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Effects of reactive oxygen species on lymphokine-activated killer cells in patients with bladder cancer[1]

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KEY WORDS reactive oxygen species; lymphokine-activated killer cells; cell division; immunologic cytotoxicity; bladder neoplasms

ABSTRACT

AIM: To investigate the effect of reactive oxygen species on the proliferation of lymphokine-activated killer (LAK) cells in patients with bladder cancer and their cytolysis to bladder tumor cells. **METHODS**: Sodium nitroprusside (SNP) was used as nitric oxide (NO) donor. The superoxide anion ($O_2^{-\cdot}$) was generated in the complete medium (CM) supplemented with *N*-methylphenazonium methyl sulfate (PMS) 3 - 120 μmol/L and nicotinamide adenine dinucleotide (NADH) 18 - 600 μmol/L. The hydroxyl radical ($^{\cdot}OH$) was produced by adding ascorbic acid (AA) 0.5 - 400 μmol/L and ferrous sulfate (Fe^{2+}) 0.05 - 40 μmol/L in CM. LAK cell proliferation and cytotoxicity were assayed in the presence of NO, $^{\cdot}OH$, or $O_2^{-\cdot}$. Bladder cancer cell lines BIU-87 and EJ were cultured as target cells and cytotoxicity of LAK cells were determined by MTT assay. **RESULTS**: The proliferation of LAK cells induced by interleukin-2 (IL-2) was inhibited by hydroxyl radical from 48 h to 96 h in a dose-dependent fashion and was inhibited to 34.5 % compared with control at 96 h in the concentration of ascorbic acid 400 μmol/L and ferrous sulfate 40 μmol/L. The inhibition induced by $^{\cdot}OH$ can be overcome by certain concentrations of mannitol or editic acid. On the contrary, the proliferation of LAK cells induced by IL-2 was stimulated by certain concentrations of NO or $O_2^{-\cdot}$. The stimulation induced by $O_2^{-\cdot}$ can be overcome to control level by superoxide dismutase (SOD) 3×10^5 U/L. Exogenous $O_2^{-\cdot}$ resulted in an increase in cytotoxicity of LAK cells against BIU-87 and EJ cells. However, the LAK cells cytotoxicity treated with hydroxyl radical or SOD showed no difference as compared with the control. **CONCLUSION**: NO and $O_2^{-\cdot}$ enhanced the proliferation and activation and $O_2^{-\cdot}$ up-regulated antitumor cytotoxicity of LAK cells in patients with bladder cancer. The growth of LAK cells induced by IL-2 was down-regulated by hydroxyl radical. The effects of these reactive oxygen species on the proliferation of LAK cells induced by IL-2 were different.

INTRODUCTION

Although lymphokine-activated killer (LAK) cells have been shown to exert a potent cytotoxicity on many histologically different tumors, the results of using LAK in clinical practice are not very satisfactory. Therefore, it becomes very important to understand the modulation mechanism of LAK cells.

Previous investigators showed that the proliferation and cytotoxicity of immunocytes may be regulated by some kinds of reactive oxygen species (ROS). The interaction of ROS and cells seems to represent a general signaling in several natural antitumor systems[1]. Superoxide anion ($O_2^{-\cdot}$) is involved in the cytotoxicity of activation of peripheral blood neutrophils[2]. Activated macrophages released macrophage-derived tumoricidal mediators like nitric oxide (NO) and $O_2^{-\cdot}$ which exhibited potent cytotoxic activity against AK-5 cells *in vitro*[3]. However, it is not clear what the effects of ROS are on the proliferation and cytotoxicity of LAK cells in patients with bladder cancer.

This study was undertaken to investigate the role of NO, hydroxyl radical ($^{\cdot}OH$), and $O_2^{-\cdot}$ in the generation of LAK cells in patients with bladder cancer and the cytotoxicity of LAK cells against bladder cancer cell lines.

