The effects of PI3K/Akt/mTOR Signaling Pathway Inhibitors on Immune Evasion Mechanisms of Acute Myeloid Leukemia Cell Line

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Research Article

Keywords: PI3K/Akt/mTOR, immune evasion, AML, Idelalisib, MK-2206, Everolimus

DOI: https://doi.org/10.21203/rs.3.rs-404383/v1

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Abstract

Purpose: One of the most important immune escape mechanisms of AML is the up-regulation of immune checkpoint ligands. Herein, we have investigated the relationship between the inhibition of these pathways and the expression regulation of the immune checkpoint ligands in AML cells.

Methods: HL-60 cell line was treated with different inhibitors including idelalisib as PI3K inhibitor, MK-2206 as Akt inhibitor, and everolimus as mTOR inhibitor either in single or combination therapy. Cell viability and apoptosis were evaluated using MTT and flow cytometry assays, respectively. The relative expression of PD-L1, galectin-9, and CD155 was determined by Real-Time PCR.

Results: Our findings demonstrated decreasing in proliferation and increasing in apoptosis of HL60 cells after treatment with idelalisib, MK-2206, and everolimus. As expected, combined treatment showed more growth inhibition when compared to single treatment. Interestingly, our results demonstrated that the expression of PD-L1 and Gal-9 but not MK-2206, was decreased after treatment with idelalisib and everolimus. Regarding CD155, the expression of this molecule was downregulated after treatment with everolimus, but not idelalisib and MK-2206. However, combined treatment of HL-60 cells with two or three inhibitors decreased the expression levels of PD-L1, Gal-9 and CD155 checkpoint ligands.

Conclusion: We showed that PI3K/Akt/mTOR pathway inhibitors not only serve as cytotoxic drugs, but also regulate the expression of immune checkpoint ligands and interfere with the immune evasion mechanisms of AML leukemic cells. Combinational therapy approaches to block these pathways might be a promising and novel therapeutic strategy for AML patients via interfering in immune escape mechanisms.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematopoietic malignancy defined by abnormal proliferation and differentiation of hematopoietic myeloid precursor cells, leading to accumulation of blast cells in bone marrow, blood, and other tissues (Saultz and Garzon 2016, Luppi et al. 2018). Among hematological malignancies, AML is the most common disorder in adult, and the prevalence is enhancing with the age of the population. AML consists of subtypes that are biologically, molecularly, and clinically different and complex (Deng et al. 2017, Luppi et al. 2018). Most AML patients react well to treatment with earlier therapy, but, nearly 15–30% of them are predominantly resistant to common chemotherapy (Feldman 2015, Sandhöfer et al. 2015). Furthermore, a number of patients who achieve complete remission are associated with the increased levels of clinical relapse (Feldman 2015, Sandhöfer et al. 2015, Khaled et al. 2016). So, to find the new targeted therapies with an improved cure rates and lower toxicity, the development of specific small molecules which target the main signaling pathways is an exciting alternative therapeutic strategy (Bertacchini et al. 2015, Sandhöfer et al. 2015).

Abnormal intracellular signaling is frequently observed in AML patients, including the phosphatidylinositol-3-kinase-Akt-mechanistic/mammalian target of rapamycin (PI3K-Akt-mTOR)
pathway, which appears to be essential in both normal and leukemic hematopoiesis (Polak and Buitenhuis 2012, Brenner et al. 2016). This pathway is involved in several cellular activities, such as cell cycle progression, cell survival, proliferation, apoptosis, differentiation, protein synthesis, metabolism, migration, autophagy, angiogenesis, and resistance to chemotherapy (Bertacchini et al. 2015, Brotelle and Bay 2016, Nepstad et al. 2018). Prior studies have indicated that the PI3K/Akt/mTOR pathway is over-activated and correlated with reduced overall survival in AML, and then continued proliferation of leukemic cells in nearly 50–70% of cases (Dos Santos et al. 2006, Bertacchini et al. 2015). The discovery of small molecule inhibitors as selective inhibitors for this pathway with a high therapeutic index, provides a new strategy for the treatment of AML patients (Dos Santos et al. 2006, Polak and Buitenhuis 2012). During the last decades, the efficacy of PI3K/Akt/mTOR inhibitors are discussing in the preclinical and clinical studies on AML (Bertacchini et al. 2015). In this regard, there are some ongoing clinical trial studies in phase 1 and 2 applying idelalisib, MK2206, and everolimus in AML either as monotherapy or combination with other therapeutic approaches.

