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ORIGINAL ARTICLE

Heteroleptic Ruthenium Polypyridyl Complex Had Differential Effects on the Production of Pro-inflammatory Cytokines TNFα, IL1β, and IL6 by the Mammalian Macrophages *In Vitro*

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Abstract— Modulation of the immune system has gathered more attention in the field of medicine due to the immense potential that it presents. Our immune system has important roles against cancer to infectious diseases, as well as in the development of autoimmune disorders. Therefore, being able to manipulate our immune system cells would enable us to determine the type and strength of the immune response to certain danger stimuli. Macrophages play an important role in the regulation of the immune system by producing cytokines, chemokines and by presenting antigens to other immune system cells to enable their activation; in our study, we focused on their *in vitro* activity in terms of pro-inflammatory cytokine production. In order to screen new immunomodulatory or immunostimulatory drug candidates, we examined the effect of ruthenium polypyridyl-based complex K30 that is used in solar cells as photosensitizer. Due to its electron transfer capacity, this material has potential to change the electron transfer reactions therefore could alter the function of the cells through metabolic changes at a cellular level. Our results suggest that K30 was differentially regulating the secretion levels of the pro-inflammatory cytokines by the LPS-activated mammalian macrophages, while it did not stimulate the macrophages by itself. K30 has an anti-inflammatory potential while lacking the immunostimulatory effect in our *in vitro* results and has potential to be used as anti-inflammatory drug molecule in metallic implants of the fractured bones to prevent damaging inflammatory environment and enable more efficient transplant and healing.

KEY WORDS: TNFα; IL-6; IL-1β; Inflammation; Macrophage; Immunomodulation; Anti-inflammatory molecules; Innate immunity.

INTRODUCTION

Our immune system can recognize an infectious agent or cancer tissue and initiate a proper response against them based on the signaling cues it receives from the macro-environment. Certain disease conditions develop because of the lack of a proper immune response against the danger that our system faces. In some cases, our immune system starts a reaction against the danger but it activates the type of cells that could not fight against the invaders or tumor cells efficiently. In other cases, such as during an

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Abbreviations: TNFα, tumor necrosis factor α; IL-6, interleukin 6; IL1β, interleukin 1β; RAW 264.7, mouse macrophage cell line; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide

autoimmune reaction or in an inflammatory disorder our immune system cells damage the body due to excessive inflammation. In order to circumvent these problems, there has been an increasing body of research to better understand the way our immune system works to be able to manipulate it according to our needs [1–13].

Immunomodulatory molecules are able to regulate the activity of the immune system cells and we need to develop a selection of this kind of molecules to be able to use them appropriately against different types of conditions. In this study, a ruthenium polypyridyl-based complex K30 was tested for its immunostimulatory as well as immunomodulatory potential [14]. There have been studies supporting their high potential in solar cells [14–16]. Rationale behind our hypothesis was that since these molecules are able to regulate the electron transfer processes in solar cells, they should be able to regulate it at cellular level to such an extent that they may change the metabolic activity of the immune cells. Therefore, overall activity of these cells could be regulated. Since studies support that metabolic activities define the type and strength of the immune responses [17].

Macrophages are the main inflammatory cells of the innate immune system. Upon activation, these cells can produce tumor necrosis factor α (TNFα), interleukin 6 (IL-6), and interleukin 1β (IL1β) to fight against certain danger stimulus [18–29]. Either against an infectious agent or tumor cell or a self-antigen in case of autoimmune reactions; macrophages play a key role in the regulation of the immune responses through the production of the cytokines. Pro-inflammatory cytokines change the activity of the resident cells of the endangered tissue and other immune system cells to fight more efficiently against danger [18–29]. One of the key cytokines produced by macrophages is TNFα. This cytokine regulates the function and polarization of other immune cells [18–29]. It can also cause tissue damage since it induces cell death on certain cell types that do not get enough of the survival signals. Macrophages, endothelial, and epithelial cells can produce IL1β that also has inflammatory properties [18–29]. Macrophages produce another pro-inflammatory and in some cases wound healing and anti-inflammatory cytokine, IL-6. While some of the pro-inflammatory functions of TNFα and IL1β overlap with those of IL6, they lack the ability to induce the antibody secretion by B cells, as well as induction of the wound healing in damaged tissues [18–29]. IL6 can lead to the activation of a different type of immune response than TNFα and IL1β. Macrophages are the major immune cells that can secret these cytokines, therefore *in vitro* activity of K30 was examined on these cells [18–29].

