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Increased CD200 expression in acute myeloid leukemia is linked with an increased frequency of FoxP3$^+$ regulatory T cells

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CD200 is a type-1a transmembrane cell-surface glycoprotein that is normally expressed in critical tissues such as the central nervous system and testis, as well as certain leukocytes, including T and B lymphocytes, where its role is to promote peripheral tolerance and protect immune privileged sites.[1] CD200 has no known intracellular signaling motif, but induces immunosuppression through engagement with CD200R, a cell-surface receptor homolog, which is expressed on leukocytes of myeloid lineage, including mast-cells, macrophages, basophils, dendritic cells as well as certain T-cell populations.[2] CD200, which is frequently overexpressed in acute myeloid leukemia (AML) patient blasts and associated with a worse outcome,[3] has the potential to induce the formation of CD4$^+$CD25^{++}FoxP3$^+$ regulatory T cells (Tregs),[4] a subset of immunosuppressive T cells that are linked with a poor prognosis in AML.[5] Most importantly, Tregs have been documented to suppress the anti-leukemia response *in vitro* from AML patients,[6] suggesting that these cells have an important role in regulating AML patient tumor immunity. We therefore investigated whether CD200 protein expression on human AML blasts was associated with an increased frequency of Treg cells in AML using a cohort of 40 AML patients (at the point of diagnosis before to any treatment; Supplementary Table S1 for patient demographic). AML blast CD200 protein expression and patient Treg frequency was analyzed by flow cytometry (for full gating strategy, refer to Supplementary Figure S1). The data show that CD200 blast expression level correlated significantly with the frequency of Treg cells (Figure 1a). This association was found to be independent of white blood cell count (Figure 1b), suggesting that CD200 expression on AML blasts promotes Treg formation. We also examined whether CD200 expression was differentially expressed on AML blasts with a putative leukemic stem cell phenotype (CD34$^+$CD38$^-$),[7] but could find no evidence for this within the six samples examined (Supplementary Figure S2).

Given that the depletion of Tregs has been shown to improve T-cell-mediated therapy, as well as improving antitumor activity in leukemia patients that are in complete remission;[8,9] we investigated whether Tregs from CD200^{hi} patients were functionally immunosuppressive. We examined the ability of purified Tregs to suppress naïve responder T-cell (CD4$^+$CD25$^-$) proliferation following CD3/CD28 co-stimulation (refer to Supplementary Materials and Methods). The data demonstrate that Tregs isolated from CD200^{hi} AML patients were capable of suppressing T-cell proliferation at responder to Treg ratios of >1 to 0.01 (Figure 1c). Reciprocal analysis of Tregs from CD200^{lo} patients could not be carried out because of the extremely low frequencies of these cells in CD200^{lo} patients (Figure 1d). In fact, Treg frequencies in CD200^{lo} AML patients were uniformly lower than in healthy donor controls, suggesting that CD200 has an influence on Treg induction in this context.

Since mouse models have suggested that CD200-induced Tregs are likely to mediate Th1 immunosuppression,[10] a cytokine response with a prognostic link in leukemia,[11] we measured the Th1 cytokine response (TNFα, IL2 and IFNγ production) in CD200^{hi} patients before and after Treg depletion. Representative flow cytometric plots confirmed Treg depletion using magnetic separation (Figure 2a). However, removal of Tregs alone was insufficient to significantly improve the Th1 cytokine response as detected by intracellular cytokine staining (Figure 2b). Elsewhere we report that, in addition to its known influence on Treg production, CD200 is also directly immunosuppressive through engagement with CD200R