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MATERIALS AND METHODS

Reagents Superoxide dismutase (SOD, 1600 U/mg) was purchased from the Institute of Xiahe Bioproducts (China). *N*-Methylphenazonium methyl sulfate (PMS) and nicotinamide adenine dinucleotide (NADH) were provided by Fluka (Swizerland). RPMI-1640 medium was obtained from GIBCO. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and *L*-glutamine were obtained from Sigma. Recombinant interleukin-2 (IL-2) was provided by Changchun Institute of Biological Products (Ministry of Public Health, China). Ascorbic acid, fetal calf serum (FCS), mannitol, edetic acid, sodium nitroprusside (SNP), and ferrous sulfate was purchased from Sino-American Biotechnology Company.

Cultivation of LAK cells Peripheral blood mononulear cells (PBMC) obtained from fresh, heparinized periperal blood of 26 patients with pathologically diagnosed transitional cell carcinoma of bladder were isolated by Ficoll-paque (1.077 kg/L, Shanghai 3rd Chemical Reagents Ltd) density-gradient centrifugation (440 × *g*). Interface cells were aspirated and washed 3 times with Hanks' solution. The PBMC were suspended (1×10^9/L) in complete medium (CM) consisting of RPMI-1640, benzylpenicillin 100 kU/L, streptomycin 100 kU/L, gentamycin 50 kU/L, *L*-glutamine 2 mmol/L, sodium pyruvate 1 mmol/L, and 15 % heat-inactivated FCS. The cells were allowed to settle in 25-cm^2 cell culture flasks at 37 ℃ in 5 % CO_2 for 2 h. The non-adherent PBMC (2×10^8/L) were transferred into another 25-cm^2 flask and further cultivated in CM supplemented with IL-2 1 MU/L for 96 h.

Cell proliferation assay The non-adherent PBMC (1.5×10^4 cells per well in 300 μL CM + IL-2) were plated in 96-well plates in the presence of various concentrations of three groups treatment: (1) SNP (NO donor); (2) either ·OH alone or ·OH combined with mannitol or edetic acid; (3) either O_2^{-}· alone, O_2^{-}· plus SOD or O_2^{-}· plus inactive SOD (SODi) which was boiled at 100 ℃ for 10 min. Each concentration has 6 wells. The cells were serially counted in a hemocytometer chamber. The tests were repeated at least three times.

Target cells Human bladder transitional cell carcinoma cell lines BIU-87 and EJ were kindly provided by the Institute of Urology at Beijing Medical University. The cells were maintained in CM at 37 ℃ in a 5 % CO_2 air environment. Tumor cells were harvested by overlaying the monolayer with a solution of 0.05 % trypsin (Sigma) and edetic acid 0.53 mmol/L and then resuspended in CM. The cells were plated in 96-well plates at 6×10^4 cells per well in 150 μL CM for cytotoxicity assay.

Cytotoxicity assay Human bladder transitional cell carcinoma cell lines EJ cells used as the target cells were placed in 96-well plates at 6×10^4 cells per well in 150 μL CM and cultured for 24 h. The cytotoxicity of LAK was determined by MTT assay[14]. After removing the medium, the LAK were added to target cells in effector/target (E/T) ratios = 20 : 1 in 250 μL CM supplemented with various concentrations of either ·OH alone or ·OH combined with mannitol or edetic acid and either O_2^{-}· alone, O_2^{-}· plus SOD or O_2^{-}· plus SODi. The cell mixtures were then incubated at 37 ℃ for 4 h before washing the plate with RPMI-1640 and 2 % FCS. The cytotoxicity assay was repeated for 4 independent experiments (4 wells each). The absorbance (A) at 570 nm in each well was determined with a microplate autoreader (Nanjing, China). The percent of lysis = $[1-(A_{target\ cell} + A_{LAK\ cell})/A_{target\ cell}] \times 100$ %.

Generation of NO, ·OH, or O_2^{-}· SNP was used as NO donor. The O_2^{-}· was generated in the CM supplemented with NADH 18 – 600 μmol/L and PMS 3 – 120 μmol/L. The ·OH was produced by adding ascorbic acid (AA) 0.5 – 400 μmol/L and ferrous sulfate (Fe^{2+}) 0.05 – 40 μmol/L in CM[15].

Statistical analysis Statistical analysis of all LAK cell proliferation experiments was performed by analysis of variance. The statistical analysis of all LAK cell cytotoxicity against tumor cells was carried out by the u test.

