

## CD200 Suppresses the Natural Killer Cells and Decreased its Activity in Acute Myeloid Leukemia Patients

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### Abstract

Immunosuppression is greatly involved in tumor escape from immune surveillance in acute myeloid leukemia (AML) patients. Being an immunosuppressive molecule, CD200 is upregulated in some hematological malignancies. CD200 also represents an independent prognostic factor in AML. In the present study, we assessed the influence of CD200 expression level in AML cases by flow cytometry on natural killer (NK) cell activity and evaluate its prognostic implications. In this study it was reported that CD200high patients showed a reduction in the frequency of activated NK cells (CD56dim) compared with CD200low patients. Survival analysis showed that the patients with CD200High expression had significantly shorter OS (median, 18 months) than the patients with CD200Low expression (median, 25 months) ( $P = 0.0188$ ) with hazard ratio of 0.4860 (95%CI: 0.2261–1.0447). Interferon- $\gamma$  level was highly expressed in AML cases with CD200low when compared to CD200high ( $P > 0.0001^*$ ). Generally, our findings suggest that CD200 overexpression suppresses NK cell antitumor response in AML patients and hence increased risk relapse in AML patients.

**Keywords:** Cd200; Natural killer cells; Cd56; Aml

### Introduction

CD200 is a trans-membrane cell surface glycoprotein belonging to the type-I immunoglobulin superfamily [1]. It is related to the B7 family of co-stimulatory receptors, with two extracellular domains, a single transmembrane region and cytoplasmic tail without signal motif [2]. Expression of CD200 is normally seen in some population of T and B lymphocytes, neurons and endothelial cells. The expression of CD200R1 which is the receptor for CD200 is frequently restricted to monocyte/macrophage lineage and certain population of T cells led to cytokine profiles from Th1 to T-regulatory cells [3]. Immunosuppression through engagement with CD200R, a cell surface receptor is expressed on leukocyte of myeloid lineage comprising macrophages, mast cells, dendritic cells, basophils and T-cell population [4].

In several human cancers, CD200 expression and function has been reported before [5] and its appearance in acute myeloid leukemia (AML) was reported by Tonk et al., [6] as there is overexpression in CD200 in hematological malignancies including AML and in solid tumors. In addition overexpression of CD200 in AML is a poor prognostic indicator, since the expression of this protein is a common character of cancer stem cells and it is closely related to the progress of the tumors [7]. However, the expression of CD200 and immunosuppression has an important role in the progression of the disease. Stem cells and other critical tissues are protected from immune damage by CD200 that has a central role in immune tolerance [8].

Leukemic cells express leukemia-associated antigen, MHC, co-stimulatory molecules and ligands for natural killer (NK) cells activating receptors, therefore leukemic cells are susceptible to attack by T and NK cells [9]. NK cells, T cells and dendritic cells are the most important players in immune surveillance of leukemic cells as their properties as anti-leukemia have been detected in different strategies of immune-therapy [10].

A significant impairment in NK cell function occur in several hematological malignancies as AML related to escape of immune surveillance [11]. NK cells have lytic cell activity against leukemic cells is inversely proportional to the progress and relapse rate of the disease. So, NK cells can provide a helpful prognostic marker to AML [12]. Lytic activity of NK against AML blast has inversely proportional to the progress and relapse rate of the disease which provide a helpful prognostic markers for AML as NK cell cytolytic activity is inversely correlated with progress of the disease and relapse rate. In AML it has been proposed that impaired NK cell cytokine production is accompanied with early relapse in spite of remission status [12]. So, in this study, we assessed the effects of CD200 that expressed on blasts cells in AML cases on the natural killer (NK) cell activity and evaluate its prognostic implications.

### Subjects and Methods

From a group of 102 newly diagnosed AML patients, Seventy eight patients presented with positive CD200 were enrolled in this study, their mean ages was  $43.18 \pm 11.8$  years range from (19-69), 49 cases (62.8%) were males and 29 cases (37.2%) were females with male to female ratio (1.7:1). The diagnosis and classification of AML patients were based on the French-American-British (FAB) and World Health Organization (WHO) criteria combined to immunophenotyping and

cytogenetic analysis [13,14]. Karyotyping was performed and chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN; 1985). Patients were divided into three risk groups of favorable, intermediate, and adverse for cytogenetic abnormalities according to the revised Medical Research Council prognostic classification [15]. For patients with AML, treatment with 7 days of cytarabine (Ara-C) and 3 days of daunorubicin has been a standard remission induction regimen used by the Cancer and Leukemia Group B (CALGB), Eastern Cooperative Oncology Group (ECOG) and others.

