of daratumumab. Peripheral blood and bone marrow samples were analyzed before and during the treatment. This study was approved by the Institutional Review Board of the Kameda Medical Center and conducted in accordance with the Declaration of Helsinki. Patients' characteristics and treatment details are summarized in Table 1. The majority of patients (82%) received more than two prior therapies with the median of four prior lines of therapy. Almost all patients were refractory to proteasome inhibitors (PI) and IMiD. Fourteen patients (32%) received a PI-based regimen, 28 (64%) received an IMiD-based regimen, and two (4%) received other daratumumab-containing regimens. Twenty-seven patients (61%) had a partial or better response (responders), whereas 17 patients (39%) did not respond (non-responders).

A previous report had demonstrated a significant positive association between CD38 expression levels in myeloma cells and the efficacy of daratumumab monotherapy.[6] We therefore analyzed CD38 expression levels in bone marrow myeloma cells before the treatment to investigate whether they could predict the extent of response to daratumumab alone or in combination with IMiD or PI. CD38 mean fluorescence intensity (MFI) was accessed in the neoplastic plasma cell population ($CD38^{high}$/$CD138^{high}$/$CD56^+$ or $CD56^-$/$CD19^-$) (Figure 1A). As previously reported, there was marked heterogeneity in CD38 MFI values. Pre-treatment CD38 MFI levels were significantly higher in responders than in non-responders (Figure 1B). Although 20 patients showed a rapid response even after one cycle of daratumumab (early responders), CD38 MFI was significantly higher in the early responders than in others, indicating that the early cytotoxic effect occurred due to the direct antibody effect (Figure 1B). Therefore, even when combined with IMiD or PI in a real-world setting, pre-treatment CD38 MFI of myeloma cells may be an early predictor of the response to daratumumab. However, patients with relatively low CD38 MFI also presented a clinical response, suggesting the existence of indirect mechanisms.

Next, we examined lymphocyte subsets, including $CD38^+$ Tregs, before and after the treatment to investigate the immunomodulatory mechanism of daratumumab. As reported previously,[8] we confirmed that absolute $CD8^+$ T-cell numbers significantly increased after daratumumab treatment. Moreover, we found that the absolute numbers of $HLA\text{-}DR^+$-activated T cells were also significantly higher after the treatment. However, these increased numbers of $CD8^+$ and $HLA\text{-}DR^+$ T cells did not correlate with clinical response (*data not shown*). It has been shown that CD38 expression levels correlate with FOXP3 expression in Tregs of multiple myeloma patients,[9] and $CD38^{high}$ Tregs are more immunosuppressive *in vitro* than CD38-negative Tregs.[8,9] To confirm the effect of daratumumab on these Tregs, we examined the changes in the circulating Treg numbers after daratumumab treatment. Tregs were identified as a fraction of the $CD4^+CD25^{high}CD127dim$ population (Figure 1C).[10]

Table 1. Patients' characteristics.

Parameter	N of patients (N = 44)
Median age, years (range)	77 (50-92)
Male (%)	24 (55%)
Female (%)	20 (45%)
Paraprotein type	
IgG	19 (43%)
IgA	9 (21%)
IgD	1 (2%)
LCD	15 (34%)
Previous therapies	4 (1-11)
> 2 lines of therapy	36 (82%)
≤ 2 lines of therapy	8 (18%)
Double refractory	42 (95%)
Regimen	
PI-based	14 (32%)
IMiD-based	28 (64%)
Others	2 (4%)
Response	
≥ PR (responder)	27 (61%)
< PR (non-responder)	17 (39%)

N: number; LCD: light chain disease; PI: proteasome inhibitor; IMiD: immunomodulatory drug; PR: partial response.

