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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 function¹ and the suppression of Th1 cytokine (TNF α , IL-2 and IFN γ)-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 TNF α -, IL-2- and IFN γ -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 TNF α -, IL-2- and IFN γ -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 IFN γ . Interestingly, CD200^{lo} patients displayed a higher IFN γ 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 TNF α -, IL-2- and IFN γ -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 TNF α , IL-2 and IFN γ 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 TNF α , IL-2 and IFN γ 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

antigens (PPP) by ELISPOT (Supplementary Materials and Methods).¹¹ We observed a significant 75% reduction in the frequency of IFN γ -secreting T-cells towards PPP in CD200^{hi} vs CD200^{lo} AML (Figures 1d and e). CD200 expression level did not influence the overall frequency of CD3⁺ lymphocytes (Supplemental Figure S3), demonstrating that the difference observed in these cohorts was due to T-cell inhibition in CD200^{hi} patients and not due to a decrease in overall T-cell frequency or increase in AML blasts. Thus, we show for the first time (in any context) that CD200 expression in AML is associated with the suppression of Th1 memory T-cell quality and function. Not only does this finding demonstrate that the memory T-cell response in CD200^{hi} patients is suppressed through an antigen-specific mechanism, but suggests that CD200 expression may exacerbate the susceptibility of leukemia patients to common microbial infections, which may impair patient recovery.⁴ This notion is supported by a study from Snelgrove *et al.*, who demonstrated in a murine model that CD200 expression suppresses T-cell responses towards influenza.¹²

To demonstrate that this immunosuppression was functionally driven by CD200, we asked whether blocking CD200 could also recover the AML Th1 memory T-cell response. Figures 2a and b show a significant recovery of memory T-cells secreting IFN γ for CD200^{hi} patients in an ELISPOT assay, raising the possibility that AML blast CD200 was directly interacting with memory T-cells via CD200R. Flow cytometric data confirmed CD200R expression on memory T-cells from AML patients (Figure 2c), supporting previous literature using healthy subjects.¹³ To rule out the possibility of indirect suppression through antigen-presenting cells, we next carried out a refined assay in which a CD4⁺ T-cell clone (Belx2)¹⁴ was co-cultured with K562 cells, which differed solely in their expression of CD200 (Supplemental Figure S4).¹⁵ We showed a significantly impaired TNF α response in CD200^{hi} K562 cells compared with control co-cultures (Figure 2d). We also observed a significant suppression in IL-2 and IFN γ production with a loss of multi-functionality in terms of TNF α and IFN γ production in the presence of CD200⁺ cells (Supplemental Figure S4), though the frequency of Belx2 cells producing IFN γ and IL-2 was minimal compared with TNF α under these assay conditions. Adding anti-CD200 to the CD3/CD28-stimulated assay could significantly recover TNF α production in CD200⁺ co-cultures to the same level as CD200⁻ co-cultures (Figure 2d), thus demonstrating that blockade of CD200 alone is sufficient to recover memory T-cell activity. The data also show that the intensity of TNF α was decreased (though not significantly) in CD200⁺ cultures, which was fully recovered by the addition of

anti-CD200 (Figure 2e). This finding indicates that CD200 can suppress both the magnitude and intensity of the memory Th1 response in AML and that blocking CD200 in this disease may be therapeutically advantageous.

Previously, we have shown that CD200 on AML cells directly impairs NK cell function.¹⁵ However, CD200 expression may not always promote immunosuppression in every context. One study using a CD200⁺ mouse plasmacytoma model showed that CD200 had the capacity to decrease production of the suppressive