RESULTS

Effect of ·OH on LAK cell growth Increasing concentrations of ·OH (from AA 0.5 μmol·L^{-1}/ Fe^{2+} 0.05 μmol·L^{-1} to AA 400 μmol·L^{-1}/Fe^{2+} 40 μmol·L^{-1}) and ·OH combined with mannitol (0 – 20 mmol·L^{-1}) or edetic acid (0 – 40 μmol·L^{-1}) were added to LAK cells for up to 96 h. Hydroxyl radical significantly inhibited proliferation of LAK cells started from the 48th hour (Fig 1, $P < 0.01$). The lower concentrations of ·OH exhibited no inhibition on proliferation, but the higher concentrations of ·OH exhibited inhibition in a dose-dependent manner (Fig 2). The inhibition induced by ·OH can be overcome ($P < 0.05$) by certain

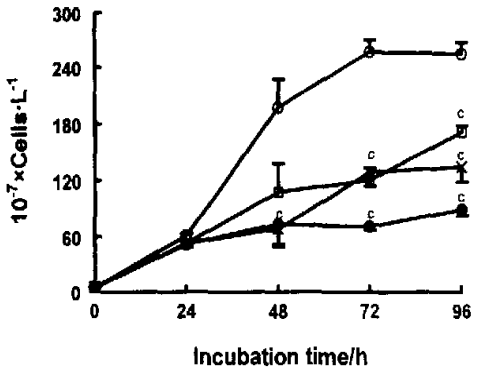

Fig 1. *Effect of ·OH on LAK cell proliferation induced by* IL-2 1 MU·L^{-1}. The LAK cells were treated with *IL-2 alone* (○), *IL-2 plus AA 400* μmol·L^{-1}/Fe^{2+} *40* μmol·L^{-1}(●), IL-2 plus AA 200 μmol·L^{-1}/ Fe^{2+} 20 *μmol·L*$^{-1}$(×), *IL-2 plus AA 100* μmol·L^{-1}/Fe^{2+} *10* μmol·L^{-1}(□). *n* = 6 wells and repeated for 3 *independent experiments.* $\bar{x} \pm s$. $^cP < 0.01$ *vs* IL-2 alone.

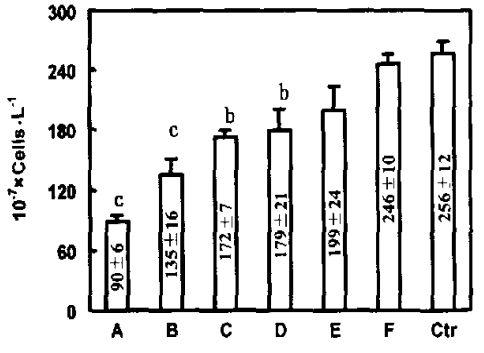

Fig 2. Inhibition of ·OH on LAK cell proliferation induced by IL-2 1 MU·L^{-1}. LAK cells were cultured for 96 h with IL-2 plus AA 400 μmol·L^{-1}/Fe^{2+} 40 μmol·L^{-1}(A), AA 200 μmol·L^{-1}/Fe^{2+} 20 μmol·L^{-1}(B), AA 100 μmol·L^{-1}/Fe^{2+} 10 μmol·L^{-1}(C), AA 50 μmol·L^{-1}/Fe^{2+} 5 μmol·L^{-1}(D), AA 5 μmol·L^{-1}/Fe^{2+} 0.5 μmol·L^{-1}(E), AA 0.5 μmol·L^{-1}/Fe^{2+} 0.05 μmol·L^{-1}(F) and AA 0 μmol·L^{-1}/Fe^{2+} 0 μmol·L^{-1}(Ctr). n = 4 wells and repeated for 4 independent experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* IL-2 alone (Ctr).

concentrations of mannitol (5 – 20 mmol·L^{-1}) or edetic acid (10 – 40 μmol·L^{-1}).

Effect of NO on LAK cell growth LAK cells were treated with IL-2 1 MU·L^{-1} plus different concentrations of SNP (0 – 400 nmol·L^{-1}). The proliferation of LAK cells was stimulated by SNP at concentrations of SNP 25 – 400 nmol·L^{-1} (Fig 3). The strongest stimulation was achieved at the concentration of SNP 100 nmol·L^{-1}.