A written consent from all patients and an approval from the local ethical committee were obtained. This research was carried out in the period between (Jan 2012-Jun 2014).

Patients were reclassified into two groups as regard the level of CD200 group I: AML patients with high CD200 (CD200high) they were 46 patients (28 males and 18 females) and group II that included patients with Low CD200 (CD200low), they were 32 AML patients (21 males and 11 females).

## Methods

The diagnosis of AML depends on basis of full history taking emphasis on presence of leukemia associated symptoms (fever, pallor, bleeding tendency, bone aches). Through clinical examination was done lying stress on the presence and extent of leukemia involvement including organomegaly (liver and spleen), lymphadenopathy and CNS infiltration. Laboratory investigations included complete blood count by ADVIA 120 Hematology System (Bayer Corporation, Tarrytown, NY, USA) with examination of peripheral blood and bone marrow aspirate with check of Leishman stained films, cytochemical examination of peripheral blood and/or bone marrow smears for myeloperoxidase, CSF examination of blast cells, evaluation of liver and kidney function using Dimension® RxL Max® (Integrated Chemistry System, Siemens, USA).

Cytogenetic analysis was done using G banding technique: BM and PB samples on lithium heparin were studied by G banding analysis [16].

## Immunophenotyping by flow cytometry

Immunophenotyping of BM sample were performed on EDTA-anticoagulant BM aspirate using flow cytometry (FACScalibur Becton-Dickinson (BD), San Diego, USA). Samples were processed within 12 hours of collection.

For AML diagnosis, the following panel of monoclonal antibodies (MoAbs) was used. The MoAbs were conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or Peridinin-chlorophyll proteins (PerCP) were directed to CD33, CD13, CD14, CD64, CD117, CD19, CD20, CD22, CD45, HLA-DR, CD34, CD3, CD5, CD7 and TDT. 100µl of BM sample were added to 10µl of MoAb and the cell suspensions were then incubated for 15 min at 4°C. Cells were washed with phosphate buffer saline (PBS) and resuspended in PBS containing 2% fetal calf serum. One ml lysing solution (1:10) were added for 10 min. at room temperature and then centrifuged for 5 min at 350g. After two cell washes with PBS, the cell pellet was resuspended in 500µl PBS. Then, the sample became ready for acquisition and analysis by flow cytometry.

Cell surface immunophenotyping was performed for CD200 on blast cells in acute myeloid leukemia. CD200 (PerCP) antibody was

obtained from R&D system (Minneapolis, USA). Gating on myoblasts and lymphoid cells was based on CD45 versus side scatter analysis. The expression of CD200 on AML blast can be detected by excluded lymphocytes from analysis based on low side scatter and high CD45 expression, also CD200 expression evaluated as percentage and designated as either AML blasts with CD200 low relates to data of minimum percentage from (10%-50%), and CD200 high range from (51%-100%) of gated AML blast cells.

Sample positive for certain markers (cut off point) was by its expression >20% of gated cells except CD34 and HLA-DR were positive with >10% of the cells expressed those markers. Data acquisition was carried out on FACScalibur flow cytometry and analyzed using CellQuest software BD.

For the detection of NK cells, the samples were added to two tubes, which contained the following antibodies and fluorochromes (BD): in Tube #1, CD45 (FITC), CD19 (PE) and CD7 (PerCP); in Tube #2, CD3 (FITC), CD56 (PE) and CD16 (PerCP). The specimens were then incubated in the dark at 4°C for 20 min, resuspended and washed again in PBS. Data (20,000 events per tube) was acquired and analyzed using CellQuest Software. Antigen expression was depicted as mean fluorescence intensity (MFI) on dual parameter scattergrams in comparison to the cell stained with isotype controls antibody. NK cells were identified by first gating on CD45+ cells with low side scatter (lymphocytes), then subgating on CD3+ and/or CD7+ cells, and selecting the CD56+CD16+ cells.