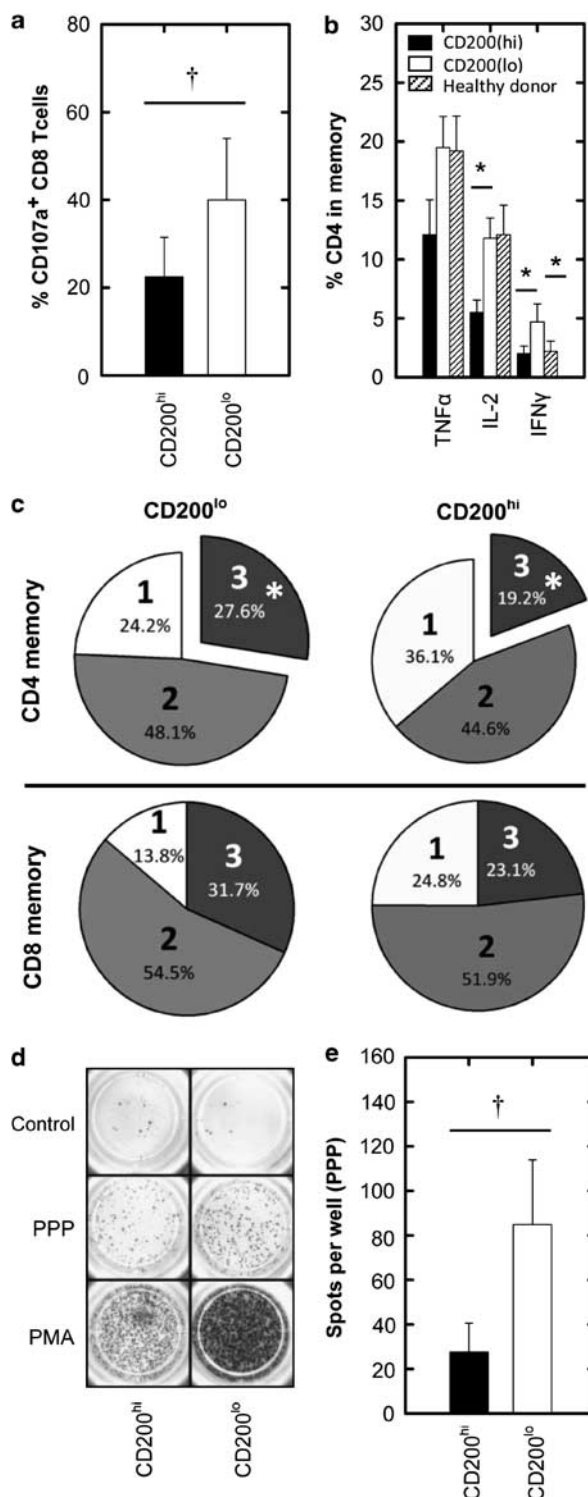
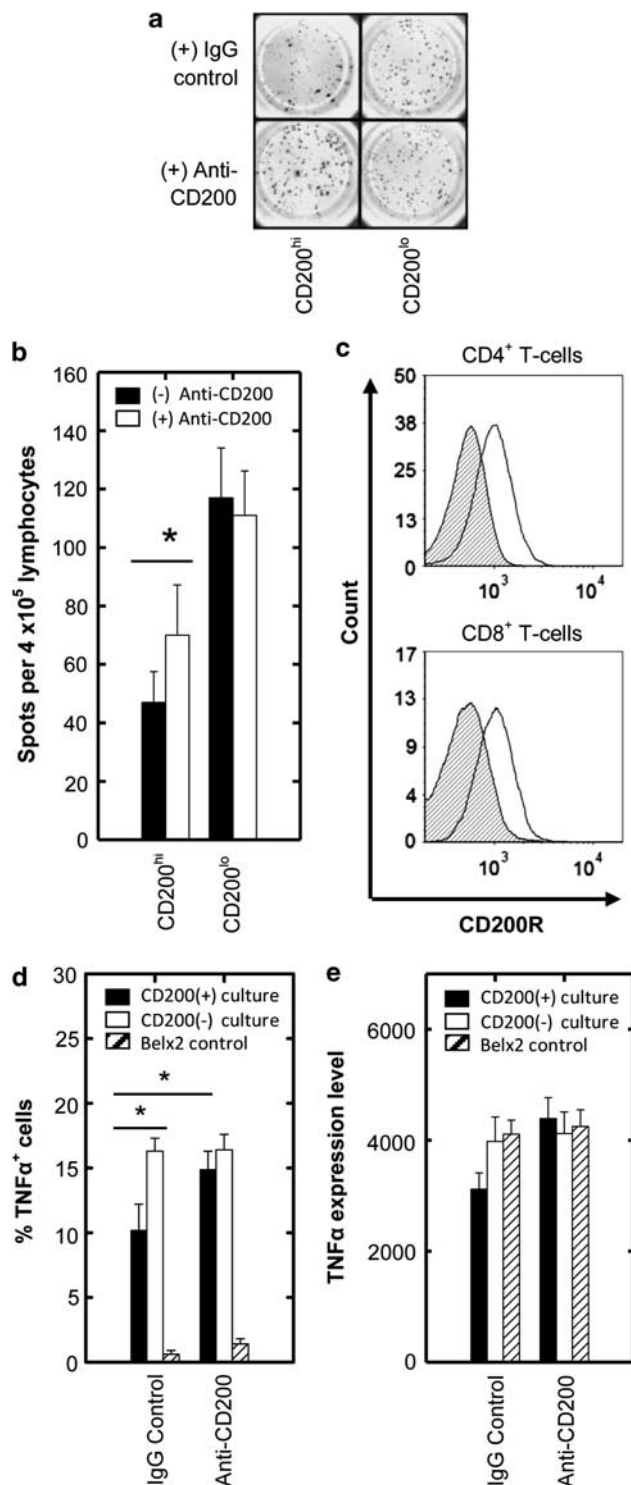


