

# Influence of IFN $\alpha$ -2b and BCG on the release of TNF and IL-1 by Kupffer cells in rats with hepatoma

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## INTRODUCTION

Kupffer cells are residential macrophages in the liver, which play a critical role in the maintenance of normal liver function and in immunal surveillance of hepatocellular carcinoma (HCC) and other cancers<sup>[1]</sup>. The biological immune modulants have been used for treating patients with HCC and other cancers<sup>[2]</sup>. In our previous studies, the combined use of biological immune modulants showed better effects. The normal rats and hepatoma rats induced by DEN (Diethylnitrosamine) were treated by either IFN $\alpha$ -2b or BCG or both, the number of KCs and the amount of H<sub>2</sub>O<sub>2</sub> released increased obviously, while the combined use of IFN $\alpha$ -2b and BCG showed the best results<sup>[3,4]</sup>. This study was focused on the influence of biological immune modulants on the release of TNF and IL-1 by KCs in rats with hepatoma.

## MATERIALS AND METHODS

### Animals

Male Wistar rats weighing 150 g-200 g, were provided by the Experimental Animal Center of the Chinese Academy of Medical Sciences. The rats were given drinking water containing 0.008% DEN for 12 weeks, then randomly divided into four groups, they were given IFN $\alpha$ -2b (Schering Plough Co. USA) at a dose of 16U/kg, BCG (Biological Product's Research Institute, Shanghai) at a dose of 0.1 mg/kg, and combination of the two intraperitoneally, the controls were given the same

volume of normal saline at various survival periods (12th week, 16th week).

### Isolation of KCs

Under anesthesia, the rats were exsanguinated and the livers were perfused *in situ* via the portal vein with Hank's balanced salt solution (HBSS). The livers were dissected free and passed through 60  $\mu$ m brass screens into HBSS. Following centrifugation at 500  $\times$  g for 10 min (4°C), the pellet was resuspended in 30 mL HBSS containing 0.05% collagenase (TYPE I, Sigma) and 0.1% pronase (TYPE E, Sigma), and incubated for 30 min in a 37°C agitating water bath, following centrifugation at 500  $\times$  g for 2 min to sediment the hepatocytes, an enriched nonparenchymal cell pellet was obtained by centrifugation of the supernatant at 500  $\times$  g for 35 min. Erythrocytes were lysed by incubation for 3-5 min with ammonium chloride (0.83%). The cells were washed twice with HBSS and resuspended in standard RPMI-1640. After 4 h incubation at 37°C in 100 mm plastic petridishes, the nonadherent cells were removed by three successive wash with warm HBSS. The adherent cells, phagocytosing latex beads (Sigma) were designated as Kupffer cells. After 10 min incubation at 37°C in Trypsin-EDTA (0.05%/0.02%), KCs were liberated from the plastic dishes by vigorous pipetting, washing, and counted. The purity of KC was >95% by latex bead ingestion and viability was >90% as indicated by trypan blue exclusion (0.4% trypan blue stain in 0.85% saline). The KCs of control hepatoma rats (at the 18th week) were isolated and treated with IFN $\alpha$ -2b (2500 U/mL), BCG (2 mg/L) or both respectively. The human hepatoma cell line SMMC-7721 cells and KCs were cocultured in the RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 4 h. Supernatants were collected, and stored at -20°C until use.

### IL-1 activity assay

IL-1 activity in the culture supernatants was assayed by the enhancement of thymocyte proliferation to concanavalin A (ConA). Briefly, thymocytes were obtained from female C57BL/6N mice at 5-7 weeks of age, cell suspensions of thymocytes were prepared by pressing the thymus tissue through a 50  $\mu$ m wire mesh, then filtering the tissue fragments through a 30  $\mu$ m nylon mesh into RPMI-1640 medium supplemented with 2.4 g/L ConA. The

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cells were resuspended to  $1.5 \times 10^6$  cells/mL, and 100  $\mu$ L of cell suspensions were placed in each well of 96-well microtiter plates, 100  $\mu$ L of supernatants were added to each well and the plates were incubated for 72 h. Each cell culture well was pulsed  $3.7 \times 10^{10}$   $\mu$ Bq  $^3$ H-thymidine during the final 8 h of incubation and harvested onto glass fibers, using an automatic cell harvester,  $^3$ H-thymidine incorporation was determined by a liquid scintillation spectrometer, the enhancement of thymocyte proliferation of the unknown supernatant IL-1 levels was determined by the ratio of radioactivity incorporated in the experimental and control groups (SI).

