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Ruthenium Based Photosensitizer Exerts Immunostimulatory and Possible Adjuvant Role on the Mammalian Macrophages *In vitro*

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Abstract. Immune system cells play a crucial role against different types and scales of danger in our body. Macrophages, one of the major innate immune system cells, can recognize the danger and produce different kinds of inflammatory signals as well as present the antigen to the other immune system cells to produce a proper immune response. The new era of medicine is focusing on the regulation of the immune system cells in order to eliminate the chronic inflammation induced by infections or to eliminate the tumors with the patients' own immune system, that would bring out a more sustainable and permanent cure.. In order to screen new candidates in the light of this approach, we tested the immunomodulatory and immunostimulatory capacities of ruthenium polypyridyl based complex K28 that is used in solar cells.

Our results suggest that K28 was able to stimulate the pro-inflammatory activity of the mammalian macrophages based on the TNFa and IL1b production levels. K28 has potential to be used as an adjuvant based on our *in vitro* results.

***Keywords*:** TNF-a, IL-6, IL-1b, inflammation, macrophage, immunomodulation, adjuvants, innate immunity.

Makrofajlar Üzerinde İmmünostimülatör ve Adjuvan Etkisi Olan Rutenyum Bazlı Işığa Bağlı Molekül

Özet. Immune system cells play a crucial role against different types and scales of danger in our body. Macrophages, one of the major innate immune system cells, can recognize the danger and produce different kinds of inflammatory signals as well as present the antigen to the other immune system cells to produce a proper immune response. The new era of medicine is focusing on the regulation of the immune system cells in order to eliminate the chronic inflammation induced by infections or to eliminate the tumors with the patients' own immune system, that would bring out a more sustainable and permanent cure. In order to screen new candidates in the light of this approach, we tested the immunomodulatory and immunostimulatory capacities of ruthenium polypyridyl based complex K28 that is used in solar cells.

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***Anahtar Kelimeler*:** TNF-a, IL-6, IL-1b, inflammation, macrophage, immunomodulation, adjuvants, innate immunity.

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1. INTRODUCTION

An infectious agent or tumor cell would activate our immune system through the metabolic by-products as well as through their toxins. These stimulatory molecules are the danger signals that can be recognized by our immune system cells, especially by the innate immune cells through their pattern recognition receptors. Upon activation, depending on the signaling cues that they receive these cells produce different kinds and amounts of cytokines or chemokines to further activate other immune system cells and eventually eliminate the danger. In most cases of the diseases, this process is either not initiated or has not been propogated properly therefore our body gives a less efficient type of a reponse that can lead to further growth of the tumor cells as well as infectious agents. This lack of a proper response can lead to inefficient vaccinations as well, and adjuvants are used to start a proper immune response. In some cases, our immune system cells overreact and damage our own tissues which is obvious in case of inflammatory disorders or autoimmune diseases. The field of medicine is trying to comprehend and manipulate the immune system no matter what the problem might be; either lack of a proper response or excessive inflammation and tissue damage. Characterization and screening of the immunostimulatory or immunomodulatory agents to fight against certain diseases would increase the success rates against them [1-13].

In our study we utilized from a ruthenium polypyridyl based complex K28 [14]. Ruthenium based complexes and their derivatives have been used in solar cells in order to improve their photovoltaic performances [15-16]. Their ability to get involved in the electron transfer processes was the basis of our screening on the macrophages. Since our cells utilize from the electron transfer chain reactions to produce energy and to regulate the metabolism which very well defines the cellular responses and processes as well as the systemic immune reactions in case of the immunity [17].

Tumor necrosis factor a (TNF-a),interleukin 6 (IL-6), and interleukin 1b (IL1b)are the pro-inflammatory cytokines secreted by the mammalian macrophages upon stimulation [18-29].Through the production of these cytokines, macrophages regulate the immune responses against infections or tumor cells. These cytokines affect the activity of the tissue resident cells and other immune system cells in the breached tissue as well as in our blood, to eradicate the danger [18-29].Due to their inflammatory capacities they are coined as pro-inflammatory cytokines. Macrophages are the major producers of the TNFa, which modulates the activity of the other immune system cells [18-29]. Due to its ability to stimulate the cell death, this cytokines' elevated levels can lead to tissue damage. IL1bis produced by macrophages, endothelial and epithelial cells and it also has pro-inflammatory roles [18-29]. IL-6is another type of pro-inflammatory cytokine that is primarily produced by macrophages and endothelial cells. This cytokine differs from TNFa and IL1b in terms of its ability to activate the antibody secreting B cells and induce the production of the acute phase proteins by the liver [18-29]. Therefore, IL-6 leads to activation of different types of immune responses. Since macrophages are the primary cell types that secret these pro-inflammatory cytokines, we screened the*in vitro* activity of K28 on these cells [18-29].

