The immunosuppressive ligands PD-L1 and CD200 are linked in AML T cell immunosuppression: identification of a new immunotherapeutic synapse

Long term remission in acute myeloid leukemia (AML) is generally not durable only being achieved in <50% of patients.[1] Consequently there is a need to establish new treatments to prevent relapse. A promising approach is to augment the anti-tumor immune response in these patients; however, it is well established that over-expression of immunosuppressive molecules such as CD200 on the surface of AML cells directly suppresses the anti-tumor response.[2–4] Nevertheless, blocking CD200:CD200R, only partially restores T cell activity, suggesting that alternative immunosuppressive mechanisms need to be explored if the anti-tumor response in AML is to be optimally exploited.[5]

Recently, promising clinical outcomes using humanized antibodies targeting PD-1 have been reported for melanoma and even for non-small cell lung cancer.[6] PD-1 suppresses immunological function via interaction with its cognate ligand PD-L1 (aka B7-H1, CD274) and previous work has indicated that PD-L1 may also suppress immunological function in AML.[7,8] Here we investigate whether the PD-1:PD-L1 axis cooperates with CD200 in mediating immunosuppression in AML patients.

Initially, we investigated whether CD200 and PD-L1 were co-expressed in AML blasts. Gene expression data from 158 AML diagnostic samples were analyzed and stratified into

$CD200^{hi}$ and $CD200^{lo}$ based on upper and lower quartiles of expression.[3] As shown in Figure 1A, $CD200^{hi}$ AML patients had 10-fold higher levels of PD-L1 mRNA compared to $CD200^{lo}$ patients. Flow cytometric analysis of CD200 and PD-L1 protein expression on AML blast cells confirmed this association at the protein level (Figure 1B). Taken together, these data establish that the immunosuppressive ligands CD200 and PD-L1 are co-expressed on patient AML blast cells, indicating that CD200 and PD-L1 could cooperate in AML cell mediated immunosuppression.

In common with other malignancies, robust $CD8^+$ T cell responses are thought to be important in AML anti-tumor immunity.[9] We previously demonstrated that CD200 overexpression in AML suppresses memory $CD8^+$ T cell effector function.[4] To investigate whether CD200 together with PD-L1 had the potential to inhibit $CD8^+$ T cell effector function, we first determined whether these cells expressed the respective negative co-receptors CD200R and PD-1in AML patients. This analysis showed expression of both CD200R and PD-1 on $CD8^+$ T cells from AML patients, interestingly, higher expression levels of PD-1 were observed for $CD200^{hi}$ AML patients (Supplemental Figure 1). To further characterize $PD\text{-}1^+$ T cells, we analyzed several AML patient $CD8^+$ T cell subpopulations, including; $CD57^+$ $CD28^-$ (late differentiated, poor anti-tumor function in AML)[10] and $CD57^-$ $CD28^+$ (early differentiated, important for robust anti-tumor function).[11] Figure 2A shows that the mean frequency of $CD57^+$ $CD28^-$ $PD\text{-}1^+$ and $CD57^-$ $CD28^+$ $PD\text{-}1^+$ $CD8^+$ T cells was almost twice that for $CD200^{hi}$ patients compared with $CD200^{lo}$. These findings show for the first time a link between CD200 expression level on AML blast cells and the frequency of $PD\text{-}1^+$ late differentiated and $PD\text{-}1^+$ early differentiated $CD8^+$ T cells, illustrating that CD200 and PD-1 are linked at multiple levels of $CD8^+$ T cell differentiation.

The above data suggested that stimulation of the CD200:CD200R immune-axis may have the capacity to induce PD-1 expression on target $CD8^+$ T cells. To investigate this, we assessed whether CD200:CD200R stimulation was directly capable of mediating PD-1 up-regulation on target $CD8^+$ T cells. We carried out a refined co-culture assay in which a $CD8^+$ T cell clone (7E7)[12] was incubated with $CD200^+$ or $CD200^-$ K562 cells.[3] CD200R expression on 7E7 T cells was confirmed by flow cytometry (Supplemental Figure 2). In these assays, 7E7 T cell PD-1 expression was monitored by flow cytometry. Figure 2B illustrates that in the presence of $CD200^+$ K562 cells, the frequency of $PD\text{-}1^+$ 7E7 T cells was significantly increased 1.5-fold compared with 7E7 T cells co-cultured with $CD200^-$ K562 cells and exceeded the level of PD-1 up-regulation achieved through CD3/CD28 receptor co-stimulation of 7E7. To verify these data, a CD200 blocking antibody[3] was added to the $CD200^+$ K562 7E7 T cell assay. Figure 2C illustrates a significant reduction in the frequency of $PD\text{-}1^+$ 7E7 T cells in the presence of the CD200 blocking antibody, demonstrating that PD-1 expression on target $CD8^+$ T cells can be reduced through CD200:CD200R blockade in a $CD200^{hi}$ setting. Taken together, our findings illustrate, for the first time, that CD200:CD200R interaction has the capacity to increase the frequency of $PD\text{-}1^+$ $CD8^+$ T cells.