## ELISA analysis for IFN-γ

Serum samples were collected and stored at -20 until analysis. The levels of Interferon-γ (IFN-γ), in the serum were assayed using specific ELISA kits according to the manufacturer's instructions (ELISA kits from eBioscience, San Diego, CA, USA). All assays were performed in duplicate. The results are expressed in pg/ml.

## Statistical analysis

All statistical calculations were done using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

Data were statistically described in terms of mean ± SD. Comparison of quantitative parametric variables between two studied groups was done t-student test. Chi-square and correlation coefficient study were done. The Kaplan-Meier's method was used to construct survival curves; Overall survival was the primary outcome studies and was calculated from the date of first diagnosis to death from any cause. A probability value (p value) less than 0.05 was considered statistically significant.

## Results

To characterize the expression of CD200 in this study, we analyzed 102 de-novo AML patients only 78 patients were CD200 positive, they were 49 males and 29 females with ratio 1.7:1.

Patients with denovo AML were classified according to the level of CD200 expression into CD200 high (CD200high) group which included 46 patients (58.9%) and CD200 low (CD200low) group which included 32 patients (41.1%). In AML cases with CD200high expression, There was highly significant different in the level of LDH when compared with cases with CD200low (Table 1).

As regard to the frequency of NK cells presence in AML cases that identified by assessing the percentage of CD56/CD16 by flow cytometry, it was found that AML cases with CD200high had a significant decrease in the frequency of NK cells when compared with AML cases with CD200low. There were no statistically significant differences between CD200high AML and CD200lowAML cases as regard the MFI levels of NK cells ( $t= 0.7315$ ,  $P=0.4667$ ), the NK cells in the two group expressed the same type of NK cells that characterized with CD56dim expression. NK cells in PB were mainly cytotoxic and most of them produce cytokines. IFN- $\gamma$  was an important cytokines secreted by NK cells. There was highly statistically significant difference in IFN- $\gamma$  levels between CD200high AML and CD200lowAML cases ( $t=27.9101$ ,  $P<0.001$ ) (Table 2).

As regard karyotyping, a lower frequency of CD200 positivity was found in favorable and relatively in intermediate cytogenetic groups (1/8, 12.5%) and (22/39, 56%), and high frequency was found in unfavorable cytogenetic group (15/20, 75%). A significant difference was found between the frequency of the cytogenetics risk groups of CD200 positive cases when compared to CD200 low cases ( $p = 0.0102$ ).

There was statistically negative correlation ( $r=-0.699$ ,  $P<0.001$ ) between the positivity of CD200 on AML blast cells and the number of NK cells (CD56 positive cells) in these AML cases (Figures 1 and 2).

With a median follow-up of 21 months (range, 3–36 months), Survival analysis showed that the patients with CD200high expression had significantly shorter OS (median, 18 months) than the patients with CD200Low expression (median, 25 months) ( $P = 0.0188$ ) with hazard ratio of 0.4860 (95%CI, 0.2261–1.0447).

	CD200High (46)	CD200Low (32)	Test of significant t	P
Age (year), mean $\pm$ SD	45.36 $\pm$ 12.17	41.64 $\pm$ 13.91	1.2520	0.214
@Sex (male: female)	28/18	21/11	0.04	0.812
WBC ( $\times 10^9/L$ ), mean $\pm$ SD (range)	46.63 $\pm$ 27.93 (8 – 108)	50.59 $\pm$ 22.93 (11 – 91)	0.6615	0.510
Hb g/dL	7.68 $\pm$ 2.11 (5 – 10)	6.93 $\pm$ 2.06 (3 – 10)	1.559	0.123
Plt $\times 10^9/ml/L$	40.47 $\pm$ 19.66 (11 – 78)	33.96 $\pm$ 17.9 (7 – 81)	1.4914	0.1400
§LDH (U/L), median (range)	1025 (164–3151)	654 (178–1575)	-3.62	0.0001*
@Auer rod positive/negative	36/10	14/18	9.77	0.002*
Cytogenetic risk				
Favorable	1/8 (12.5%)	7/8(87.5%)	Fisher test	0.0106*
intermediate	22/39(56%)	17/39(43.5%)		
Unfavorable	15/20(75%)	5/20(25%)		
NA	8/11	3/11		