In this study, we used a ruthenium complex, K28, whose activity was previously shown in the solar cells. We tested its immuno-stimulatory and immuno-modulatory roles by utilizing from RAW 264.7 a well characterized mouse macrophage cell line. Pro-inflammatory TNF-a, IL-6 and IL1b secretion levels were measured in the presence of K28 and we used lipopolysachharide (LPS) as the stimulant to mimic the danger signal on the macrophages.

For the first time in our study, we are presenting the immunostimulatory and therefore adjuvant potential of the ruthenium complex K28 on the mammalian macrophages.

2. MATERIAL AND METHODS

*2.1. In vitro*Cell activation studies:

- Cell Culture: RAW 264.7 cells were purchased from ATCC and grown in Roswell Park Memorial Institute media (RPMI 1640) media with %10 fetal bovine serum, %1 antibiotics (100 ug/ml penicillin and 100 ug/ml streptomycin) and sodium pyruvate. Cells were incubated in 37 ºC % 5 CO_2 incubator. Cell media was refrehsed once in every 4 days until they reach confluency to be used in the experiments.

-Preparation of K28:K28 was prepared according to the procedure specified in the reference number 14. K28 is water soluble and therefore it was dissolved in sterile distilled water before usage.

-K28 and Lipopolysachharide (LPS) stimulation of the mammalian macrophages: RAW 264.7 cells were put in 10^6 cells/well concentration in 1ml fresh complete RPMI as described above into 24-well plates, then they were rested overnight in 37 ºC 5% CO2 incubator. We tested 1ug/ml and 10ug/ml K28's effect on RAW 264.7 cells in the presence and absence of inflammatory stimulator LPS. 1ul of LPS (1mg/mL, Enzo Life Sciences, Salmonella minnesota R595) was put into 1 mL media of overnight rested cells. The same volume of distilled water was put into control negative and LPS only control wells. Then cells were treated with K28,and LPS for 24 hours in 37 ºC 5% incubator. Afterwards supernatants of each well were collected into eppendorf tubes and centrifuged at 2000RPM to get rid of any cellular debris, then supernatants were transferred into fresh eppendorf tubes and kept at -80ºC before further examination. All experimental conditions were tested as a triplicate and these triplicate trials were conducted at least in four different experiments. In order to measure IL1b production by RAW 264.7 cells, freshly prepared 5mM ATP (Fisher Scientific) was put into each well 2 hours before the harvest. The same experimental set up as stated above was used.

-TNFa, IL6 and IL1b ELISAs: TNFa, IL6 and IL1b production was measured by using enzyme-linked immunosorbent assay (ELISA). For each cytokine type (BD Biosciences, CA, USA) ELISA kit was used by following the manufacturers instructions. Maxisorb 96 well plate (Krackeler) was first coated overnight with hamster anti-mouse cytokine (0.5 ug/mL in bicarbonate buffer pH=9.5, 100 uL /well). After getting rid of the solution the plate was washed 3 times with 0.05% Tween 20 PBS. Then plate was blocked with 200 uL blocking buffer (1% BSA PBS) in each well after 3 hours of incubation at room temperature. After washing the plate3 times samples were put as 100 uL into each well and incubated overnight at 4°C. Afterwashing the plate 3 times 100 uL biotin human anti-mouse cytokine (0.5 ug/mL 10% FBS PBS'te) was put into each well and plate was incubated at room temperature for 2 hours. After discarding the solution the plate was washed 3 times and 100 uL of Streptavidin HRP solution was put into each well and plate was incubated for 2 hours at room temperature. Then plate was washed 3 times and 100 uL TMB substrate (BD OptEIA) was put into each well and 50 uL of 1 M sulfuric acid was used to stop the reaction and absorbance was measured at 450nm. By using known concentrations of each cytokine's as standard the concentration of TNFa, IL1b and IL6 in each sample was calculated.

-Cell counting and proliferation: After removing the supernatant media the cells are resuspended in 1ml PBS and counted by mixing 10 uL of cells and 90 uL of Trypan Blue (0.1 uM) and counting them by a hematocytometer and microscope.

2.2 Statistical analysis: GraphPad-Prism Software 5, CA was used for statistical analysis and for each data set there was nine independent results and unpaired two tail t-test was executed to draw statistical significance.

