

# Low Dose of Lenalidomide Enhances NK Cell Activity: Possible Implication as an Adjuvant

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## ABSTRACT

**Background:** Lenalidomide, a synthetic immunomodulatory drug, has a wide range of features including anti-angiogenic and anti-proliferative properties. To date, researchers have shown that lenalidomide is capable of ameliorating the immune system factors and antitumor responses. Most researchers have reported that lenalidomide enhances the immune response in certain cancer patients through several pathways including the stimulation of Natural Killer cells; notwithstanding, it is still crucial to investigate the effect of lenalidomide on the activity of NK cell cytotoxicity both *in vitro* and *in vivo*. **Objective:** To evaluate the *in vitro* impact of lenalidomide, of different doses, on NK cytotoxicity activity and an *in vivo* investigation to find the adjuvant behavior of lenalidomide. **Methods:** NK cytotoxicity was measured with the lactate dehydrogenase (LDH) release assay via K562 cells. Lenalidomide was prepared at 1 mM, 2 mM, 4 mM and 8 mM for *in vitro* study. In addition, the adjuvant properties of lenalidomide were assessed in ten mice groups using NS3 HCV DNA vaccine model of antigen pcDNA3.1(+)/NS3. **Results:** The results showed that, comparisons to other doses, 4 mMol of lenalidomide was able to noticeably increase NK cytotoxicity activity. Furthermore, the animal model indicated that lenalidomide stimulated NK cytotoxicity *in vivo*, augmenting it from 16.67% ± 2.07% for the control group to 38.17% ± 2.87% for the lenalidomide-treated. **Conclusion:** Treatment by lenalidomide and pcDNA3.1(+)/NS3 improves NK cytotoxicity up to 66.80% suggesting that lenalidomide can be used in parallel with such therapeutic vaccines as cancer vaccine or virus vaccines.

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**Keywords:** Lenalidomide, NK cell, Adjuvant, LDH test

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## INTRODUCTION

Lenalidomide Immunomodulatory drug (IMiDs) (Revlimid TM) is a synthetic derivative of thalidomide approved by Food and Drug Administration (FDA) for multiple myeloma (MM) and myelodysplastic syndrome (MDS) (1). Davies *et al.* (2) inspected the potential immunomodulatory effects of thalidomide therapy in patients with multiple myeloma. They demonstrated that the *in vitro/in vivo* role of NK cell cytotoxicity in thalidomide-treated patient increased IL-2 levels and improved the innate and adaptive immune system. In so far as chemical structure is concerned, thalidomide shares certain features with lenalidomide. Unlike thalidomide, however, lenalidomide is not teratogenic in rabbit models and is more potent to promote the immune system, a fact attributed to the low dose of lenalidomide used by adjuvant. Reviewing the literature, it becomes patent that the side effects of lenalidomide, as a cancer vaccine adjuvant, are but few and far between (1,3).

Previous studies have shown that lenalidomide is an immunomodulator which can affect both the cellular and humoral limbs of the immune system in patients suffering from lymphoma (4–6). So far, a myriad researchers have indicated that IMiDs enhances the innate immune response in the MM bone marrow microenvironment. Furthermore, IMiDs can improve adaptive T-cell immunity through polarizing T-cell immunity toward T helper response (3,7). The immunopotentiating aspects of lenalidomide have been recently observed in patients with multiple myeloma as it increased the active Natural Killer (NK) cell cytotoxicity, and augmented the serum levels of activation markers, cytokines and growth factors (8).

On the other hand, it has been proven that NK cells in early innate immune responses can recognizably affect the recruitment and function of both cytokines and cytotoxicity during viral infections, resulting in more antigen-specific T cell responses in different viral doses (9,10). Lately, it has been reported by Kerry *et al.* (11) that the interplay between the inhibitory and activating signals might play a crucial part in NK cell activation and functioning, hence the fact that studying the NK cell activation and function mechanism in controlling the viral infections can provide more insight to developing NK cell-based therapies (12). In addition, it is well-established in the literature that NK cells are able to kill infected cells which lacked the expression of major histocompatibility complex (MHC) class I antigens. Most NK cells exist in the bone marrow, peripheral blood, lymph nodes and spleen which are well placed to respond to vaccine antigens (13,14). One of the advantages of NK cells is their ability to migrate toward inflammation sites such as infectious or tumor cells; what is more, NK cells have a crucial part in immunosurveillance against infection or tumor formation (10,11). They are also able to rapidly respond to effector functions immediately following stimulation, it means resting NK cells can exert against pathogens but antigen naive T and B cells can proliferate and differentiate to effector and memory cells only after becoming fully matured (15). Research on the anticancer mechanism shows that when both the innate and adaptive immune systems decline and tumors develop, NK cells and their receptors can still be targeted via many a therapeutic approaches.