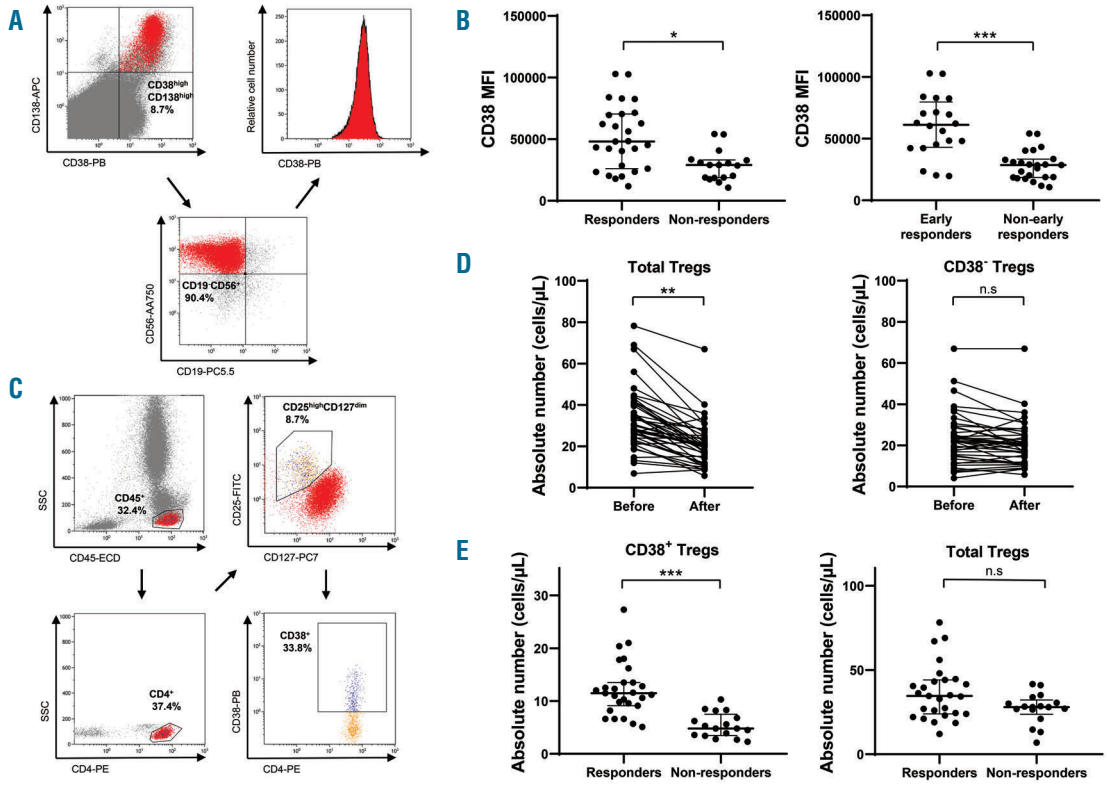

Figure 1. CD38 expression levels in myeloma cells and the frequency of circulating CD38-positive regulatory T cells (Tregs) are associated with the response to daratumumab. Bars indicate the median with interquartile range. Significance of differences between the indicated groups was assessed by the Mann-Whitney U-test. *0.01≤*P*<0.05; **0.001≤*P*<0.01; ***P<0.001; n.s: not significant. (A) Flow cytometry analysis of CD38 mean fluorescence intensity (MFI) in the clonal plasma cell population. Clonal plasma cells were defined as the $CD38^{high}/CD138^{high}/CD56^{+}$ or $CD56^{-}/CD19^{-}$ population. Representative flow cytometry histograms are shown. The percentages of the gated subsets are shown. Flow cytometry was performed using the antibodies from Beckman Coulter on a Navios cytometer (Beckman Coulter). (B) Comparison of CD38 MFI in myeloma cells of responders (defined as those with partial response or better) and non-responders. (C) A representative example of the gating strategy used for circulating $CD38^{+}$ Tregs is shown. Tregs were identified as a fraction of the $CD4^{+}CD25^{high}CD127^{dim}$ population. The percentages of the gated subsets are shown. (D) Difference between the frequency of the total Tregs and CD38-negative Tregs before and four weeks after daratumumab treatment. y-axis: absolute numbers of Treg cells (cells/μL). (E) Comparison of the numbers of circulating $CD38^{+}$ and total Tregs between responders and non-responders.