3. RESULTS

We used sterile distilled water (the solvent of the K28) in our negative control wells and it did not have any cytotoxic effect and did not stimulate the macrophages by itself which confirms that it was not contaminated neither the macrophages that we used in our experiments (Figure1-4). When we used LPS in our positive control wells compared to only distilled water treated negative control wells there was a significant and substantial production of TNFa, IL1b and IL6 (Figure 2-4). We applied K28 on the cells without adding LPS, a danger mimic, to test the immunostimulatory (adjuvant) potential of it ,. There was a significant increase in TNFa and IL1b production levels their production levels when 10ug/ml of K28 was applied to the cells compared to the negative control wells (Figure 2 and 3). This indicates K28's immunostimulatory function at 10ug/ml on the macrophages. In addition, K28 was not able to induce IL6 production by macrophages in the absence of any other stimulant (Figure 4). LPS was used as a danger mimic and K28 was applied to the macrophages in the presence of LPS in order to test its ability to regulate the function of the activated cells. There was no change in the production level of TNFa, IL1b and IL6 cytokines by the LPS activated macrophages in the presence of K28 except LPS treated positive control ones (Figure 2-4). These results suggest that K28 was not able to modulate the activated immune system cells' pro-inflammatory cytokine production levels. However, no change was observed in the cell viability of all the experimental afer Trypan Blue staining (Figure 1). Due to its immunostimulatory capacity K28 can be used as an adjuvant candidate.

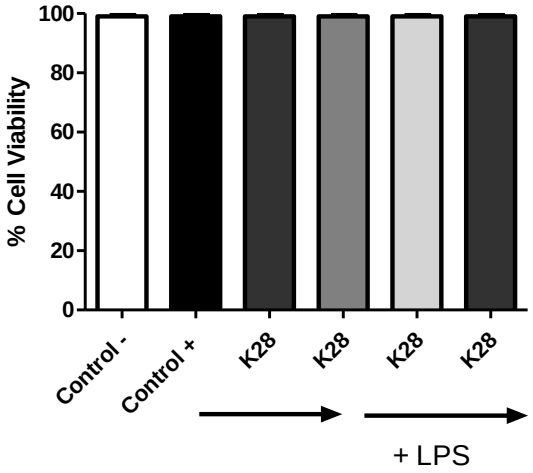

Figure 1. Percentage of viable cells were counted with Trypan blue staining after stimulating RAW macrophage cells for 24 hours with 10 and 100 ug/ml of K28. $1X10^6$cells/ml cell concentration was used and distilled water was used for negative control, 1ug/ml of LPS and distilled water was used for positive control and 10 and 100 ug/ml of the chemicals dissolved in distilled water with or without LPS were applied to the cells. Student t test was applied for statistical analysis,*p<0.001,** p<0.0005,***p<0.0001 N=9. Data are presented mean SEM.

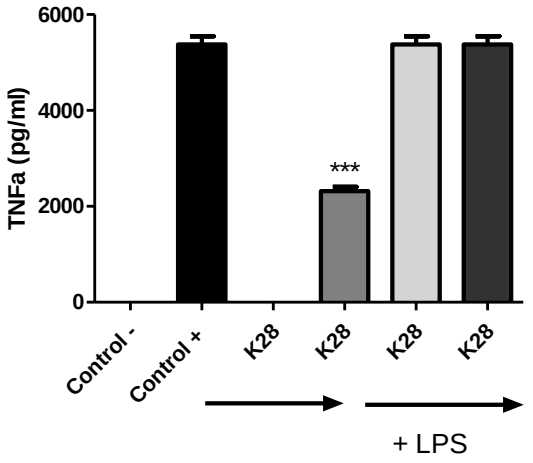

Figure 2. TNFa level was quantified by ELISA onthe supernatants of RAW macrophage cells stimulated for 24 hours with 10 and 100 ug/ml of K28. $1X10^6$cells/ml cell concentration was used and distilled water was used for negative control, 1ug/ml of LPS and distilled water was used for positive control and 10 and 100 ug/ml of the chemicals dissolved in distilled water with or without LPS were applied to the cells. Student t test was applied for statistical analysis, p<0.001,** p<0.0005,***p<0.0001 N=9. Data are presented mean SEM.

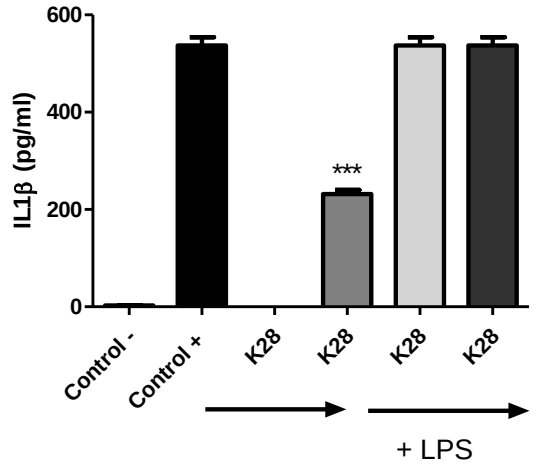

Figure 3. IL1b level was quantified by ELISA onthe supernatants of RAW macrophage cells stimulated for 24 hours with 10 and 100 ug/ml of K28. 1X106cells/ml cell concentration was used and distilled water was used for negative control, 1ug/ml of LPS and distilled water was used for positive control and 10 and 100 ug/ml of the chemicals dissolved in distilled water with or without LPS were applied to the cells. 5mM freshly prepared ATP solution was applied to the cells 2 hours before the harvest to stimulate this cytokine's secretion. Student t test was applied for statistical analysis, $p<0.001$,** $p<0.0005$,***$p<0.0001$ N=9. Data are presented mean SEM.