On the other hand, it has been extensively understood that the immune system can identify and control tumor cells described as the process called tumor immunesurveillance (Smyth et al. 2006). In addition, leukemic cells use a variety of strategies to evade immune responses, resulting to tumor progression and relapse (A Knaus et al. 2017). There are several mechanisms for tumor immune escape, such as the modulation of tumor surface antigen, low immunogenicity of tumor cells, recognition of tumor-specific antibodies as autoantigens, and tumor-induced immunosuppression, the latter of which has been the most widely studied mechanism to date (A Knaus et al. 2017, Jiang et al. 2019, Taghiloo and Asgarian-Omran 2020). Immunosuppression mechanisms involve expression of the immunosuppressive receptors or their ligands, such as programmed death-1/programmed death-ligand 1 (PD-L1/PD-1), T-cell immunoglobulin and mucin domain-3/galectin-9 (Tim-3/Gal-9), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T cell immunoreceptor with Ig and ITIM domains (TIGIT), and lymphocyte-activation gene 3 (LAG-3) which are known as the immune checkpoint molecules (Teague and Kline 2013, Jiang et al. 2019, Rajabian et al. 2019). The interaction of immune checkpoint receptors with their ligands causes immune cell exhaustion, which is associated with impaired function of these cells and tumor progression (Sakuishi et al. 2010, Taghiloo and Asgarian-Omran 2020). Nevertheless, there is still no detailed information on the intracellular mechanisms and signaling pathways of these processes in AML patients.

To explore the association of the signaling pathways with the tumorigenesis and immune evasion mechanisms, we have investigated the relationship between the blockade of the PI3K/Akt/mTOR signaling pathways and the expression of the immune checkpoint ligands on AML cells as one of the crucial immune escape mechanisms.

Materials And Methods

Reagents
Idelalisib, MK-2206 and everolimus were purchased from Cayman chemical company (Michigan, United States) as PI3K inhibitor, Akt inhibitor, and mTOR inhibitor, respectively. These chemicals were dissolved in dimethyl sulfoxide (DMSO) and stored frozen in aliquots.

**Cell line and culture**

HL-60, a human AML FAB-M2 cell line, was obtained from Pasteur Institute of Iran (Tehran, Iran). Cells were maintained in RPMI-1640 (Biowest, Nuaille, France) medium supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Nuaille, France), 100 units/mL of penicillin, and 100 µg/mL of streptomycin. For experimental procedures, cells were seeded into culture flasks and grown in a humidified atmosphere of 5% CO2 at 37°C. To define the optimal concentration of drugs, cells were treated at concentrations up to 64 µM of idelalisib, MK-2206, and everolimus for 24 and 48 hours in 96-well culture plates. Half-maximal inhibitory concentration (IC50) values were calculated for all drugs using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA) curve-fitting software.

**Cell viability assay**

Cell viability was evaluated by MTT assay. Briefly, leukemic cells were seeded in 96-well plates at 15000 cells/well, treated with signaling inhibitor drugs and then incubated at 37°C for 48 hours. All assays were done in triplicate. After incubation time, MTT reagent was added to each well at final concentration of 0.5 mg/ml. Following incubation for 4 hours at 37°C, the microplates were centrifuged at 300 g for 10 min, the supernatants were discarded and 150 µl of DMSO was added to each well. Crystals of formazan were dissolved by shaking of microplates, and absorbance was measured using a microplate spectrophotometer (Synergy H1 BioTek, Winooski, USA) at 570 nm in a 630 nm. Determination of cell viability was calculated as follows: percentage of cell viability = (absorbance \text{ sample} - absorbance \text{ blank})/(absorbance \text{ control} - absorbance \text{ blank}) x 100%.