Notably, the absolute number of $CD38^{+}$ Tregs among the patients was highly variable (Figure 1D). After the administration of daratumumab, $CD38^{+}$ Tregs were almost undetected, suggesting a possibility that daratumumab eliminated $CD38^{+}$ Tregs, or that the lack of CD38 detection was due to competition of the CD38 detection antibody for binding sites with daratumumab.[11] We also utilized a CD38 multi-epitope antibody; however, CD38 expression in Tregs was also undetected (*Online Supplementary Figure S1*). Importantly, the absolute total number of Tregs significantly decreased four weeks after the start of the treatment. Moreover, CD38-negative Treg numbers remained relatively stable after daratumumab treatment (Figure 1D). These results suggested that daratumumab primarily targeted $CD38^{+}$ Tregs. Therefore, as $CD38^{+}$ Treg number increased, daratumumab-induced depletion of Tregs became more pronounced. Thus, immunomodulatory effects are more likely to be induced in patients with higher $CD38^{+}$ Treg numbers. Indeed, the absolute number of $CD38^{+}$ Tregs before the treatment was significantly higher in responders than in non-responders (Figure 1E). However, the absolute total number of Tregs was not associated with clinical response (Figure 1E). These results indicate that the frequency of $CD38^{+}$ Tregs differed among the patients and that they affect the response to daratumumab.

As mentioned above, CD38 expression levels rapidly declined after daratumumab treatment.[6,7] However, some patients presented with durable and deep responses to daratumumab, likely due to immunomodulatory effects. Indeed, among the 24 responders who were followed up for ≥6 months, 17 showed a long-lasting response (≥6 months, durable responders). Importantly, the absolute number of $CD38^{+}$ Tregs was significantly higher in these responders (Figure 2A). However, CD38 MFI of myeloma cells was not associated with durable response (Figure 2B). Furthermore, the reduction in total numbers of Tregs was significantly higher in the durable responders than in others, suggesting the elimination of $CD38^{+}$ Tregs (Figure 2C). These results indicate that patients with more $CD38^{+}$ Tregs have durable responses to daratumumab. Finally, to investigate the role of $CD38^{+}$ Tregs in disease progression, we analyzed the frequencies of $CD38^{+}$ Tregs in healthy volunteers and patients with monoclonal gammopathy of undetermined significance, smoldering myeloma, newly diagnosed myeloma, and relapsed/refractory myeloma. $CD38^{+}$ Treg numbers were significantly higher in the relapsed myeloma group than in the other disease or control groups (Figure 2D), suggesting that $CD38^{+}$ Tregs play an important role in the progression of myeloma. This circumstance provides a rationale for targeting $CD38^{+}$ Tregs using daratumumab