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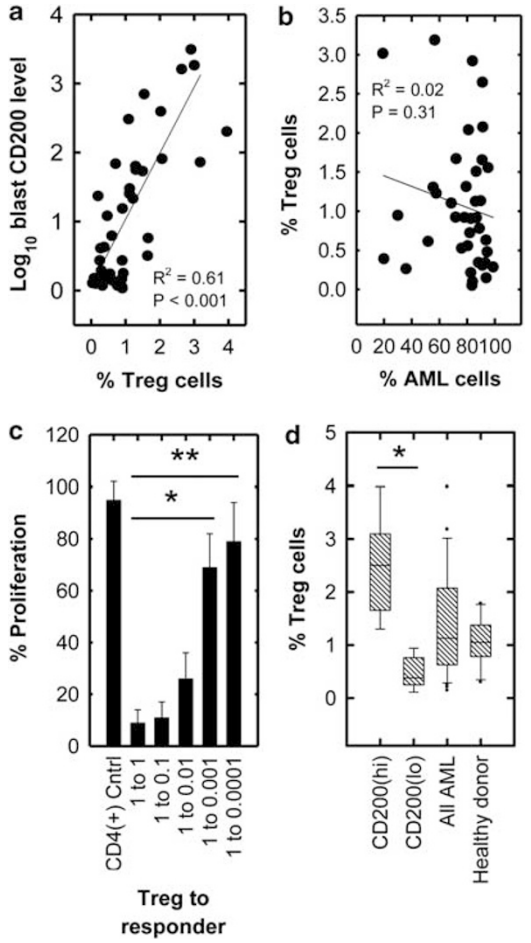

Figure 1. Assessment of AML blast CD200 expression with respect to Treg frequency and function. Treg cells from 40 AML patients at diagnosis were identified by flow cytometry based on the frequency of $CD3^+$ $CD4^+CD25^{++}$ T cells that were >95% $FoxP3^+$ (refer to Supplementary Figure S1). (**a**) Linear regression analysis comparing AML blast CD200 expression level with the frequency of Tregs from 40 diagnostic AML samples. (**b**) Correlation of AML blast frequency with Treg cell frequency. To assess the suppressive function, Tregs and naïve T cells ($CD4^+CD25^-$) were isolated from $CD200^{hi}$ AML patients by magnetic separation and added together at increasing ratios before CD3/CD28 co-stimulation. Suppression was measured by the ability of Tregs to inhibit naïve $CD4^+$ T-cell proliferation (refer to Supplementary Materials and Methods). (**c**) Suppression of naïve $CD4^+$ T-cell proliferation by AML Treg cells at indicated Treg to responder ratios (data represent mean ± 1 s.d., $n=4$). (**d**) Treg cell frequency stratified with respect to AML blast CD200 expression. $^*P<0.05$ and $^{**}P<0.01$ analyzed by one-way analysis of variance with Tukey's multiple comparison test.

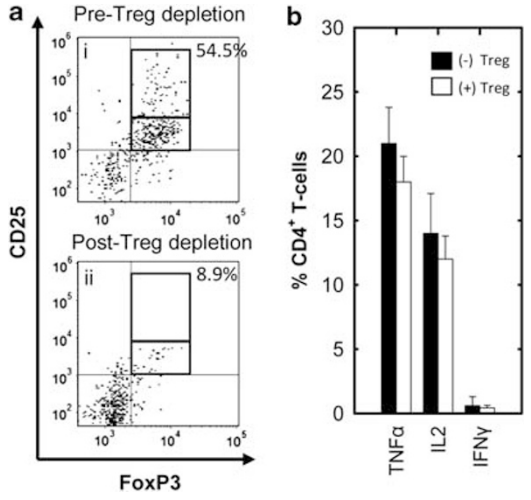

Figure 2. Assessment of AML patient Treg depletion in recovering the Th1 response in $CD200^{hi}$ patients. Tregs cells were depleted from $CD200^{hi}$ AML patients by magnetic separation. (**a**) Representative flow cytometric data depicting T cells (i) before depletion of Tregs ($CD3^+CD4^+CD25^{++}FoxP3^+$) and (ii) following Treg depletion from a $CD200^{hi}$ AML patient. AML cells were stimulated with PMA/ionomycin for 6 h at 37 °C before intracellular Th1 cytokine staining was performed. Flow cytometry was used to assess Th1 cytokine levels within the $CD45^{++}CD3^+CD4^+$ fraction. (**b**) Summary data showing the Th1 response from $CD200^{hi}$ AML patients ± Treg cells ($n=6$).

on T cells in AML.[12] However, in the present study we show that removal of Treg cells alone is unlikely to show effect in these assays when large numbers of $CD200^+$ blast cells are present. These data would predict that Tregs have little impact on immunosuppression at diagnosis when the disease burden is high, as the dominant mode of T-cell suppression would be mediated by $CD200^{hi}$ blasts directly inhibiting Th1 responses.[12] Conversely, following chemotherapy, the reduction of tumor burden would be predicted to make Treg-mediated immunosuppression cells the dominant factor.