**Cell apoptosis assay**

HL-60 cell line was incubated for 48 hours with signaling inhibitors. After that, apoptosis-mediated cell death was examined by a dual-color FITC-labeled Annexin V/propidium iodide (PI) apoptosis detection kit (Thermo Fisher Scientific, San Diego, CA, USA) according to the manufacturer’s protocol. Briefly, 250000 treated and untreated cells were collected after incubation time, and then washed in phosphate-buffered saline (PBS) twice and incubated with binding buffer containing 5 µL of FITC-conjugated Annexin V for 10 min at room temperature. Following incubation, cells were washed in PBS twice, then were incubated with binding buffer containing 10 µL of PI (20 µg/mL). Finally, the externalization of phosphatidylserine and the permeability to PI were evaluated using a Partec PAS flow cytometer system (Partec GmBH, Munster, Germany). Cells in early stages of apoptosis were positively stained with Annexin V, whereas cells in late apoptosis or necrosis were positively stained with both Annexin V and PI.

**RNA isolation and semi-quantitative Real-Time PCR**

To measure the gene expression of PD-L1, Gal-9, and CD155, total RNA was extracted using Denazist Asia kit (Mashhad, Iran) based on the manufacturer’s instruction. The RNAs were then reverse transcribed
to cDNA by Yekta-tajhiz cDNA synthesis kit (Tehran, Iran), in brief by Moloney murine leukemia virus reverse transcriptase, coupled with random hexamers under standard conditions. Semi-quantitative Real-Time PCR (qRT-PCR) was carried out in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR green as the detection dye (Ampliqon, Copenhagen, Denmark). The specific primer pairs were shown in Table 1, and the primer efficiencies were confirmed by standard curves following the reaction. Finally, the transcript levels of PD-L1, Gal-9, and CD155 were normalized to β-actin and the relative expression of each molecule was calculated using the $2^{-\Delta\Delta Ct}$ method.

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5'-3')</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galectin-9</td>
<td>F: TTTCTGGGACTATTCAAGGAG  R: GAAGTGGAAGGCAATGTCA</td>
<td>137 bp</td>
</tr>
<tr>
<td>PD-L1</td>
<td>F: CTATGGTGTTGGCAGACTACAA  R: CTGCTTCGACGATTGACTTCG</td>
<td>159 bp</td>
</tr>
<tr>
<td>CD155</td>
<td>F: GGACGGCAGAAATGTGAC  R: CCAGTTGTTATCATAGCCAGAG</td>
<td>124 bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: CCTTCCTGGGATGGAGCGTCT    R: TGGGTGCCAGGGCAGTGAT</td>
<td>174 bp</td>
</tr>
</tbody>
</table>

F, Forward primer; R, Reverse primer

### Statistical analysis

Statistical analyses were performed with GraphPad Prism 6 and SPSS20 softwares. Quantitative data are expressed as means ± SD. Analysis was performed using Kolmogorov-Smirnov test to determine the normality distribution of the obtained data, and one-way analysis of variance (ANOVA) followed by the Dunnett test for multiple comparisons. P-values less than 0.05 were considered statistically significant.

### Results

#### PI3K/Akt/mTOR inhibitors cause growth inhibition in HL-60 cells

The effects of PI3K/Akt/mTOR inhibitors on growth inhibition were examined in HL-60 cell line. Cell viability was measured via MTT assay following 48 hours of exposure to various concentrations of idelalisib, MK-2206, and everolimus. As shown in Fig. 1, after single exposure to idelalisib, MK-2206, and everolimus at concentrations up to 64 µM, HL-60 cells indicated a dose-dependent reduction in viability, with an IC$_{50}$ value of 5 µM, 5 µM, and 2 µM, respectively. In the next step, we measured the anti-
proliferative activity of single or combined treatment by MTT assay using calculation of the relative cell proliferation index for all samples. In this experiment, we found that the proliferation of HL60 cells was significantly reduced by treatment with idelalisib, MK-2206, and everolimus (p < 0.0001, Fig. 2, supplementary data). As expected, co-treatment with these drugs led to an enhanced cell growth inhibition when compared to single treatment (Fig. 2).