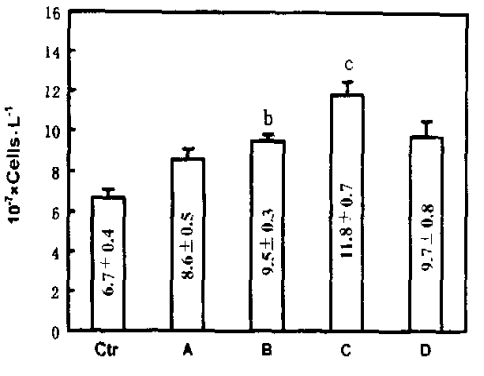

Fig 3. Growth of LAK cell treated with NO. The LAK cells were treated with IL-2 0 U·L^{-1}(Ctr), or IL-2 1 MU·L^{-1} *alone* (A) *plus SNP 25 nmol·L*$^{-1}$(B), *plus* SNP 100 nmol·L^{-1}(C), and plus SNP 400 nmol·L^{-1} (D). *n = 6 wells and repeated for 6 independent* experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* IL-2 alone.

Effect of $O_2^{\cdot-}$ on LAK cell growth LAK cells were treated with IL-2 1 MU·L^{-1} plus different concentrations of $O_2^{\cdot-}$ (NADH 18 – 600 μmol·L^{-1} and PMS 3 – 120 μmol·L^{-1}). The proliferation of LAK cells was stimulated by $O_2^{\cdot-}$ at concentrations of NADH 18 – 36 μmol·L^{-1} and PMS 3.5 – 7.5 μmol·L^{-1} from 72 to 96 h (Fig 4). The strongest stimulation was

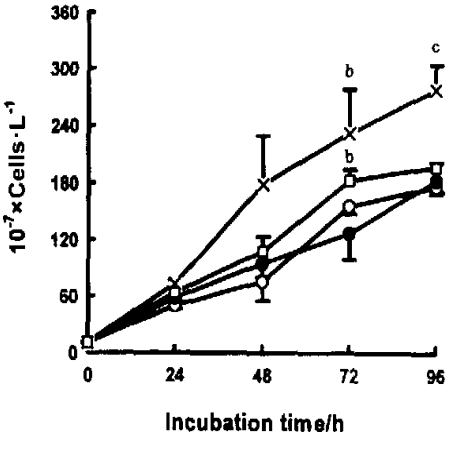

Fig 4. Growth of LAK cell treated with $O_2^{\cdot-}$. The LAK cells were treated with IL-2 1 MU·L^{-1} alone (○), or plus NADH 72 μmol·L^{-1}/PMS 15 μmol·L^{-1}(●), plus NADH 36 μmol·L^{-1}/PMS 7.5 μmol·L^{-1}(×) and plus NADH 18 μmol·L^{-1} NADH/PMS 3.5 μmol·L^{-1}(□). n = 6 wells and repeated for 6 independent experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* IL-2 alone.

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achieved at the concentration of NADH 36 μmol·L^{-1} and PMS 7.5 μmol·L^{-1}. In the medium concentration groups, there was no stimulation. On the contrary, the obvious inhibition appeared in the higher concentration (Fig 5). This stimulation by $O_2^{-\cdot}$ can be overcome to control level by adding SOD (3×10^5 U·L^{-1}) but SODi can not (Fig 6).