In this study, we investigated the effects of lenalidomide on NK cell activity of mice *in vitro* and *in vivo*. Further evaluated was the adjuvanticity impact of the lenalidomide on improving the NK cell activity in the presence of HCV NS3, as a model antigen.

## MATERIALS AND METHODS

### *In vitro* study

**Reagents.** Lenalidomide was provided by NATCO and dissolved in dimethyl sulphoxide (DMSO) to a stock concentration of 10 mM and stored at 20°C.

**Spleen cell preparation.** Mice were sacrificed and, under sterile conditions, spleens were removed and single-cell suspension was prepared in RPMI 1640 without phenol red (Gibco, UK). Red blood cells were osmotically lysed using ammonium chloride buffer (NH<sub>4</sub>Cl 0.16 M, Tris 0.17 M). Cells were washed twice with RPMI 1640, and counted; the viability was then specified by trypan blue (0.4% w/v) exclusion.

**Cell culture.** K562, a lymphoblastoid human erythroleukemia cell line, was used as a highly sensitive *in vitro* and *in vivo* target for the natural killer assay and was cultured in RPMI-1640 supplemented with 10% fetal calf serum. Once specified, cells were treated with lenalidomide. In order to achieve the optimum concentration, lenalidomide was prepared at 1 mM, 2 mM, 4 mM, 8 mM.

**LDH assay.** Splenocytes were treated with different doses of lenalidomide and incubated for 24 hours at 37°C. To evaluate NK cells' cytotoxic activity, 10 mice spleens and K562 cells were combined as effector and target cells, respectively. The ratios of 25/1 and 50/1 (target/effector) were used on 96-well round-bottom plates. The plates were incubated at 37°C and 5% CO<sub>2</sub> for 6 hours. The cytotoxicity of NK cells was measured through the use of cytotoxicity detection kit (Roch, Germany) according to the kit's protocol. The cytotoxicity was determined for each sample as the triplicates mean (16).

### *In vivo* study

**Animals and drug.** Balb/c mice were purchased from Pasteur Institute of Iran (Tehran, IRAN). For individual experiments, 6- to 8-weeks female mice were used. All the mice were maintained in a pathogen-free mouse facility according to institutional guidelines. For *in vivo* studies, the drugs were dissolved in a concentration of 5 mg/ml in 0.5% DMSO in PBS and stored at 4°C throughout the experiment.

**DNA Vaccine construct preparation.** 1095-1384 amino acids of NS3, which contain conserve epitopes and encodes immunogenic, were subcloned to the pcDNA3.1(+). Recombinant vector pcDNA3.1(+)/NS3 was grown in Escherichia coli, and the strain TOP10F', in Laurie broth medium, was supplemented with Ampicillin (17). A large scale purification was conducted using Plasmid Mega Kit (Qiagen, Valencia, CA). As a model antigen, 100 mg/mouse pcDNA3.1(+)/NS3 was injected to 10 mice intramuscularly (i.m.) on days 0 and 14.