In this study, a ruthenium complex, K30, was utilized. In previous studies, its activity and application were shown in the solar cells. A well-characterized mammalian macrophage cell line, RAW 264.7, was used to examine K30'simmunostimulatory and immunomodulatory potentials. ELISA was performed to measure the pro-inflammatory TNFα, IL-6, and IL1β secretion levels by lipopolysaccharide (LPS) danger mimic activated macrophages in the presence of K30. To our knowledge, this study is the first one to present immunomodulatory and therefore anti-inflammatory drug potential of the ruthenium polypyridyl complex K30 on the mouse macrophage cell line.

MATERIAL AND METHODS

In Vitro Cell Activation Studies

Cell Culture. RAW 264.7 mouse macrophage cell line was purchased from ATCC and grown in Roswell Park Memorial Institute media (RPMI 1640) media with %10 fetal bovine serum, %1 antibiotic (100 μg/ml penicillin and 100 μg/ml streptomycin) and sodium pyruvate. 37 °C % 5 CO_2 incubator was used for the cell growth processes and experiments.

Preparation of K30. Procedure specified in the reference number 14 was followed for the production. Distilled sterile water was used to dissolve it.

K30 and Lipopolysaccharide Treatment of the Mammalian Macrophages. RAW 264.7 cells were used in 10^6 cells/well concentration in 1 ml fresh complete RPMI as described above in 24-well plates. Cells were rested overnight in 37 °C 5% CO_2 incubator before the experiments. A total of 1 μg/ml and 10 μg/ml K30 were used on RAW 264.7 cells in the presence and absence of the lipopolysachharide (LPS). 1 μl/ml of LPS (1 mg/mL, Enzo Life Sciences, Salmonella minnesota R595) concentration was used. K30 and LPS treatments were conducted for 24 h in 37 °C 5% incubator. Afterward, supernatants of each well were collected and kept at − 80 °C before ELISAs. Triplicates were set-up for each experimental condition and these triplicate trials were repeated at least in four different independent experiments. In order to measure the IL1β secretion levels by the cells, freshly prepared 5 mM ATP (Fisher Scientific) was put into each well 2 h before the harvest. The same experimental set-up as stated above was used.

TNFα, IL6, and IL1β ELISAs. TNFα, IL6, and IL1β productions were measured by using enzyme-linked immunosorbent assay (ELISA). For each cytokine type, manufacturer's instructions were followed by using BD Biosciences, CA, USA ELISA kits.

Cell Counting and Proliferation. Trypan Blue was used to count the dead and live cells and draw the cell viability after 24 h.

Statistical Analysis

GraphPad Prism Software version 5 was utilized for plotting the graphs and statistical analysis.

RESULTS

Sterile distilled water (the solvent of K30) was used in our negative control wells. Even at the highest volumes that it was used, it did not cause cell death (Fig. 1). Furthermore, it did not stimulate the macrophages by itself which confirms that it was not contaminated and neither the macrophages that we used in our experiments since they were dormant in terms of cytokine secretion in the absence of any stimuli (Figs. 2, 3, and 4). LPS was used in our positive control wells and compared to the only distilled water treated negative control wells; the production of TNFα, IL1β, and IL6 was significant and substantial (Figs. 2, 3, and 4). In the absence of danger