### TNF assay

The levels of TNF in culture supernatants were determined by a TNF specific ELISA obtained from Institute of Military Medical Sciences. The kit was composed of 96 determinations and the assays were performed exactly as recommended by the manufacturer. All samples were assayed in triplicate.

### Statistical analysis

The results were presented as the mean of triplicates  $\pm$  SEM, and statistical significance was assessed by Student's *t* test.

## RESULTS

The influence of biological immune modulators on the release of TNF by KCs of normal rats *in vivo* (Table 1). The KCs of normal rats could release a little TNF and IL-1 when cocultured with human SMMC-7721 hepatoma cells, the TNF and IL-1 released by KCs of normal rats treated with biological immune modulators *in vivo* increased obviously. The effect of combined use of IFN $\alpha$ -2b and BCG exhibited the best effects, the combination of IFN $\alpha$ -2b and BCG increased the production of TNF by 3.5 times and the activity of IL-1 by 80%. BCG was better than IFN $\alpha$ -2b in increasing the release of IL-1, and IFN $\alpha$ -2b was better in increasing the production of TNF.

**Table 1 The influence of biological immunal modulators on the release of TNF and IL-1 by KCs of normal rat *in vivo***

Groups	TNF (ng/L)	IL-1 (SI)
Control	56.2 $\pm$ 10.5	1.40
BCG	79.4 $\pm$ 17.6 <sup>a</sup>	2.27 <sup>b</sup>
IFN $\alpha$ -2b	134.9 $\pm$ 51.4 <sup>b</sup>	1.84 <sup>a</sup>
IFN $\alpha$ -2b+BCG	199.5 $\pm$ 71.5 <sup>b</sup>	2.54 <sup>b</sup>

*n* = 18, <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 vs control.

The influence of biological immune modulators on the release of TNF and IL-1 by KCs of rat with hepatoma *in vivo* is shown in Table 2. The influence of biological stimulants on the release of

TNF and IL-1 by KCs of rats with hepatoma *in vivo* was similar to those of normal rats. The combined use of IFN $\alpha$ -2b and BCG exhibited the best effect, the amount of TNF released by KCs treated with IFN $\alpha$ -2b and BCG increased by 5.6 times (12th week) and by 4.5 times (16th week), the activity of IL-1 increased by KCs treated with IFN $\alpha$ -2b and BCG increased by 48% (12th week) and 78% (16th week).

**Table 2 The influence of biological immunal modulators on the release of TNF and IL-1 by KCs of rats with hepatoma *in vivo***

Groups	2nd week		16th week	
	TNF (ng/L)	IL-1 (SI)	TNF (ng/L)	IL-1 (SI)
Control	50.1 $\pm$ 9.4	1.57	79.4 $\pm$ 10.6	1.58
BCG	234.4 $\pm$ 63.1 <sup>b</sup>	2.58 <sup>b</sup>	148.5 $\pm$ 47.2 <sup>a</sup>	2.74 <sup>b</sup>
IFN $\alpha$ -2b	251.2 $\pm$ 82.1 <sup>b</sup>	2.33 <sup>b</sup>	166.0 $\pm$ 30.5 <sup>a</sup>	2.42 <sup>b</sup>
IFN-2b+BCG	281.8 $\pm$ 63.3 <sup>b</sup>	2.80 <sup>b</sup>	354.8 $\pm$ 96.4 <sup>b</sup>	4.08 <sup>b</sup>

*n* = 18, <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 vs control.