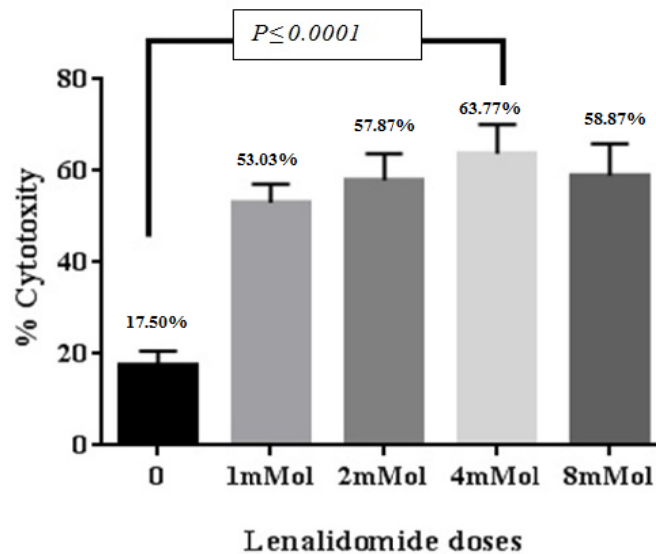
**NK cell assay.** A group of 10 Balb/c mice were treated (i.p.) with a daily dose of 5 mg/kg lenalidomide. In the model of antigen group (pcDNA3.1(+)/NS3 and lenalidomide), and following each pcDNA3.1(+)/NS3 injection for intermittent days, lenalidomide was given three times on days -1, 0 and 1, respectively. We focused on NK cell stimulation by the erythroleukemic cell line K562, as a prototypical example of a MHC class I-deficient tumor cell target which is only targeted by NK cells. Spleens were harvested a week after the final lenalidomide injection (18). The cytotoxicity of NK cells was measured using the LDH activity of cellular culture supernatants as was previously mentioned; further employed was a LDH cytotoxicity detection kit according to the procedures stated by the manufacturer (LDH Cytotoxicity Detection Kit, Roche, Germany).

**Statistical analysis.** The data analysis was conducted via Graph Pad Prism software Version 6 and the statistical analysis was performed through the use of the one-way ANOVA, where the significance was defined as  $P \leq 0.05$ . In the following sections, data are shown as means  $\pm$  standard deviation (SD).

## RESULTS

### The effect of lenalidomide on NK cells *in vitro*.

All samples successfully responded to lenalidomide following a 24-hour *in vitro* stimulation of splenocytes with lenalidomide. In the process of stimulation, lenalidomide was used in different doses including 1 mM, 2 mM, 4 mM and 8 mM. Results showed that 4 mM was more effective than other doses as the measured cytotoxicity of NK cells was the highest in this case. Figure 1 shows that *in vitro* treatment with lenalidomide can increase NK cell cytotoxicity compared to base level of NK cell cytotoxicity in the control group. The optimum dose of lenalidomide was 4mM because the cytotoxic activity of NK cell was the highest *in vitro*. This indicates the significance of lenalidomide dose as far as immune system response is concerned.



**Figure 1.** LDH assay for evaluation the effect of lenalidomide on the mouse NK cells *in vitro*.

### Effect of lenalidomide on NK cells *in vivo*.

After seven days of lenalidomide injection, and using K562 cell line as the target cell, it was confirmed that lenalidomide alone has a direct effect on the cytotoxic capacity of NK cells *in vivo*. Results in Figure 2 indicate that lenalidomide is able to increase NK cytotoxicity *in vivo* from  $16.67\% \pm 2.07\%$  to  $38.17\% \pm 2.87\%$ . In the attempt to analyze the adjuvant behavior of lenalidomide by means of pcDNA3.1(+)/NS3 model, NK

cytotoxicity was enhanced up to 66.80% ± 3.69%, as illustrated in Figure 3. The analysis showed that lenalidomide can noticeably improve the NK cell activity *in vivo*.

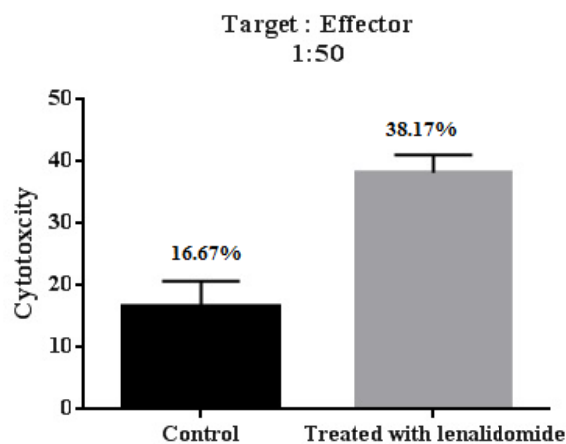


Figure 2. LDH assay for evaluation the effect of lenalidomide on the mouse NK cells *in vivo*.

