# Selective cytotoxicity and anticancer activity: ROS-induced cell death facilitated by metal complex

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**Abstract**. Hepatocellular carcinoma (HCC) recurrence rate consists of 10%-20% and remains the leading cause of cancer-related death. We have successfully designed the protocol for adoptive immunotherapy to liver cirrhotic patients with HCC. In our study we have shown that activated lymphocytes, containing much of immune cells of innate immunity, from healthy volunteers, have a vigorous anticancer effect. However, the volume and viability for the treatment with activated lymphocytes might lead to limited effects. We aimed to develop a new therapeutic approach for the efficient expansion of such innate components of cellular immunity in combination with metal complex. Human lymphocytes were cultured for 7 days by appropriate protocol with cytokines. The phenotype and characterization of activated lymphocytes were identified by flow cytometric analysis and the cytotoxicity against tumor was determined. After being cultured for 7 days the proportion of cell fractions in the expanded activated lymphocytes varied among individuals. The average proportion of immune cells of innate immunity which were activated were in a high level. The addition of metal complex exhibits enhanced expression of surface receptors and intracellular cytokines which involved in antitumor process. Activated lymphocytes in combination with metal complex showed vigorous anticancer ability in vitro, of HepG2, an HCC cell line. These findings suggest that adoptive immunotherapy using activated lymphocytes might be a promising approach for inducing innate immunity to decrease the incidence of cancer recurrence and repeatedly applied in the clinical setting.

## 1 Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide and one of the most common malignancies [1]. The current standard of care for HCC is trans arterial chemoembolization (TACE), local ablation therapy, and surgical resection. Even with curative treatment, recurrence is common, and the long-term results are still insufficient [2]. The outcome of HCC is correlated with numerous host and tumor

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variables. Recent developments in immunotherapy imply that the prognosis of cancer is influenced by several immune regulators. The immunological landscape and immunotherapy for HCC, suggest that novel treatment methods are urgently needed.

In HCC, the liver is enriched with various immune cells of innate immunity, among which natural killer (NK) cells are important in host defense and maintaining immune balance. NK cells are innate immune lymphocytes that are capable of lysing cancerous cells without prior sensitization [3]. They are critical to the body's defense against viruses and cancer as they mediate defense through both direct cell lysis and modulation of other immune cells of innate immunity via cell contact and release of pro-inflammatory cytokines, such as IFN-gamma and TNF alpha, upon activation [4].

Due to these inherent functions, NK cells have attracted attention as a promising immunotherapeutic for cancer and viral therapies [5]. NK cells are highly plastic, and their activation is tightly regulated by a balance of activating and inhibitory receptors and by the cytokine profile in their environment [6]. NK cells are stimulated by cytokines generated by activated immune cells of innate immunity in an inflammatory milieu, which leads to NK cell proliferation, cytokine generation, and cytotoxic action [7]. As a result, comprehending how these cytokines work in concert with one another to affect NK cell activation is crucial for maximizing its potential in cancer immunotherapies.

It is commonly known that TF control and coordinated cytokine signals from hematopoi etic stem cells (HSCs) drive the gradual process of NK cell differentiation [8,9,10,11]. Multilineage myeloid cells and lymphocytes, which are essential for preserving the proper quantity and functionality of immune cells, are mostly derived from HSCs [12]. The steady acquisition of particular receptors define the development and function of innate cells. Extrinsic cytokine signals are critical for regulating the formation and function of innate cells. Among many sources of HSC establishment recent studies showed that apheresis from healthy donors could be used for innate cells development.

We recently presented a novel study that shown, for the first time, that metal complex exhibits cancer cell-selective cytotoxicity in vitro by causing ROS buildup and cell death in cells [13]. There are some limitations of this study. We primarily used MCF-7 to assess the anticancer impact of metal complex; however, metal complex actions may be mediated by other pathways (apart from ROS generation) connected to intracellular reduction.

Using apheresis from healthy individuals, we established this method by estimating the lymphocytes' capacity to respond to multiple cytokines and an extra metal complex to produce innate immune cells in an ex-vivo setting. Our goal in doing this study was to develop a novel treatment strategy for the effective growth of innate immune components.

The aim of study is to improve the lymphocyte activation ability by addition of metal complex in the cell culture for treatment against hepatocellular cancer in human.

## 2 Materials and Research Methods

### 2.1 Isolation and Cell Culture

Apheresis obtained from healthy volunteers were isolated by gradient centrifugation with Lympholyte and suspended in either X-VIVO 15 mediums (Lonza, Walkersville, Inc., MD) with the addition of heat-inactivated 5% AB-serum. Cytokines as IL-7, IL-15, Flt3 and SCF were added to all culture media, which were maintained at 37°C in a 5% CO2 incubator during the indicated period. The medium was replaced after 3 days, and cell concentrations were adjusted depended on cell numbers. On day 6, metal complex was added to the cultures for pre-activation for 24 hours in the cultures. The cell viability was assessed by using the trypan blue dye exclusion test.