To investigate the consequences of CD200 and PD-L1 co-expression on T cell activation, we created a series of K562 lines expressing CD200 or PD-L1 or both molecules in combination (Figure 2D). The 7E7 $CD8^+$ T cell clone produces tumor necrosis factor alpha (TNFα) upon stimulation,[12] and was used as an endpoint for 7E7 T cell activation in our assays. Figure 2E shows that both CD200 and PD-L1 induced a similar (>50%) reduction in the frequency of

activated 7E7 T cells (compared with co-cultivation with K562 control cells expressing neither molecule); however, when both CD200 and PD-L1 were co-expressed 7E7 T cell activation was almost ablated (~90% reduction). Moreover, the strength of the TNFα response was significantly reduced in co-culture assays where either CD200 or PD-L1 were present, indicating a direct effect at the level of $CD8^+$ T cell function (Supplemental Figure 3). These data demonstrate that CD200:CD200R and PD-L1:PD-1 engagement on T cells can act in tandem to produce a greater immunosuppressive effect on $CD8^+$ T cells when expressed on leukemia cells. This is of particular importance in AML, where both CD200 and PD-L1 are frequently co-expressed.

Previous studies in AML indicate that multiple immunosuppressive mechanisms may work in conjunction; for example co-expression of PD-1 and the negative regulatory receptor, Tim-3, identify a dysfunctional $CD8^+$ T cell population;[13] whilst in other contexts it has been shown that dual blockade of PD-L1:PD-1 and CTLA-4 is required to restore $CD8^+$ effector T cell anti-tumor responses.[14]. Here we propose that stimulation of the CD200:CD200R immune-axis augments the frequency of $PD\text{-}1^+$ $CD8^+$ T cells and that these in turn engage with PD-L1 on AML blasts, exacerbating immunosuppressive effects. Interestingly in AML, overexpression of both CD200 and PD-L1 in have been linked to a worse patient prognosis,[15,16]. Given the recent progress in PD-1 targeted immunotherapy (e.g. Nivolumab and Pembrolizumab) and also Samalizumab for CD200:CD200R blockade,[6,17] we propose a novel CD200/PD-L1 immunotherapeutic synapse in AML which should be targeted by combining CD200:CD200R and PD-L1:PD-1 blockade for future immunotherapy of AML.

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Author contributions

SJC designed and performed the experiments, analyzed all data and co-wrote the manuscript. MNG assisted with PD-L1 cloning and retroviral transduction. RR assisted with 7E7 T cell expansion. SK and AKB provided resources and clinical insight. SM, AT and RLD contributed to experimental design and co-wrote the manuscript.

Conflicts of interest

The authors declare no potential conflicts of interest.

On line supplementary information

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

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Figure legends

Figure 1. Assessment of CD200 and PD-L1 co-expression on AML patient blasts. The co-expression of CD200 and PD-L1 in AML patients was compared at the transcript level by microarray and at the protein level by flow cytometry. **(A)** Affymetrix gene expression data (U133 plus_2.0) from 158 AML patients was analyzed using GeneSpring v12.6 (Agilent Technologies). Gene expression data were normalized to median gene expression and expressed as Log_2 as previously described.[15] AML patients (Supplemental Table 1) were stratified to $CD200^{hi}$ and $CD200^{lo}$ (probe set; 209582_s_at) based on normalized expression level (n = 39 for each) as previously described.[4] Data were consistent for the alternative probe for CD200, 209583_s_at (not shown). Data illustrate a significant increase in PD-L1 normalized expression (probe sets; 223834_s_at and 227458_s_at) for $CD200^{hi}$ AML patients (mean ± s.e). **(B)** The association between CD200 and PD-L1 expression level on AML patient blast cells was analyzed by flow cytometry. AML blast cells were identified through CD45/CD34 bivariate analysis as previously described.[2] The data illustrate a positive correlation between CD200 and PD-L1 protein expression (normalized mean fluorescence intensity; MFI)[2] on AML patient blast cells; Pearson's correlation coefficient, $r^2 = 0.4901$, $p<0.01$ (n = 14). *$p<0.05$ analyzed by one-tailed unpaired t test. See Supplemental Methods for detailed methods.

Figure 2. Functional assessment of the relationship between CD200:CD200R and PD-L1:PD-1 co-expression. (A) Using flow cytometry, the percentage of PD-1^+ $CD8^+$ T cell subsets was evaluated between $CD200^{hi}$ and $CD200^{lo}$ AML age matched patients, median age; 53 (range, 35-64) and 54 (range, 17-70) respectively (Supplemental Table 1). **(B)** Assessment of PD-1 expression induction upon co-culture of the $CD8^+$ T cell clone 7E7 with either $CD200^+$ or $CD200^-$ K562 cells.[3] Assays were performed with or without prior CD3/CD28 receptor co-stimulation of 7E7 using 5µg/10^6 cells anti-CD3 (OKT3) and anti-CD28 (28.2) as previously described.[11] **(C)** Effect of 5µg/10^6 cells un-conjugated anti-human CD200 (MRC OX-104), on the frequency of PD-1^+ 7E7 $CD8^+$ T cells following co-culture with $CD200^+$ or $CD200^-$ K562 cells. Isotype matched IgG antibody was used as a control. **(D)** Flow cytometric analysis of K562 cells ($CD200^-$PD-$L1^-$) following retroviral transduction with CD200 and/or PD-L1 to create, $CD200^+$PD-$L1^-$ (CD200 single positive), $CD200^+$PD-$L1^+$ (double positive) or $CD200^-$PD-$L1^+$ (PD-L1 single positive) K562 cells. **(E)** The effect on 7E7 $CD8^+$ T cell activation of co-culture with K562 cells expressing CD200 and/or PD-L1. This was assessed by secretion of TNFα (intracellular cytokine staining)[4] in CD3/CD28 activated 7E7 $CD8^+$ T cells following co-culture with K562 cells (n = 9). Data are mean ± 1s.e. *$p<0.05$ analyzed by one-way ANOVA with Tukey's multiple comparison test. †$p<0.05$ and ††$p<0.01$ analyzed by one-tailed paired t-test.