**PI3K/Akt/mTOR inhibitors induce apoptosis in HL-60 cells**

Since apoptosis may contribute to a decrease in cell viability, we also examined apoptosis induction of idelalisib, MK-2206, and everolimus in HL-60 cells. Flow cytometric analysis was carried out with Annexin V-FITC/PI staining of HL-60 cells. As indicated in Fig. 3A and 3B, the apoptotic level of HL-60 was increased after treatment with inhibitors when compared with untreated cells, but single treatment with any of the three drugs could not induce a significant difference in apoptosis of HL-60 cells. Combined treatment with all three drugs led to an increased cell apoptosis comparing to either single treatment or co-treatment with two drugs (Fig. 3A and 3B, p = 0.01, supplementary data). Notably, the apoptotic cells were mainly in the late-stage of apoptosis.

**The expression of immune checkpoint ligands were reduced after treated with PI3K/Akt/mTOR pathways inhibitors**

To further understand the molecular events underlying the immune escape mechanisms of AML, the effects of PI3K/Akt/mTOR inhibitors on the expression of key immune checkpoint molecules including PD-L1 as PD-1 ligand, Gal-9 as Tim-3 ligand, and CD155 as TIGIT ligand were studied on HL-60 cell line. The relative expression of PD-L1, Gal-9, and CD155 mRNA was evaluated in all samples by a semi-quantitative Real-Time PCR method using β-actin as an internal control. Our results demonstrated that the expression of PD-L1 was significantly reduced on HL-60 cells after treatment with idelalisib, and everolimus alone when compared to untreated group (p = 0.003 and p < 0.0001, respectively), but the difference was not significant for MK-2206 group (Fig. 4A, supplementary data). Interestingly, the expression of PD-L1 was more decreased after co-treatment with two or three drugs (p < 0.0001, Fig. 4A, supplementary data). Similar to PD-L1, our results showed the down-regulation of Gal-9 immune checkpoint molecule on HL-60 cells after treatment with idelalisib, and everolimus alone (p < 0.0001, Fig. 4B), but not for MK-2206 (Fig. 4B, supplementary data). However, after combined treatment of HL-60 with two or three inhibitors, Gal-9 expression was strongly reduced (p < 0.0001, Fig. 4B). Our data indicated that the expression of CD155 was significantly decreased on HL-60 treated with everolimus (p = 0.02, Fig. 4C), but not with idelalisib and MK-2206 (Fig. 4C). Nevertheless, CD155 expression was vigorously decreased after combined treatment with two or three inhibitors (p < 0.0001, Fig. 4C, supplementary data). Finally, the current results were analyzed to find any correlations between the expressions of these immune checkpoint ligands. Our results indicated significant positive correlations between the expression of PD-L1, Gal-9, and CD155 on HL-60 after single treatment with each inhibitors.
Understanding the immune escape mechanisms of cancerous cells in tumor microenvironment is crucial to develop and improve new approaches for tumors targeted therapy (Teague and Kline 2013, Beatty and Gladney 2015). There are numerous mechanisms for tumor cells to escape from the host immune system, which one of the most important of them is the expression of immune checkpoint molecules (Blank et al. 2005, Drake et al. 2006, Beatty and Gladney 2015). This mechanism includes the over-expression of immune checkpoint receptors on immune cells and their ligands on tumor cells, such as PD-1/PD-L1, Tim-3/Gal-9, and TIGIT/CD155 (Blank et al. 2005, Beatty and Gladney 2015). These interactions play a pivotal role in the immune suppressing behavior of the tumor microenvironment, which may also inhibit the antitumor immunity and eventually lead to the immune escape of tumor cells (Dong et al. 2016, Jiang et al. 2019, Peng et al. 2019). Targeting these pathways is an appealing approach for cancer immunotherapy, but the clinical efficacy remains poor in some tumors. These conditions need a greater comprehension of the complex and diverse molecular mechanisms and the intracellular signaling pathways in tumor cells (Dong et al. 2016, Jiang et al. 2019). On the other hand, blockade of signaling pathways using small molecule inhibitors is a new promising strategy for cancer treatment. Various small molecule inhibitors are currently being evaluated in clinical trials for hematopoietic and non-hematopoietic malignancies. However, little is known about their possible associations with the immune evasion mechanisms. Accordingly, in