### 2.2 Flow Cytometry

An FACSCanto was used to perform flow cytometric studies. The following monoclonal antibodies (mAbs) were utilized to stain the surface of the lymphocytes: phycoerythrin (PE)-conjugated anti-CD56 mAb (clone B159; BD Biosciences), biotinylated anti-TRAIL (biotin conjugated anti-TRAIL) mAb (clone RIK-2; eBioscience, Inc., San Diego, CA), and fluorescein isothiocyanate (FITC)-conjugated anti-CD3 mAb (clone HIT3a; BD Biosciences). Allophycocyanin (APC)-streptavidin was used to visualize the biotinylated mAb (BD Biosciences, San Diego, CA). Excluded from the analysis were any dead cells found using propidium iodide staining and light scattering.

### 2.3 Measurement of cell death

In a 6-well plate, 15 × 104 cells were seeded per well of HepG2. Following a 24-hour incubation period, the plate was incubated for an additional 24-hour period with an aqueous solution that either included activated lymphocytes exclusively or in conjunction with 24 μM of metal complex. Following incubation, Dulbecco's phosphate-buffered saline (DPBS, FUJIFILM Wako Pure Chemical Corporation) was used to harvest the cells using TrypLE Express (Thermo Fisher Scientific, Waltham, MA) and wash them. Following cleansing, the cells were once more suspended in DPBS supplemented with 10 μg/mL propidium iodide (PI: Sigma-Aldrich). PI (ex. 488 nm, em. 644/22 nm)] was used to analyse cell mortality using the MoFlo Astrios EQ (Beckman Coulter, Inc., Fullerton, CA, USA).

### 2.4 Evaluation of cell cycle

For a duration of 24 hours, HepG2 cells were cultivated on a 6-well plate with 15 × 104 cells per well. The cells underwent a 24-hour incubation period prior to harvesting, after being treated with activated cells alone and a metal complex (12 and 24 M). TrypLE Express was used to harvest the cells after incubation, and DPBS was used twice for washing. At 4 °C, the cells were fixed for the entire night in 70% ethanol. Following the removal of the ethanol, the cells underwent two DPBS washes. The cells were subsequently resuspended in DPBS with 10 μg/mL of PI and 0.25 mg/mL of RNase A (Sigma). The samples spent thirty minutes being incubated in the dark. Prior to testing, a 40-μm nylon mesh filter was used to filter the samples. PI (ex. 488 nm, em. 620/29 nm)] was used to measure the fluorescence of the cells using the Bio-Rad S3eTM Cell Sorter. A minimum of 10,000 cells yielded values, and the information was presented as mean ± SD.

### 2.5 Study statistical techniques

When applicable, linear regression analysis or the unpaired or paired Student's t
test were used to statistically analyse the data. P-values less than 0.05 were considered to be statistically significant.

## 3 Results and Discussion

### 3.1 Generation of immune cells of innate immunity

We first analyzed the state of proliferation activity of immune cells of innate immunity according to the count of cells on the day of 1, 3 and on the day of harvest (day 7) after they had been cultured (Figure 1). In lymphocytes obtained from healthy volunteers we did not

observe the decrease of whole cell number. On the other hand, $CD56^{+}CD3^{-}$ cells proportions were 5.5% (range, 0.7-10.1) on day 0 and they increased on day 14 after culture to 29.2% (range, 3-52). At the same time, we found the gradual growth $CD56^{+}CD3^{+}$ and $CD56^{-}CD3^{+}$ cell proportions which consisted of 10% (range, 2.5 – 45.8) and 49% (range, 12.3 – 79.3) on day 7. With this, the additional metal complex was influenced on cell proportions as $CD56^{+}CD3^{-}$, $CD56^{+}CD3^{+}$ and $CD56^{-}CD3^{+}$, which consisted of 15.4% (range, 3.1 – 39.7), 10% (2.2 – 37) and 58.4% (17.9 – 91.8), respectively. Cell conditions also well maintained the viability 95% (range, 89-97) in group of additional metal complex of the expanded cells on the day of harvest.