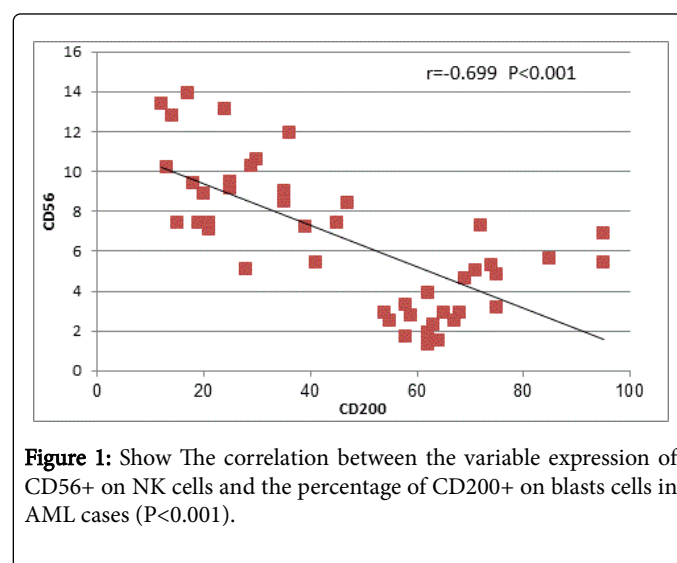
**Table 1:** Demographic data of AML groups.

§: Mann-Whitney test @: Chi-Square test \*: Highly significant

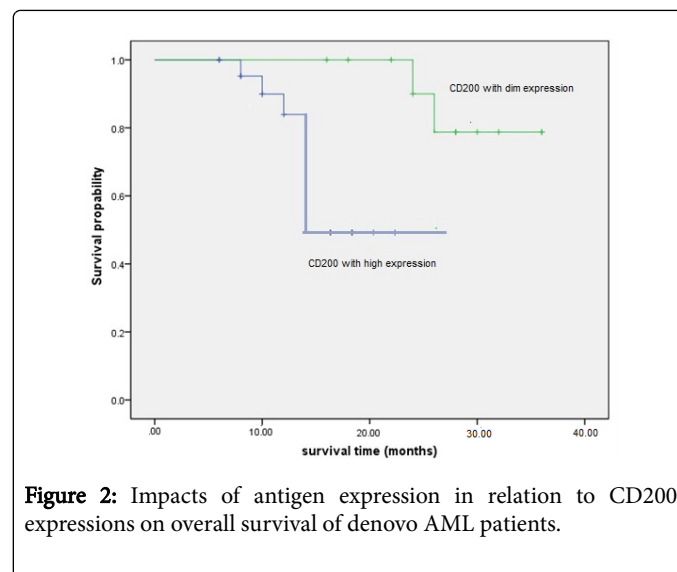
	CD200High (46)	CD200Low (32)	t	P
Frequency of NK cells	3.7 $\pm$ 1.7	9.3 $\pm$ 2.4	12.0725	0.0001*
MFI of NK cells expression	94.69 $\pm$ 35.1	89.45 $\pm$ 24.2	0.7315	0.4667
IFN- $\gamma$ pg/ml	51.52 $\pm$ 8.3	380.3 $\pm$ 79.5	27.9101	0.0001*

**Table 2:** The frequency, MFI and activity of NK cells in AML cases as regard CD200 expression.

\*: Highly significant



**Figure 1:** Show The correlation between the variable expression of CD56+ on NK cells and the percentage of CD200+ on blasts cells in AML cases ( $P<0.001$ ).



**Figure 2:** Impacts of antigen expression in relation to CD200 expressions on overall survival of denovo AML patients.

## Discussion

CD200 is an immunosuppressive glycoprotein that transduces inhibitory signals upon interaction with CD200R [2]. NK cells usually

exert direct cytotoxic activity and also activating other immune cells. In AML patients, it was detected that there were impaired in the cytotoxic activity of NK cells [17]. Also there were changes in the expression of both receptors and ligands which substantially impair killing mediated by NK cells [18].

In this study we examined the presence and role of CD200 and its influence as negative immune regulator according to fluctuation in its level and compared the NK cell frequency and activity in AML patients. In our study we found that 46 AML patients had positive CD200 with high expression and 32 patients with positive CD200 with low expression.