this study we aimed to find any associations between the PI3K/Akt/mTOR signaling pathways and the expression of immune checkpoint ligands PD-L1, Gal-9, and CD155 on AML cells. Our results revealed that the blockade of PI3K/Akt/mTOR pathways could be a potential target for regulating the expression of PD-L1, Gal-9, and CD155 on HL-60 myeloproliferative cell line. The PI3K/Akt/mTOR pathway is of particular interest among the main abnormal intracellular signaling pathways engaged in hematological malignancies (Barrett et al. 2012, Bertacchini et al. 2015, Sandhöfer et al. 2015). Over-activation of PI3K/Akt/mTOR suggests an essential role for this axis in tumor progression of several hematological malignancies, including AML, chronic myelogenous leukemia (CML), and acute lymphoblastic leukemia (ALL), as well as in lymphoproliferative disorders (Barrett et al. 2012, Deng et al. 2017, Herschbein and Liesveld 2018). Recent studies have demonstrated that the PI3K/Akt/mTOR pathway is activated, in 50–80% of AML patients (Dos Santos et al. 2006, Martelli et al. 2010). Moreover, practically high levels of mTOR signaling is displayed in all AML patients (Park et al. 2010). Because of these discrepancies, it is assumed that dual or triple blockade of PI3K/Akt/mTOR pathway could be more effective than single blockade in AML. Previous reports suggested that the blockade of these pathways reduce cell viability and induce apoptosis of AML cells (Martelli et al. 2007, Deng et al. 2017, Hao et al. 2019). Similar to these finding, we showed that the combination blockade of PI3K/Akt/mTOR pathways by small molecule inhibitors reduces the cell viability and enhances the apoptosis on HL-60 cell line. Since the PI3K/Akt/mTOR pathway is involved in activation of some oncogenes participated in AML oncogenesis, we postulated that this pathway might regulate the immune escape mechanisms and especially the expression of immune checkpoint ligands in AML. Our findings indicated a strong correlation between the expression of PD-L1, Gal-9, and CD155 and over-activation of the PI3K/Akt/mTOR pathway in AML. Previous reports showed the associations
between over-expression of immune checkpoint ligands and tumor immune escape (Lin et al. 2015, Qin et al. 2015, Taghiloo et al. 2017). Interaction of the PD-1/PD-L1, Tim-3/Gal-9, and TIGIT/CD155 pathways were able to regulate the host immune response and T-cell exhaustion in tumor microenvironment (Pardoll 2012, Allahmoradi et al. 2017, Silva et al. 2017, Taghiloo et al. 2017). Over-activation of PD-L1 and Gal-9 and CD155 is reported in a substantial number of solid and hematopoietic malignancies, but little is known about the expression of CD155 on leukemia cells (Pardoll 2012, Folgiero et al. 2015, Dong et al. 2016, Taghiloo et al. 2017, Zhao et al. 2017). Previous findings demonstrated that the activation of receptor-mediated signaling pathways, such as PI3K/Akt/mTOR, JAK/STAT, MEK/ERK, and NF-κB promote the immune escape mechanisms by regulating the expression of PD-L1 in several tumors (Ritprajak and Azuma 2015, Lastwika et al. 2016, Peng et al. 2019). In this regard, several reports indicated that the up-regulation of PD-L1 is heavily dependent on PI3K/Akt/mTOR signaling pathway in glioma, lung cancer, and pancreatic cancer (Parsa et al. 2007, Zhang et al. 2013, Zhang et al. 2017). In contrast with these reports, PD-L1 expression is also correlated with the MEK/ERK signaling pathway in myeloma cells, but not the PI3K/Akt/mTOR signaling pathways (Liu et al. 2007). It also should be noted that the expression regulation of these ligands is very complex and most probably depends on the status of underlying signaling pathways. Interestingly, most of the research in this area is related to PD-L1 expression and not much information is available about other checkpoint ligands like Gal-9 and CD155. To our knowledge, there are no evidences to investigate the association between the expression of immune checkpoint ligands with PI3K/Akt/mTOR signaling pathways in hematological malignancies. Here, our data showed that the combination blockade of PI3K/Akt/mTOR pathway by small molecule inhibitors is associated with the down-regulation of PD-L1, Gal-9, and CD155 in AML cell line. These results highlight the importance of combination blockade of PI3K/Akt/mTOR pathway as a promising target for potentiating anti-tumor immune responses by regulating the expression of immune checkpoint ligands on AML cells.