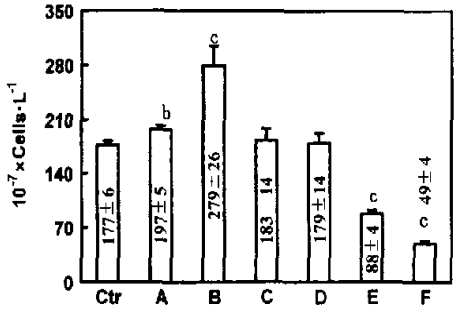

Fig 5. Concentration-dependent proliferation of LAK cell in combination with $O_2^{-\cdot}$ and IL-2. LAK cells were cultured for 96 h with IL-2 1 MU·L^{-1} plus NADH 0 μmol·L^{-1} and PMS 0 μmol·L^{-1}(Ctr), NADH 18 μmol·L^{-1} and PMS 3.5 μmol·L^{-1}(A), NADH 36 μmol·L^{-1} and PMS 7.5 μmol·L^{-1}(B), NADH 72 μmol·L^{-1} and PMS 15 μmol·L^{-1}(C), NADH 150 μmol·L^{-1} and PMS 30 μmol·L^{-1}(D), NADH 300 μmol·L^{-1} and PMS 60 μmol·L^{-1}(E) and NADH 600 μmol·L^{-1} and PMS 120 μmol·L^{-1}(F). n = 4 wells and repeated for 4 independent experiments. $^bP<0.05$, $^cP<0.01$ *vs* Ctr.

The role of ROS on cytotoxicity of LAK cells against tumor cells Superoxide anion increases cytotoxicity of LAK cells against BIU-87 and EJ cells (Tab 1). This increased cytotoxicity can be overcome to the control level by adding SOD 3×10^5 U·L^{-1} but SODi can not. However, ·OH, or ·OH with mannitol and edetic acid or SOD has no influence on cytotoxicity.

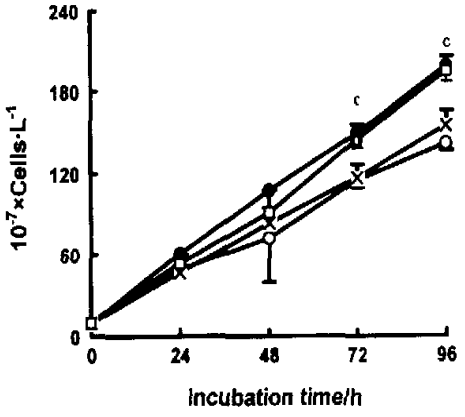

Fig 6. Proliferation of LAK cell treated by $O_2^{-\cdot}$ and SOD. The LAK cells were treated with IL-2 1 MU·L^{-1} alone (○), IL-2 1 MU·L^{-1} plus NADH 36 μmol·L^{-1}/PMS 7.5 μmol·L^{-1}(●), IL-2 1 MU·L^{-1} plus NADH 36 μmol·L^{-1}/PMS 7.5 μmol·L^{-1} combined with SOD 3×10^5 U·L^{-1}(×), or IL-2 1 MU·L^{-1} plus NADH 36 μmol·L^{-1}/PMS 7.5 μmol·L^{-1} combined with inactivated SOD 3×10^5 U·L^{-1}(□). n = 6 wells and repeated for 6 independent experiments. $^cP<0.01$ *vs* IL-2 alone.

DISCUSSION

ROS produced by immnocytes play an important role in host defense against tumors induced by cytokines[6]. Oral administration of IL-1 beta significantly increased the proliferation of peripheral blood mononuclear cells stimulated with concanavalin A, and the $O_2^{-\cdot}$ production stimulated neutrophils in newborn calves[7]. Nitric oxide synthesis is strongly induced during IL-2 treatment in mice and human[8]. Moon *et al* found that Aflatoxin B1 (AFB1) decreased phagocytosis and the production of $O_2^{-\cdot}$ and *in vitro* antitumor activity of *in vivo* AFB1-treated macrophages was reduced against target cell, L929[9]. Our results showed that proliferation of LAK

Tab 1. Effect of ROS on the cytolysis of LAK cells against bladder tumor cells BIU-87 and EJ. The LAK cells were assayed for their ability to lyse BIU-87 and EJ cells in presence of $O_2^{-\cdot}$ (NADH 36 μmol·L^{-1}/PMS 7.5 μmol·L^{-1}), ·OH (AA 200 μmol·L^{-1}/Fe^{2+} 20 μmol·L^{-1}), $O_2^{-\cdot}$ + SOD (3×10^5 U·L^{-1}), $O_2^{-\cdot}$ + SODi or SOD alone (3×10^5 U·L^{-1}). n = 4 wells and repeated for 4 independent experiments. $\bar{x}\pm s$. $^bP<0.05$ *vs* control.