In summary, these findings illustrate a clear correlation between blast CD200 expression level and the frequency of immunosuppressive Treg cells. Previous studies of human AML have also reported increased frequencies of Tregs in untreated disease[13] as well as during regeneration following treatment,[14] however, the link with CD200 expression has not previously been established. Our data also indicate that while Tregs in these patients may be functionally immunosuppressive, they may only become influential following cytoreduction. Indeed, several studies have identified that increased frequencies of Tregs are associated with relapse in myeloid malignancy.[15,16] Furthermore, phase-I studies using anti-CD200 monoclonal antibody immunotherapy have shown that blocking CD200 is sufficient to reduce the Treg frequency in chronic lymphocytic leukemia patients,[17] illustrating that blocking CD200 may be therapeutically advantageous in AML.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

SJC designed and performed the experiments/analyzed all data and co-wrote the manuscript. RKH provided statistical guidance. ECYW provided biological insight. AKB provided resources and clinical insight. SM, RLD and AT contributed to experimental design and co-wrote the manuscript.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

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Expression of CD200 on AML blasts directly suppresses memory T-cell function

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Previous studies have shown that immunosuppression in acute myeloid leukemia (AML) is associated with changes in the adaptive immune compartment. Such changes include the suppression of memory T-cell function[1] and the suppression of Th1 cytokine (TNFα, IL-2 and IFNγ)-producing cells.[2] A suppressed immune response in AML is associated with a worse patient outcome and increased risk of relapse,[3] as well as increased risk of infection impairing patient recovery.[4] The over-expression of the immunosuppressive ligand CD200 is also associated with an increased risk of relapse in AML (hazard ratio 1.7); an observation consistent with a hypothesis in which CD200 inhibits clearance of residual disease.[5,6] As memory T-cell responses are central for tumor immunosurveillance and contribute to prolonged molecular remission,[7] we carried out this study to establish how these responses were affected in AML patients over-expressing CD200 (Supplemental Table S1). We initially investigated whether CD200 expression on AML blasts influenced $CD8^+$ T-cell cytotoxic potential and the frequency of TNFα-, IL-2- and IFNγ-producing $CD4^+$/$CD8^+$ memory T-cells (Supplementary Materials and Methods and Supplemental Figure S1 for gating strategy). Using CD107a as a marker of cytotoxic function, AML cells were activated with PMA/ionomycin. We show that the frequency of $CD107a^+CD8^+$ memory T-cells was significantly reduced by ~50% for $CD200^{hi}$ patients when compared with $CD200^{lo}$ AML, demonstrating that cytotoxic memory T-cell activity was compromised in $CD200^{hi}$ patients (Figure 1a). Furthermore, the frequencies of TNFα-, IL-2- and IFNγ-producing $CD4^+$ memory cells were also reduced by ~50% for $CD200^{hi}$ patients when compared with $CD200^{lo}$ AML (Figure 1b), significantly so in the case of IL-2 and IFNγ. Interestingly, $CD200^{lo}$ patients displayed a higher IFNγ response, not only with respect to $CD200^{hi}$ patients but also in comparison to healthy donors, suggesting a role for this cytokine in AML, which is attenuated by CD200. No difference was observed for TNFα-, IL-2- and IFNγ-producing $CD8^+$ memory cells between $CD200^{hi}$, $CD200^{lo}$ and healthy donors (data not shown). CD200 has also been reported to mediate suppression of the Th1 response in chronic lymphocytic leukemia as well as solid tumors,[8,9] suggesting that CD200-mediated Th1 suppression is a central mechanism in cancer immunomodulation.

The ability to simultaneously produce TNFα, IL-2 and IFNγ is an important indicator of 'T-cell quality' in anti-tumor/viral responses.[10] We therefore simultaneously measured the production of all these cytokines in $CD200^{hi}$ and $CD200^{lo}$ patients after PMA/ionomycin stimulation (Supplemental Figure S1). A significant reduction (30%) in $CD4^+$ memory T-cells capable of simultaneously producing TNFα, IL-2 and IFNγ was observed in $CD200^{hi}$ compared with $CD200^{lo}$ AML patients (Figure 1c). Although a similar reduction was observed within the $CD8^+$ memory cells, the changes in this subpopulation were less consistent and were not statistically significant (Supplemental Figure S2). To assess if CD200 expression on AML blasts influences the memory Th1 response through an antigen-specific mechanism, we compared T-cell responses with common microbial recall

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