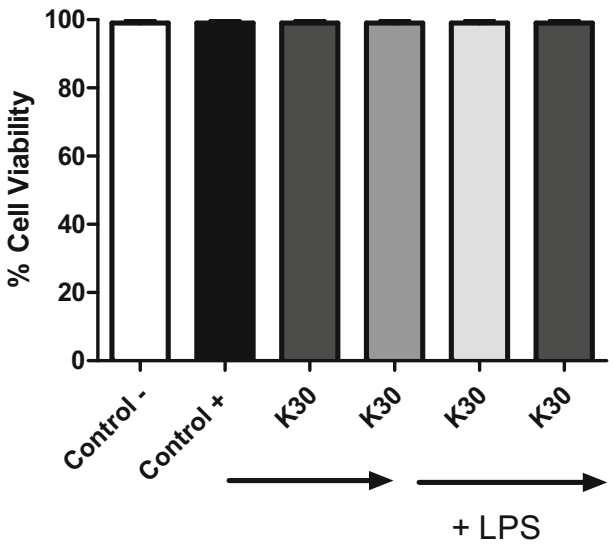

Fig. 1. Percentage of viable cells were counted with Trypan blue staining after stimulating RAW macrophage cells for 24 h with 10 and 100 μg/ml of K30. $1X10^6$cells/ml cell concentration and distilled water was used for negative control, 1μg/ml of LPS and distilled water was used for positive control, and 10 and 100 μg/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,*$p < 0.001$, **$p < 0.0005$, ***$p < 0.0001$, $N = 9$.

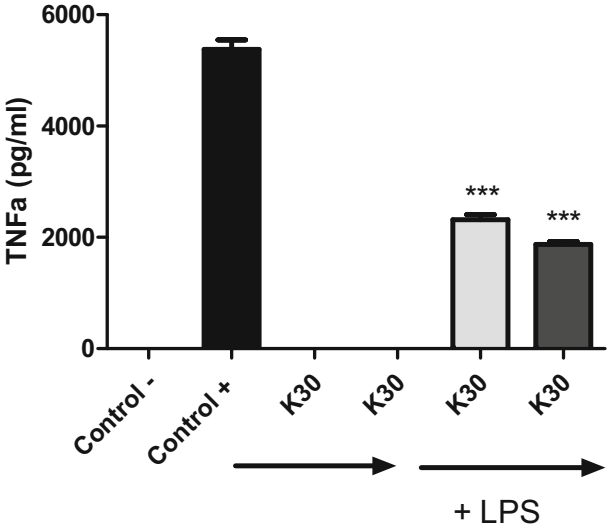

Fig. 2. TNFa ELISA for the supernatants of RAW macrophage cells stimulated for 24 h with 10 and 100 μg/ml of K30. $1X10^6$cells/ml cell concentration was used and distilled water was used for negative control, 1 μg/ml of LPS and distilled water was used for positive control, and 10 and 100 μg/ml of the chemicals dissolved in distilled water with or without LPS were applied to the cells. Student *t* test was applied for statistical analysis, $p < 0.001$, **$p < 0.0005$, ***$p < 0.0001$, $N = 9$.

mimic LPS, K30 was applied onto the cells to test the immunostimulatory (adjuvant) potential of it. K30 was not able to stimulate the cells by itself since there was no production of TNFα, IL1β, and IL6 by the macrophages, while our positive control LPS was able to stimulate macrophages to produce these pro-inflammatory cytokines (Figs. 2, 3, and 4). These results suggest that K30 does not have an immunostimulatory potential. In order to examine its ability to modulate the function of the already activated cells, K30 was applied onto the LPS-stimulated macrophages. There was a significant decrease in TNFα and IL1β levels in K30 treated LPS-activated macrophages compared to the only LPS-activated cells (Figs. 2 and 3). This decrease was dose-dependent since higher doses of K30 lead to a slightly lower TNFα and IL1β secretion levels by LPS-stimulated macrophages (Figs. 2 and 3). There was no change in the production level of IL6 cytokine by the LPS-stimulated macrophages in the presence of K30 compared to the LPS-activated positive control cells (Fig. 4). These results suggest that K30 was a potent anti-inflammatory agent while lacking the immunostimulatory potential. We did Trypan Blue staining to examine the cytotoxic activity of K30 and it did not lead to any change in the cell viability at the concentrations that we used (Fig. 1). Therefore, K30 is a water-soluble and biocompatible molecule that we can utilize from.