Bladder tumour cells	Specific cytolysis/%					
	Control	$O_2^{-\cdot}$	·OH	$O_2^{-\cdot}$ + SOD	$O_2^{-\cdot}$ + SODi	SOD
BIU-87	39.1±0.3	55.42±0.25^b	39.4±0.6	43.7±0.4	33.2±1.0^b	42.3±1.1
EJ	37.2±0.3	50.8±0.4^b	37.3±0.6	39.7±1.2	49.32±0.70^b	42.9±1.0

cells was stimulated by NO or O_2^{-} · at certain concentrations. Possible explanation for enhanced growth by NO or O_2^{-} · is an increase in protooncogenes expression and stimulation of protein tyrosine phosphorylation[10]. ROS activate NF-κB, a transcriptional regulator of genes associated with the inflammatory response in T cells[10]. However, the data presented in this study indicated that the growth of LAK cells induced by IL-2 was inhibited by ·OH in a dose-dependent manner. The mechanism for the different effects between ·OH and O_2^{-} · on the proliferation of LAK cells induced by IL-2 remains unclear.

The free radical can act as an antitumor mechanism by inhibiting cellular respiration and DNA synthesis in cancer cell[8]. Xanthine oxidase (XO) mediates anticancer activity because of its ability to generate cytotoxic ROS, including superoxide anion radical and hydrogen peroxide. Sawa *et al* demonstrate that chemical conjugation of XO significantly enhanced the tumor-targeting efficacy and the antitumor activity[11]. Inhibition of SOD causes accumulation of cellular O_2^{-} · and leads to free-radical-mediated damage to mitochondrial membranes, the release of cytochrome c from mitochondria and apoptosis of the cancer cells[12]. The cytotoxicity of LAK cells was not inhibited by SOD[13]. The present study demonstrated that the cytotoxicity of LAK cells to bladder cancer cell lines was enhanced by O_2^{-} ·, although SOD did not show inhibition to the cytotoxicity, suggesting that the production of superoxide anion from neutrophils and monocytes *in vitro* might increase the cytotoxicity and stimulate proliferation of LAK cells.

Bacillus Calmette-Guerin (BCG) intravesical instillation is widely accepted as a very effective modality in treating bladder carcinoma *in situ*. Intravesical BCG administration induced systemic production of oxygen free radicals that may reflect a systemic activation of the immune system[14]. The results here demonstrated that ROS influenced the antitumour immunity of LAK cells, indicating that ROS may be involved in the development or antitumor immunity of bladder cancer.

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活性氧自由基对膀胱癌患者淋巴因子激活的杀伤细胞的作用[1]

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关键词 活性氧自由基；淋巴因子激活的杀伤细胞；细胞分裂；免疫细胞毒性；膀胱肿瘤

目的：探讨活性氧自由基对膀胱癌患者淋巴因子激活的杀伤(LAK)细胞的增殖和抗膀胱癌细胞系活性的作用. **方法**：分别用细胞计数和 MTT 法测定 LAK 细胞的增殖和细胞毒作用. **结果**：羟自由基浓度依赖性地抑制 IL-2 所诱导的 LAK 细胞增殖. 用抗坏血酸 400 μmol·L^{-1}和硫酸亚铁 40 μmol·L^{-1}培养细胞 96 h，细胞增殖被抑制 34.5 %. 这种抑制可被一定浓度的甘露醇和依地酸(edetic acid)扭转. 一定浓度的超氧阴离子或一氧化氮释放剂硝普钠可刺激由 IL-2 所诱导的 LAK 细胞的增殖. 超氧化物歧化酶(SOD)可抵消超氧阴离子的刺激作用. 外源性超氧阴离子可加强 LAK 细胞对 BIU-87 和 EJ 细胞的杀伤. 羟自由基和 SOD 对 LAK 细胞杀伤作用则影响不明显. **结论**：超氧阴离子和一氧化氮可增强膀胱癌患者 LAK 细胞的增殖、激活和抗肿瘤的细胞毒，而羟自由基对此起抑制作用. 这两种活性氧自由基对 IL-2 诱导的 LAK 细胞增殖的作用是不同的.

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