In conclusion, our findings demonstrated that the expression of PD-L1, Gal-9, and CD155 is significantly decreased after co-treatment with small molecules, including idelalisib, MK-2206, and everolimus, as PI3K/Akt/mTOR pathway inhibitors. We showed that PI3K/Akt/mTOR pathway inhibitors not only serve as cytotoxic drugs, but also regulate the expression of immune checkpoint ligands and interfere with the immune evasion mechanisms of AML leukemic cells. Combinational therapy approaches to block these pathways might be a promising and novel therapeutic strategy for AML patients via interfering in immune escape mechanisms.

Declarations

Acknowledgments

The authors thank the investigators and study teams who were involved in this project.

Funding

This study was supported by Mazandaran University of Medical Sciences. Project number 1397.3186.
Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships.

Ethical approval

This study was found to be ethically acceptable by the Ethical Committee of Mazandaran University of Medical Sciences (IR.MAZUMS.IMAMHOSPITAL.REC.1398.075).

Author contributions

HA-O conceived the original idea and designed the experiments. ST and SN performed the experiments. ST wrote the manuscript. HA-O edited the manuscript and approved the final draft. All authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Consent to Participate

Not applicable

Consent to Publish

Not applicable

References


42. Taghiloo S, Asgarian-Omran H (2020) "Immune evasion mechanisms in Acute Myeloid Leukemia; a focus on immune checkpoint pathways." Critical Reviews in Oncology/Hematology: 103164


Figures
Determination of IC50 value for Idelalisib, MK-2206, and Everolimus HL-60 cells were treated with various concentrations (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 μM) of idelalisib (A), MK-2206 (B), and everolimus (C) for 48 hours. Cell viability was measured by MTT assay. IC50 value of idelalisib, MK-2206, and everolimus were calculated to be 5 μM, 5 μM, and 2 μM, respectively. Data are mean ± SD, representative of three independent experiments.

Figure 1
Figure 2

Effects of PI3K/Akt/mTOR inhibitors on proliferation of HL-60 cells HL-60 cells were cultured in the absence or presence of idelalisib, MK-2206, and everolimus for 48 hours either in single or combined treatment. After that, cell proliferation was determined by MTT assay. The results are expressed as the means ± SD of three independent experiments. One-way ANOVA with Tukey’s post hoc test was used for analyses. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05.
Figure 3

Effects of PI3K/Akt/mTOR pathway inhibitors on apoptosis of HL-60 cells. HL-60 cells were cultured in the absence or presence of idelalisib, MK-2206, and everolimus for 48 hours either in single or combined treatment. After that, apoptotic cells were detected by Annexin-V/PI staining assay via flow cytometry. A. Representative flow cytometric dot plot is shown. B. The percentage of apoptotic HL-60 cells is represented. The results are expressed as the means ± SD of three independent experiments. One-way
ANOVA with Tukey’s post hoc test was used for analyses. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05.

Figure 4

Effects of PI3K/Akt/mTOR pathway inhibitors on the expression of PD-L1, Gal-9, and CD155 HL-60 cells were cultured in the absence or presence of idelalisib, MK-2206, and everolimus for 48 hours either in single or combined treatment. After that, total RNA was extracted from all combinations and single-stranded cDNA was synthesized. Real-Time PCR was performed with specific primers for PD-L1, Gal-9, CD155, and β-actin. (A) Relative mRNA transcript levels of PD-L1. (B) Relative mRNA transcript levels of Gal-9. (C) Relative mRNA transcript levels of CD155. Gene expression results are represented as mean ± SD of 2-ΔΔCt after normalization with β-actin as an internal control. The results are expressed as the means ± SD of three independent experiments. One-way ANOVA with Tukey’s post hoc test was used for analyses. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05.