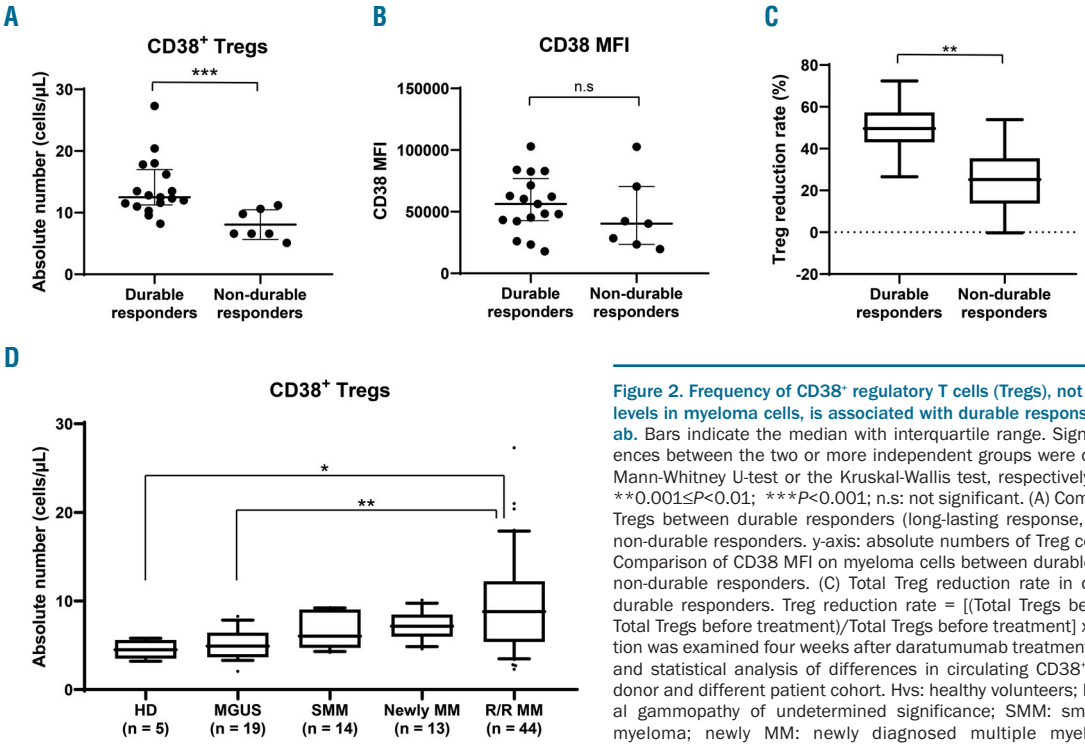

Figure 2. Frequency of CD38⁺ regulatory T cells (Tregs), not CD38 expression levels in myeloma cells, is associated with durable response to daratumumab. Bars indicate the median with interquartile range. Significance of differences between the two or more independent groups were calculated by the Mann-Whitney U-test or the Kruskal-Wallis test, respectively. *0.01≤*P*<0.05; **0.001≤*P*<0.01; ***$P<0.001$; n.s: not significant. (A) Comparison of CD38⁺ Tregs between durable responders (long-lasting response, ≥6 months) and non-durable responders. y-axis: absolute numbers of Treg cells (cells/μL). (B) Comparison of CD38 MFI on myeloma cells between durable responders and non-durable responders. (C) Total Treg reduction rate in durable and non-durable responders. Treg reduction rate = [(Total Tregs before treatment − Total Tregs before treatment)/Total Tregs before treatment] x 100. Treg reduction was examined four weeks after daratumumab treatment. (D) Frequencies and statistical analysis of differences in circulating CD38⁺ Tregs of healthy donor and different patient cohort. Hvs: healthy volunteers; MGUS: monoclonal gammopathy of undetermined significance; SMM: smoldering multiple myeloma; newly MM: newly diagnosed multiple myeloma; R/R MM: relapsed/refractory multiple myeloma.

in relapsed/refractory multiple myeloma patients.

CD38 expression in Tregs is up-regulated by IMiD *in vitro* and *in vivo*, possibly due to a negative feedback loop involved in maintaining immune homeostasis.[9,12,13] The majority of our patients were heavily treated, and almost all patients had a history of treatment with IMiD. The reason why the total number and ratio of CD38⁺ Tregs are variable is not clear; however, the results from Figure 2D suggest that CD38⁺ Tregs are involved in the refractory pathology of myeloma. Indeed, Usmani *et al*. reported a case of heavily treated myeloma with deep and durable response to daratumumab monotherapy. In that patient, immunophenotyping revealed a decrease in the numbers of Tregs during daratumumab therapy.[14]

One limitation of our study was that we could not accurately assess CD38 expression after daratumumab administration, because we did not use a non-cross-reactive CD38 antibody, such as Humax-003 or JK36.[11] However, we showed that CD38 expression levels in myeloma cells and CD38⁺ Treg before the treatment may serve as predictors of the response to daratumumab. Evaluation of the pre-treatment status may be useful because CD38 expression levels are not affected by daratumumab administration.