AML patients with CD200<sup>high</sup> showed significant increase of bad prognosis markers as old age, increased of total leucocytic count and presence of Auer rods when compared to AML cases with CD200<sup>low</sup>. CD200 expression was associated with poor prognosis in patients with AML and multiple myeloma [19].

In this study, AML patients display overexpression of CD200 (CD200<sup>high</sup>) also, demonstrated decreased NK cells more than twofold on AML patients with CD200<sup>low</sup>. This could be explained by the inhibitory effects of CD200<sup>high</sup> on NK cells in AML patients that potentially exacerbating immune dysfunction for NK cells which may suggest that CD200 directly regulate NK cells in these AML patients. These findings agreed with Coles et al. [20].

In our study the NK cells in both group of AML patients revealed no significant differences as regard their MFI, so we can concluded that the NK cells had the same subset in the two groups the CD56<sup>dim</sup> CD16<sup>+</sup> expression. This could be explained by Caligiuri, [21] who reported that the CD56<sup>dim</sup> CD16<sup>+</sup> NK cells represent at least 90% of all peripheral blood NK cells and are therefore the major circulating subset.

In healthy individuals, NK cells with this phenotype (the CD56<sup>dim</sup> CD16<sup>+</sup>) comprise the bulk of NK cells with high cytolytic activity and this was also the case for CD200<sup>low</sup> AML patients [20]. CD56<sup>dim</sup> can spontaneously lyse a target tumor cell but have limited ability to produce both cytokines and chemokines [22]. So, AML blasts with CD200<sup>low</sup> had less inhibitory effect on NK cells activity in these cases.

Aside from their cytotoxic function, NK cells have an important immune-regulatory role through the secretion of type-I and type-II cytokines. This is particularly apparent during the primary immune response in which NK cells are believed to be the principal source of IFN- $\gamma$  [23].

Cytokine secretion is generally associated with the CD56<sup>bright</sup> NK cell subset, however, CD56<sup>dim</sup> NK cells also have the capacity to produce type-I cytokines in direct correlation to cytolytic activity [23] and are capable of producing substantial amounts of IFN- $\gamma$  under similar conditions as AML [24].

INF- $\gamma$  was measured in AML cases with CD200<sup>high</sup> and CD200<sup>low</sup> to assess the activity of NK cells. INF- $\gamma$  was significantly reduced more than sevenfold in AML cases with CD200<sup>high</sup> when compared with AML cases with CD200<sup>low</sup>. This results was explained by Coudert et al. [25] who reported that CD200<sup>high</sup> AML blast cells potentially decreased the expression and activation of NK cells receptors and hence accelerated NK cells dysfunction, thus accelerate the worse diagnosis and increased risk of relapse.

The survival time in CD200<sup>high</sup> AML patients were shown to have inferior survival compared with those CD200<sup>low</sup> AML patients in our

study. This was in agreement with the study by Coles et al., [20], who reported that patients with CD200<sup>high</sup> AML, displaying both a reduced frequency and decreased levels of NCR expression, which jointly could contribute to decreased leukemic cell recognition by NK cells.

CD200 were upregulation in 43% of patients diagnosed with AML, with almost all M2 and M4 FAB types overexpressing this protein [26]. It is therefore possible that Fauriat et al. [17] mainly analyzed CD200 overexpressing patients, a cohort which we have previously identified to have a poor prognosis. Furthermore, Fauriat et al. [17], also reported that AML patients with an NK cell with dim phenotype were prognostically disadvantaged, with around 30% AML patient survival after 5 years.

Previous study suggested that blocking of CD200 by use of antibody treatment leads to inhibition and suppression of the interaction between CD200 and CD200R that activates patient NK, making CD200 a potential therapeutic target for CD200<sup>+</sup> AML as blocking and inhibition CD200 on AML blasts by inhibiting its interaction with CD200R receptor leads to restoring adaptive and innate immune response for destruction of tumor cells. So, it is very effective to use CD200 blocking antibody as a strategy in blocking remission repopulation of lymphocyte cells [27].

In conclusion, our data indicate that expression of CD200<sup>high</sup> in blast of AML patients are usually accompanied by bad prognosis and increased risk of relapse and it was an independent prognostic factor. This suggests that utilization of CD200 blocking antibody in treatment strategies as a novel therapy may be effective in remission especially if there was predescription that lymphocyte populations began to repopulate.

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