### 3.2 Activated lymphocytes express functional immune cells receptors of innate immunity.

On day 7 of culture of lymphocytes from 8 healthy volunteers we detected increased number of NKRs such as NKp46, NKp30 and NKp44 which were 87.1%, 76% and 72.8% respectively. on day 6 receptors of functional activity as TRAIL, CD226, CD137 and CD16 involved in antitumor effect were in high proportion and expression consisted of 9.2% (range, 1.27 – 14.9), 77.1% (range, 65.1 – 90.3), 13.3% (range, 6.5 – 22.7) and 55.8% (range, 37.1 – 68.2), respectively. Whereas additional metal complex procedure improved such receptors expression on day 7 and it consisted as follows TRAIL increased to 26.4% (range, 1.62 – 66.5), CD226 increased to 89% (range, 50.2 – 98.8) and CD137 increased to 19.7% (range, 14.9 – 25.7).

Intracellular staining showed high expression of secreting cytokines such as IFN gamma, IFN alpha, TNF-alpha and Granzyme B upon stimulation on day 14 (n=4) the percentage (%±SEM) was 60.2±18.7, 43.3 ±16.6, 56.7 ±24.1 and 96.5 ±25.7, and on day 17 (n=6) it was 61.9 ±12.9, 45.8 ±16.9, 65.8 ±13.6 and 91.3 ±17.3, respectively.

### 3.3 Evaluation of the mode of cell death

Using MCF-7 stained with PI, we counted dead cells to assess the mechanism of metal complex induced cell death. According to a one-way ANOVA with Tukey's post hoc test, the percentages of cell death at metal complex doses of 0 μM, 12 μM, and 24 μM were 10.6 ± 4.74%, 23.1 ± 6.46% ($p = 0.0361$), and 53.0 ± 14.1% ($p < 0.0001$), separately. Numerous cell death processes exist, including apoptosis, necrosis, and ferroptosis. After that, HepG2 was treated with ferrostatin-1, an inhibitor of ferroptosis, or Z-VAD-FMK, an inhibitor of apoptosis, in order to assess the mechanism of metal complex-induced cell death. HepG2 cells exposed to 24 μM of metal complex showed the same level of viability as the control group when pretreated with NAC, an oxidant scavenger and precursor to glutathione. A two-way ANOVA showed that the metal complex had a substantial impact on HepG2 viability, as did the inhibitors. Additionally, there was a significant interaction between the metal complex and the inhibitors. NAC dramatically counteracted the metal complex's cytotoxic effect, according to Tukey's post hoc analysis ($p < 0.0001$). In summary, even with these inhibitors, there was no discernible inhibition of metal complex-induced apoptosis, and the cell viability remained poor.

Immune cells of innate immunity have a well-established capacity to fight cancer, and numerous studies have demonstrated their ability to suppress tumor growth, mostly in relation to haematological cancers. Because innate immune cells may be produced and infused in both autologous and allogeneic environments, they represent particularly intriguing candidates for adoptive immunotherapy [14]. It is crucial to develop a clinical-grade process for producing active effectors to achieve this goal. Innate immune cells' cytotoxic activity is carefully controlled by the interplay between activating and inhibiting

signals that originate from cell surface receptors [15,16]. NKp30, NKp44, NKp46, NKG2D, and DNAM-1 are a few of the most significant receptors that actively contribute to tumor identification.

We have created a technique in the current work to produce highly activated innate immune cells that express activation receptors crucial for identifying tumor cells at considerably higher levels.

It was made clear that metal complex caused HepG2 cell death when HepG2 exposed to it was stained with PI. Furthermore, the G1 phase cell population rose dramatically, as indicated by the cell cycle analysis, indicating that the metal complex arrests the HepG2 cell cycle at this stage. The reduction in PCNA expression, a sign of increased cell division, suggested that metal complex is inhibiting MCF-7 proliferation. Furthermore, pretreatment with NAC totally inhibited the concentration-dependent anticancer action of the metal complex on HepG2 cells. This study's analysis of ROS levels made it clear that, in HepG2, but not in normal cells, STS caused an accumulation of intracellular ROS. Increased mutation rates, the development of genetic defects unique to cancer, and the promotion of the proliferation of cancer cells and malignant tumours are all generally caused by ROS [17,18].

Finally, we provide a process for generating in vitro activated lymphocytes employing a cytokine cocktail plus extra metal complex, in which the innate immune cells exhibited unique phenotypes and were preferred in modified cytokine secretion patterns. Furthermore, the addition of a metal complex enhanced the potency of these cells, making them noticeably more cytotoxic to tumour cells. Future research may clarify whether these cells can be applied to allogeneic immunotherapy.

## 4 Conclusion

As a new therapeutic approach for the efficient expansion of innate components of cellular immunity obtained from the peripheral blood, it would be possible to use activated lymphocytes in patients with HCC repeatedly in autologous immunotherapy manner.

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