Although various mechanisms of action have been reported for daratumumab, few reports have examined the factors predicting the response in the clinical practice setting. Here, we showed that pre-treatment levels of CD38 MFI are a possible predictive marker for early response to daratumumab even when combined with PI or IMiD. Moreover, we found that the frequency of CD38⁺ Tregs present before the treatment is highly heterogeneous in relapsed/refractory multiple myeloma patients and may also serve as marker of durable response. These results provide evidence to support multiple mechanisms of action of daratumumab, including antibody-dependent cellular cytotoxicity and immunomodulatory effects. Furthermore, our results indicated an association between durable response and immunomodulatory mechanisms. To obtain a deep response, a sustained response is necessary. Thus, immunomodulatory effects obtained by depleting CD38⁺ Tregs may prove to be more important than any direct effects of daratumumab. Elucidation of the accurate mechanisms of action should result in the development of effective CD38-targeting strategies, which will further contribute to improved clinical outcomes for multiple myeloma patients.

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References

1. Dimopoulos MA, Oriol A, Nahi H, et al. Daratumumab, Lenalidomide, and Dexamethasone for Multiple Myeloma. N Engl J Med. 2016;375(14):1319-1331.
2. Palumbo A, Chanan-Khan A, Weisel K, et al. Daratumumab, Bortezomib, and Dexamethasone for Multiple Myeloma. N Engl J Med. 2016;375(8):754-766.
3. Chari A, Suvannasankha A, Fay JW, et al. Daratumumab plus pomalidomide and dexamethasone in relapsed and/or refractory multiple myeloma. Blood. 2017;130(8):974-981.
4. Martin T, Baz R, Benson DM, et al. A phase 1b study of isatuximab plus lenalidomide and dexamethasone for relapsed/refractory multiple myeloma. Blood. 2017;129(25):3294-3303.
5. Nijhof IS, Groen RW, Noort WA, et al. Preclinical Evidence for the Therapeutic Potential of CD38-Targeted Immuno-Chemotherapy in Multiple Myeloma Patients Refractory to Lenalidomide and Bortezomib. Clin Cancer Res. 2015;21(12):2802-2810.
6. Nijhof IS, Casneuf T, van Velzen J, et al. CD38 expression and complement inhibitors affect response and resistance to daratumumab therapy in myeloma. Blood. 2016;128(7):959-970.
7. Krejcik J, Frerichs KA, Nijhof IS, et al. Monocytes and Granulocytes Reduce CD38 Expression Levels on Myeloma Cells in Patients Treated with Daratumumab. Clin Cancer Res. 2017;23(24):7498-7511.
8. Krejcik J, Casneuf T, Nijhof IS, et al. Daratumumab depletes CD38+ immune regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma. Blood. 2016;128(3):384-394.
9. Feng X, Zhang L, Acharya C, et al. Targeting CD38 Suppresses Induction and Function of T Regulatory Cells to Mitigate Immunosuppression in Multiple Myeloma. Clin Cancer Res. 2017;23(15):4290-4300.
10. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med. 2006;203(7):1701-1711.
11. Oberle A, Brandt A, Alawi M, et al. Long-term CD38 saturation by daratumumab interferes with diagnostic myeloma cell detection. Haematologica. 2017;102(9):e368-e370.
12. Minnema MC, van der Veer MS, Aarts T, Emmelot M, Mutis T, Lokhorst HM. Lenalidomide alone or in combination with dexamethasone is highly effective in patients with relapsed multiple myeloma following allogeneic stem cell transplantation and increases the frequency of CD4+Foxp3+ T cells. Leukemia. 2009;23(3):605-607.
13. Busch A, Zeh D, Janzen V, et al. Treatment with lenalidomide induces immunoactivating and counter-regulatory immunosuppressive changes in myeloma patients. Clin Exp Immunol. 2014;177(2):439-453.
14. Usmani SZ, Khan I, Chiu C, et al. Deep sustained response to daratumumab monotherapy associated with T-cell expansion in triple refractory myeloma. Exp Hematol Oncol. 2018;7:3.