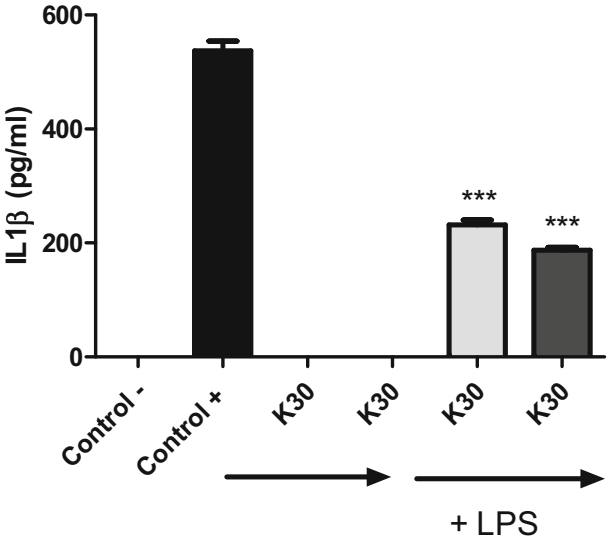

Fig. 3. IL1b ELISA for the supernatants of RAW macrophage cells stimulated for 24 h with 10 and 100 μg/ml of K30. $1X10^6$cells/ml cell concentration was used and distilled water was used for negative control, 1 μg/ml of LPS and distilled water was used for positive control, and 10 and 100 μg/ml of the chemicals dissolved in distilled water with or without LPS were applied to the cells. 5 mM of freshly prepared ATP solution was applied to the cells 2 h before the harvest to stimulate this cytokine's secretion. Student *t* test was applied for statistical analysis, $p < 0.001$, $^{**}p < 0.0005$, $^{***}p < 0.0001$, $N = 9$.

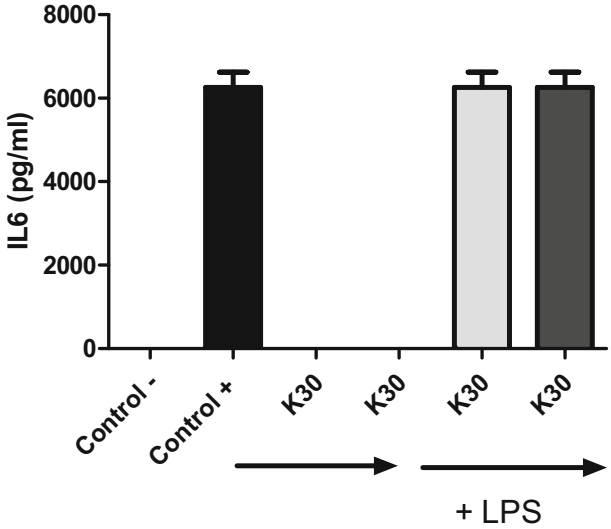

Fig. 4. IL6 ELISA for the supernatants of RAW macrophage cells stimulated for 24 h with 10 and 100 μg/ml of K30. $1X10^6$cells/ml cell concentration was used and distilled water was used for negative control, 1 μg/ml of LPS and distilled water was used for positive control, and 10 and 100 μg/ml of the chemicals dissolved in distilled water with or without LPS were applied to the cells. Student *t* test was applied for statistical analysis, $p < 0.001$, $^{**}p < 0.0005$, $^{***}p < 0.0001$, $N = 9$.