Figure 1. Cytotoxic T-cell response and Th1 memory/recall response in CD200^{hi} and CD200^{lo} AML patients. AML patient cytotoxic and intracellular Th1 cytokine memory T-cell responses were measured by flow cytometry following PMA/ionomycin stimulation (for full methods and flow cytometric gating strategies see Supplementary Materials and Methods and Supplemental Figure S1). (a) Summary data illustrating a significant difference in CD107a⁺CD8⁺ memory T-cells between CD200^{hi} and CD200^{lo} AML patients. (b) The production of TNF α , IL-2 or IFN γ for CD200^{hi}, CD200^{lo} and healthy donors for CD4⁺ memory T-cells. (c) Pie charts summarizing the proportion of CD4⁺ and CD8⁺ memory cells capable of producing one (1 = TNF α , IL-2 or IFN γ), two (2 = TNF α /IL-2, TNF α /IFN γ or IFN γ /IL-2) or three (3 = TNF α /IL-2/IFN γ) cytokines simultaneously for CD200^{hi} or CD200^{lo} (refer to Supplemental Figure S1 for Boolean analysis). The ability of T-cells from CD200^{hi} and CD200^{lo} AML patients to mount recall responses to common microbial antigens (PPP, Supplementary Materials and Methods) was assessed by IFN γ ELISPOT. (d) Representative ELISPOT wells for CD200^{hi} and CD200^{lo} patients. (e) Average spots per well for CD200^{hi} and CD200^{lo} patients. AML patient data represents mean \pm 1 s.d., $n=9$ for CD200^{hi} and $n=12$ for CD200^{lo}. $^{\dagger}P<0.05$, analyzed by one-tailed unpaired t -test; $*P<0.05$, analyzed by one-way ANOVA with Tukey's multiple comparison test.



cytokine IL-10 from tumor-associated myeloid cells resulting in an improved anti-tumor response.¹⁶ Whether this mechanism exists in human AML remains to be elucidated. Taken together, the data presented here suggests that at diagnosis, when the disease burden is high, the main mechanism of CD200 is to drive immunosuppression through direct interaction of CD200 on leukemia cells with CD200R on cells of the adaptive immune system. The situation may be different following reduction of tumor burden post-chemotherapy, where the influence of Treg cells may become a dominant factor in immunosuppression.¹⁷

Figure 2. Assessment of monoclonal anti-CD200 in relieving the inhibition of Th1 responses in CD200^{hi} AML patients with direct assessment of CD200R engagement on CD4⁺ memory T-cells. **(a)** Representative ELISPOT wells show the effect of anti-CD200 (+) or isotype control antibody (-) on IFN γ release following PPP stimulation for CD200^{hi} and CD200^{lo} AML patients. **(b)** Summary data illustrating IFN γ release in response to PPP stimulation as measured by ELISPOT for CD200^{hi/lo} AML patients +/- anti-CD200. **(c)** Representative flow cytometric plots illustrate CD200R expression on both CD4⁺ and CD8⁺ memory T-cells for AML patients (filled histograms represent immunoglobulin isotype-matched control and open histograms represent CD200R expression). Data represents mean \pm 1 s.d., $n = 7$. * $P < 0.05$, analyzed by one-tailed paired t -test. **(d)** To directly assess CD200R engagement on CD4⁺ memory T-cells, the CD4⁺ memory T-cell clone; Belx2 were co-cultured with control or CD200⁺ K562 cells. The percentage of TNF α -producing Belx2 T-cell clones in co-culture assays in response to CD3/CD28 costimulation was measured by flow cytometry following intracellular staining. Summary data illustrates the frequency of TNF α -producing T-cells in CD200⁺ and CD200⁻ K562 co-cultures +/- anti-CD200. **(e)** Summary data illustrating MFI of the TNF α ⁺ T-cell population in CD200⁺ and CD200⁻ K562 co-cultures +/- anti-CD200. Data represents mean \pm 1 s.d., $n = 6$. * $P < 0.05$, analyzed by one-tailed paired t -test.