The influence of biological immune modulators on the release of TNF and IL-1 by KCs of rat with hepatoma *in vitro* is shown in Table 3. The influence of biological stimulants on the release of TNF and IL-1 by KCs of rats with hepatoma *in vitro* was similar to the result *in vivo*. IFN $\alpha$ -2b was better than BCG in increase of release of TNF by KCs, BCG gave better results than IFN $\alpha$ -2b in increasing the activity of IL-1 released by KCs. The combined IFN $\alpha$ -2b and BCG exhibited the best effects, the amount of TNF released by KCs treated with IFN $\alpha$ -2b and BCG increased by 123.8%, the activity of IL-1 released by KCs treated with IFN $\alpha$ -2b and BCG increased by 58%.

**Table 3 The influence of biological immune modulators on the release of TNF and IL-1 by KCs of rats with hepatoma *in vitro***

Groups	TNF (ng/L)	IL-1 (SI)
Control	100.0 $\pm$ 20.6	1.42
BCG	141.3 $\pm$ 31.7 <sup>b</sup>	1.80 <sup>a</sup>
IFN $\alpha$ -2b	199.5 $\pm$ 61.2 <sup>b</sup>	1.64 <sup>a</sup>
IFN $\alpha$ -2b+BCG	223.8 $\pm$ 42.4 <sup>b</sup>	2.24 <sup>b</sup>

*n* = 18, <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 vs control.

## DISCUSSION

TNF and IL-1 are macrophage-derived cytokines, TNF is known to have cytotoxic and cytostatic effects on certain tumor cells, and with a pivotal role in inflammatory reactions and regulation of immunological response<sup>[5,6]</sup>. The actions of IL-1 have been elucidated in recent years, it is directly cytotoxic for some human tumor cells, and stimulates T cell proliferation by inducing production of interleukin 2 (IL-2) as well as increasing the number of IL-2 receptors on the T cell, and directly stimulate NK cell activity<sup>[7]</sup>.

Kupffer cells are residential macrophages in the liver, and play an important role as scavenger cells in nonspecific elimination of gut endotoxins, immune complexes and viruses<sup>[8]</sup>, especially the important role in immunal surveillance on HCC<sup>[9,10]</sup>. Kupffer cells have also been reported to release some cytokines when activated by biologically reactive substances, several reports have suggested that IFN $\gamma$  regulates monocytic function, especially in the production of TNF and IL-1. Kawada had shown that IFN $\gamma$  enhanced TNF production in the presence or absence of lipopolysaccharide (LPS), but suppressed IL-1 production by KCs<sup>[11]</sup>. Brandwein showed that IFN $\gamma$  markedly inhibits LPS-stimulated IL-1 production by mouse peritoneal macrophages<sup>[12]</sup>, but differed from the observations of Boraschi and Hart<sup>[13,14]</sup>. Amento *et al* have also observed that IFN $\gamma$  did not stimulate IL-1 production by macrophages from the human monocyte cell line U937<sup>[15]</sup>, and many reports had suggested that IFN $\gamma$  augments both TNF and IL-1 production by macrophages only in the presence of LPS<sup>[12]</sup>, this variable responses are undoubtedly related to the difference in both the interferon preparations and the responder cell types. Our study showed that IFN $\alpha$ -2b stimulated and enhanced TNF and IL-1 production by KCs in the absence of LPS, the difference may have resulted from the cell types and condition of macrophages, since KCs may always be exposed to endotoxins and activated to some degree<sup>[16]</sup>. In our previous studies, anti-tumor effects of KCs activated by various biological immunal modulants were different, but the combined use of various biological immunal stimulants showed the best effect<sup>[2]</sup>. Our previous studies of the mechanism of KCs function also showed that the combination of IFN $\alpha$ -2b and BCG exhibited the best results, the increase of the number and volume of KCs, the increase of the amount of H<sub>2</sub>O<sub>2</sub> released by KCs<sup>[3,4]</sup>. The systematic study mentioned above, regardless of

normal or hepatoma rats and both *in vivo* and *vitro* consistently confirmed the combined use of IFN $\alpha$ -2b and BCG was better than either one used alone in enhancing the activity of immune cells. These results suggested that the combined use of IFN $\alpha$ -2b and BCG to HCC patients should be advocated.

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