DISCUSSION

To our immune system, the danger can be either an infectious agent or a tumor tissue as well as a misrecognized self-antigen. Danger signals activate the immune system cells and lead to generation of the inflammatory environment. Excessive inflammation or an improper immune response may lead to tissue damage and wasting in autoimmune diseases and inflammatory disorders. Being able to modulate the function of the immune system cells would enable us to manipulate the immune response therefore a more efficient fight against cancer to the infectious and inflammatory diseases. Current approach in medicine aims to regulate the immune system cells for a better and permanent cure, and screening and characterizing new immunostimulatory or immunomodulatory drug candidates would have a major impact on our ability to fight against different types of diseases [1–13].

We expected that a ruthenium polypyridyl-based complex, K30, would change the electron transfer processes and therefore the metabolic activity in the immune cells due to their applications in solar cells [14–16]. Type of the immune response generated by our body is substantially modulated by cellular metabolism [17]. In light of this information, K30 complex's activity was measured on the mammalian macrophages by monitoring the pro-inflammatory cytokine production levels.

Pro-inflammatory cytokines tumor necrosis factor α (TNFα), interleukin 6 (IL-6), and interleukin 1β (IL1β) are primarily secreted by activated macrophages [18–29]. In order to eliminate the danger, these cytokines activate other immune system cells and lead to the development of a proper immune response [18–29]. The immune system cells' activity can be boosted by regulating these cytokines' production levels by macrophages. This kind of agent can be used as an adjuvant in vaccines. Moreover, a suppressive immunomodulatory molecule can be utilized to fight against inflammatory disorders or autoimmune diseases in order to resolve the damaging inflammatory environment. Our candidate, K30, was not able to stimulate the macrophages *in vitro* in the absence of a danger signal. This property enables its safe usage in our body since it would not revoke an unnecessary immune response during the application. Since it did not lead to cell death (Fig. 1) and is water soluble, K30 is a

biocompatible molecule. In the presence of K30, there was a significant and substantial decrease in the production of the TNFα and IL1β cytokines by LPS-activated macrophages compared to the only LPS-treated macrophages (Figs. 2 and 3). These results support that K30 is a potent anti-inflammatory agent on *in vitro* activated macrophages. While there was a decrease in TNFα and IL1β production by K30 treated LPS-stimulated macrophages, there was no change in the production levels of IL6 compared to only LPS-treated positive control wells (Figs. 2, 3, 4). Therefore, K30 differentially regulates the pro-inflammatory cytokine production by danger signal-activated macrophages. This differential regulation can be utilized in disease cases where excessive production of TNFα and IL1β has detrimental effect on the system, while IL6 production has no effect or could be beneficial [18–32]. We could also use this property to suppress TNFα- and IL1β-directed tissue-damaging Th1 cell responses while keeping the activity of Th17-directed responses that might not be detrimental [18–32]. Being able to skew the immune response from Th1 type to Th17 type can be useful to fight against certain fungal infections [18–32]. IL6 plays a crucial role in the generation of the antibody-mediated immune responses. K30 brings the opportunity of suppressing TNFα- and IL1β-mediated responses while keeping the antibody production since it does not affect IL6 production levels.

In conclusion, K30 is a biocompatible and immunologically inert ruthenium polypyridyl-based complex that can find potential applications as anti-inflammatory drug candidate. It can also be used as surface coating agent on the synthetic transplants of the bone fractures since it would prevent the rejection of the transplant by its anti-inflammatory potential. It could also enable more efficient healing after the transplant since it does not suppress the production of the IL6 by macrophages and IL6 has a substantial role in wound healing and tissue regeneration. In our current studies, K30's mechanism of action on the mammalian macrophages is investigated at the molecular level. In our future studies, we will be focusing on the delineation of the activity and effect of K30 on different immune system cells as well as its *in vivo* efficacy.

COMPLIANCE WITH ESTHICAL STANDARDS

Conflict of Interest

The author declares no conflict interest.

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