In conclusion, we show for the first time that CD4⁺ Th1 memory and memory cytotoxic responses are significantly compromised in CD200^{hi} AML patients, which may contribute to the increased risk of relapse and worse overall survival observed in these patients. Most importantly we demonstrate that CD200 on leukemia cells directly suppresses T-cell responses, supporting the use of CD200-blocking therapy for the treatment of 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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Elucidation of a novel pathogenomic mechanism using genome-wide long mate-pair sequencing of a congenital t(16;21) in a series of three *RUNX1*-mutated FPD/AML pedigrees

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Familial platelet disorder with propensity to develop myeloid malignancy (FPD/AML OMIM 601399) is an autosomal disorder characterized by quantitative and qualitative platelet anomalies and predisposition to MDS/AML, caused by germ line point mutations, deletions and duplications in *RUNX1*.^{1–4} *RUNX1* encodes a transcription factor essential for definitive hematopoiesis, containing an amino-terminal DNA binding domain (RUNT) and a carboxyl-terminal transactivation domain (TA). *RUNX1* mutations act by either a dominant negatively interfering mechanism or by haploinsufficiency. Constitutional deletions spanning *RUNX1* have been reported in isolated congenital or syndromic thrombocytopenia, stipulating copy number analysis (CNA) of *RUNX1* next to sequencing.^{5–8} Given clinical heterogeneity, vigilance in diagnosing FPD/AML is required, reducing the risk of stem cell transplantation (SCT) with carrier siblings.^{2,3} Furthermore, the identification of a *RUNX1* abnormality and the elucidation of the underlying pathogenomic mechanism may have implications for genetic counseling for recurrence risk of FPD/AML.

This study included two families with classical FPD/AML and one seemingly isolated case with storage pool deficiency (SPD), thrombocytopenia and AML. All patients were of Caucasian origin. For detailed clinical and hematological characteristics of the pedigrees, we refer to Supplemental Table S1. *RUNX1* was analyzed by Sanger sequencing of coding exons 2–9 of gDNA using intronic primers.

In pedigree 1, gDNA was extracted from peripheral blood (PB) of III-2, III-3 and IV-1. A c.508 G > A was detected in *RUNX1c* exon 4 in affected family members III-2 and IV-1 causing an amino-acid substitution p.Gly170Arg (Figure 1), which is located in the RUNT

domain. Somatic mutations within this codon have been reported in sporadic AML/MDS.⁹ RUNT domain mutations have been related to a negatively interfering mechanism in FPD/AML.¹⁰ The unaffected mother (III-3) as well as DNA from 100 controls lacked the mutation. No DNA was available of the affected daughter V-2 and of IV-3 diagnosed with AML at age of 3 years.

In pedigree 2, sequencing of PB gDNA of III-1, III-5, IV-5 and IV-6 revealed a c.784C > T substitution in *RUNX1c* exon 6 in all four affected family members causing a premature stop (p.Gln262X) that would result in a truncated *RUNX1* protein lacking the TA domain. DNA from 138 controls lacked the mutation. Similar mutations in FPD/AML and sporadic MDS/AML have been reported previously.^{3,10} Interestingly, III-5 developed refractory anemia with excess blast with acquired trisomy 21, recurrently reported in FPD/AML.^{2,4}

In the index case of pedigree 3 (III-5), sequencing of *RUNX1* on DNA of leukemic bone marrow (BM) did not reveal mutations. gDNA was analyzed using a 105K oligonucleotide array (Agilent, Santa Clara, CA, USA), Oxford design, Amadid 0190015) and the Infinium HumanHap370 Genotyping BeadChip array (Illumina Inc., San Diego, CA, USA) containing 370 000 SNPs with a median probe spacing of 8 kb for copy number changes of *RUNX1*. These analyses did not disclose unbalance of *RUNX1*. Uniparental disomy of any chromosome was excluded.

Considering a possible *RUNX1* structural abnormality, FISH analysis on leukemic BM was performed, revealing a cryptic t(16;21)(p13;q22) with *RUNX1* locus rearrangement in 15 metaphases and 200 interphases (Figure 2a). This seminal observation with the patient's hematological history prompted us to investigate *RUNX1* status in non-hematopoietic tissues. Interphase FISH analyses on cells from urinary tract epithelium (endoderm) and buccal mucosa (ectoderm), showed *RUNX1* locus rearrangement in 86% and 41% of interphases,