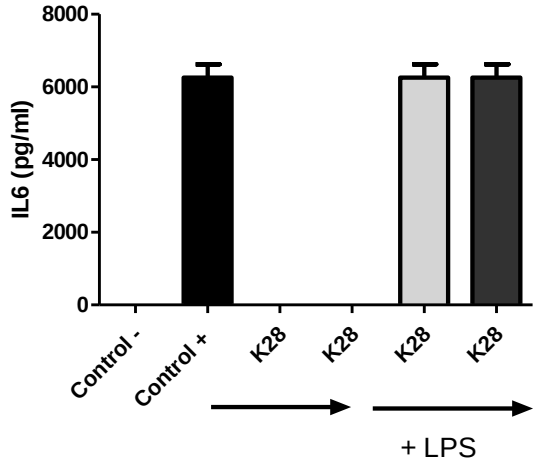

Figure 4. IL6 level was quantified by ELISA onthe supernatants of RAW macrophage cells stimulated for 24 hours with 10 and 100 ug/ml of K28. $1X10^6$cells/ml cell concentration was used and distilled water was used for negative control, 1ug/ml of LPS and distilled water was used for positive control and 10 and 100 ug/ml of the chemicals dissolved in distilled water with or without LPS were applied to the cells. Student t test was applied for statistical analysis, $p<0.001$,** $p<0.0005$,***$p<0.0001$ N=9. Data are presented mean SEM.

4. DISCUSSION

The current approach is based on the regulation or stimulation of the immune system cells in order to eradicate a danger. This danger can be either a tumor tissue or a microbe as well as an excessive damaging inflammatory response. Testing new immunostimulatory or immunomodulatory agents would open new approachesfor the field of medicine to fight against different types of the diseases [1-13].

Based on their ability in improving the photovoltaic performances in solar cells we hypothesized that a ruthenium based complex K28 could also change the electron transfer processes in the immune cells [14-16]. Immune system cells are regulated by the cellular metabolism and there has been studies supporting this notion [17]. Therefore, we tested their activity on the mammalian macrophages based on pro-inflammatory cytokine secretion levels.

Macrophages are the major cells that produce pro-inflammatory cytokines tumor necrosis factor a (TNF-a), interleukin 6 (IL-6), and interleukin 1b (IL1b) upon stimulation [18-29]. These cytokines affect the other immune system cells and produce a proper immune response to eradicate the danger. In order to beable to regulate these cytokines' secretion levels by macrophages, we could either boost the immune system cells' activity and use them as adjuvants. Furthermore, if we could suppress their production we could also utilize from this phenomena to resolve the damaging inflammatory environments created in the autoimmune diseases and in the other inflammatory disorders. K28 was able to stimulate the macrophages *in vitro* in the absence of LPS. Therefore, we can utilize from this ruthenium complex to activate the immune system cells. There was a significant and substantial increase in the production of the pro-inflammatory TNFa and IL1b levels compared to negative controls (Figure 2 and 3). However, K28 was not able to stimulate IL6 production

(Figure 4). We can utilize from this property, if we would like to generate an immune response that would not lead to antibody production as well as Th17 type of responses [18-31].Through TNFa and IL1b cytokines, we could generate an immune response to fight against intracellular pathogens or tumor cells since this would potentially lead to Th1 type of responses [18-32]. K28 was not able to regulate already activated macrophages (Figure 2-4), which supports that application of this complex would not hinder or affect other ongoing immune reactions by macrophages. K28 is water soluble and did not have any cytotoxic effect at used concentrations therefore it stands as a biocompatible adjuvant candidate.

As a result, we have a biologically safe ruthenium based complex that can be used as immunostimulant and adjuvant. Currently, we are investigating its mechanism of action on mammalian macrophages at the molecular level. In future, we are aiming to test the activity and effect of K28 on different immune system cells as well as in *in vivo* studies.

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Abbreviations

TNF-a, tumor nacrosis factor-a; IL-6, interleukin 6; IL1b, interleukin 1b; RAW 264.7, mouse macrophage cell line; ELISA, Enzyme linked immunosorbent assay; LPS, lipopolysachharide.

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