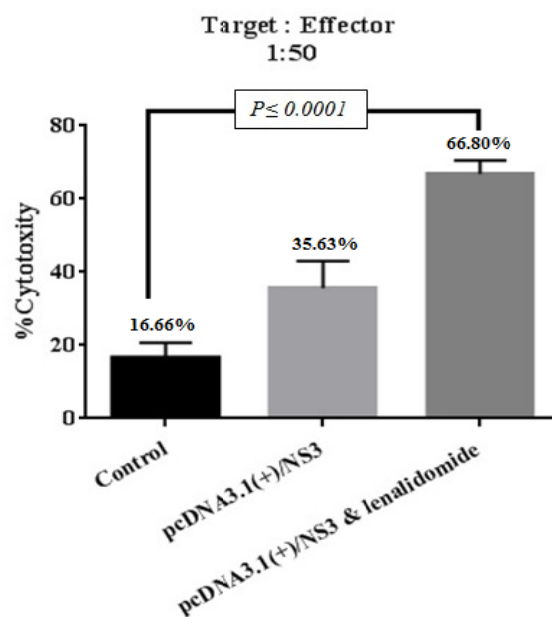


Figure 3. LDH assay for evaluation the effect of lenalidomide combined with the model of antigen on the mouse NK cells *in vivo*.

## DISCUSSION

Lenalidomide, as a chemotherapy agent, is employed in various hematologic malignancies where it stimulates the humoral and cellular immune system (4). NK cells are critical components of innate immunity considering the fact that they make up for 2% of the circulating lymphocytes. NK cells respond rapidly following contact with cancer or viral infected cells, killing them with natural cytotoxicity antibody-dependent cell-mediated cytotoxicity (ADCC) (4). Moreover, NK cells possess antitumor activity by secreting cytokines including IFN- $\gamma$  and TNF- $\alpha$  (19).

It has been reported that, in animal models, IMiDs impact on T helper cells can potentially mediate Th 1 type antitumor immunity and NK cytotoxicity in response to tumor cell vaccination (2). These findings suggest that IMiDs may provide an adjuvant function in cancer vaccine therapy (8). According to the results obtained from animal model lenalidomide, when this drug is used in the format of an adjuvant, it ameliorates the innate immune system by enhancing the activity of NK cells alone and with antigen model. It is concluded that lenalidomide can be used along with therapeutic vaccines in cancer and in vaccination against infectious agents. It is possible that lenalidomide trigger different components of immune system by initiating cytokine production, stimulating NK cell cytotoxicity and T cell activation.

The performed analysis indicated that lenalidomide can affect immune factors such as NK cells *in vitro* model which is in line with the findings of HSU *et al.* where lenalidomide elevated NK tumor cytotoxicity, *in vitro*, in patients with MM (8).

According to literature (20,21) immune response is induced in patients suffering from MM treating by azacitidine and lenalidomide via generating cancer testis antigen-specific cellular immunity. Verhelle D *et al.* showed that lenalidomide up regulates cyclin dependent kinase (CDK) Inhibitor, inhibiting proliferation in Burkitt's Lymphoma cell lines (22). In addition, one of the proposed effector mechanisms of lenalidomide is its ability to stimulate and promote antitumor immune responses. Yu-Tzu Tai *et al.* showed that lenalidomide increases tumor-specific CD8<sup>+</sup> T cell response of multiple myeloma patients via NK-mediated ADCC (23).

Furthermore, using lenalidomide in combination with azacitidine for transplantation receipts, leads to the collection of lymphocytes and a higher immune response. Similarly, in the current study, lenalidomide, as an adjuvant, in combination with pcDNA3.1(+)/NS3, was also able to boost the immune response through enhancing NK cytotoxicity in mice models.

Further studies have to be conducted so as to assay NKT cell, Treg cells and CTL cells and analyze lenalidomide effect on immune factors. This study suggests that in addition to the application of lenalidomide in tumor chemotherapy, it has the potential to be used as an adjuvant to promote immune responses and be used along with therapeutic vaccines in cancer or viral diseases.

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