Study of T-cell stimulation and cytokine release induced by *Staphylococcal enterotoxin* type B and monophosphoryl lipid A

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Abstract

Introduction: *In vitro* exposure of T cells to SEB is known to activate T cells in a subset restricted manner based on β-chain variable region (V_β) gene expression. Monophosphoryl lipid (MPL) is an adjuvant derived from a lipopolysaccharide obtained from gram negative bacteria. This study examined the ability of *Staphylococcal enterotoxin* type B (SEB), MPL and SEB + MPL to activate T cells *in vitro*.

Material and methods: Lymphocyte cells derived from lymph node Balb/c mice were exposed to series of doses of SEB, MPL and SEB + MPL (1.10, 10^2, 10^3 and 10^4 ng/µl) in order to evaluate the magnitude of proliferation and activation of lymphocyte cells on the basis of a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Results: Results of this investigation showed that the optimal concentration of SEB + MPL for activation of lymphocyte cells was 100 ng/µl ($p < 0.05$). Additionally, using Hoechst staining, we studied the ability of SEB, MPL and SEB + MPL to induce apoptosis. The results indicated no significant difference between the treatment and negative control groups. We concluded that 100 ng/µl of SEB, MPL and SEB + MPL did not generate apoptosis; they only induced the activation of lymphocyte cells. We also clarified that the combination of SEB and MPL can cause more stimulation and proliferation of mouse lymphocyte cells than any individual component or the negative control.

Conclusions: Our data suggest that MPL is a suitable adjuvant that probably leads to more sustained effect of SEB. Because the results of our cytokine assay indicate that the level of IFN-γ compared with IL-4, SEB + MPL could be a candidate for tumor therapy.

Key words: *Staphylococcal enterotoxin* B (SEB), monophosphoryl lipid A (MPL), superantigen, lymphocyte cell.

Introduction

Staphylococcus aureus enterotoxins (SEs), consisting of a group of 26 to 30 kDa proteins, stimulate T-cells in mice and humans [1-3]. *Staphylococcus aureus* enterotoxins, including *Staphylococcal enterotoxin* type B (SEB), act as superantigens and stimulate all T cells bearing reactive V_β-chains [4]. In contrast to conventional antigens (Ags), superantigens do not require processing by the antigen presenting cells

(APCs). They bind to major histocompatibility complex class II molecules (MHC-II) outside of the peptide binding groove and interact with the variable region of the β-chain of the T-cell receptor (TCR). Although recognition of conventional Ags in the context of class I and II generally requires CD8 and CD4, respectively, superantigens such as SEB induce both CD4 and CD8 T cell proliferation and effector function [5, 6]. Notably, SEs are the most common cause of food poisoning in humans [3], but the structures of T-cell receptors and mucosal cells are not identical [7].

Monophosphoryl lipid A (MPL) is an adjuvant derived from the lipopolysaccharide of *Escherichia coli*, *Salmonella Minnesota* Re595, and other gram negative bacteria. The lipid A portion of the gram-negative endotoxin has long been known to be a potent adjuvant and T-cell activator, but undesirable toxicity has limited its clinical use. Monophosphoryl lipid A, the molecule remaining after removing the phosphate and fatty acid group from lipid A, still retains the adjuvant and T-cell activator properties of lipid A but has significantly reduced toxicity [8, 9]. The adjuvant activity of MPL with viral and bacterial antigens has been investigated in murine studies; the results suggest that MPL primarily promotes a T-helper1 (Th-1) response characterized by increased interferon-γ production and induction of the IgG2a antibody isotype [10-14]. In human studies with adult volunteers receiving investigational vaccines, MPL alone or in combination with other adjuvants has been shown to be well tolerated and to enhance both humoral and cellular immune responses [15, 16].

Because MPL causes enhancement of humoral and cellular immune responses, it is employed as a proper adjuvant in vaccines, leading certainly to a more sustained immune response. Unlike other adjuvants, MPL enhances Th1 proliferation. Therefore, together with SEB, MPL could be a good candidate to examine for antitumor activity. In this research, we examined the effects of SEB and MPL individually and synergistically in lymphocyte cells.

Material and methods

Preparation of monophosphoryl lipid A and *Staphylococcal enterotoxin* type B

Monophosphoryl lipid A and SEB were purchased from Sigma-Aldrich (Munich, Germany). and we prepared a series of concentrations of 1, 10, 10^2, 10^3, 10^4 ng/µl MPL, SEB, or a combination of both of them (MPL + SEB) in phosphate buffered saline (PBS).

Mice and injection program

Female inbred BALB/c mice (6 to 7 weeks old) were purchased from the Pasteur Institute (Tehran, Iran). A total of 24 mice were divided into four six-mice groups designated as the following: group PBS, intravenous injection of PBS (negative control); group MPL, intravenous injection of 100 ng MPL; group SEB, intravenous injection of 100 ng SEB; group MPL + SEB, intravenous injection of 100 ng MPL and 100 ng SEB. These challenges were performed every 72 h for two weeks by methods described previously [22]. Animal experiments in this study were done in compliance with Tarbiat Modares University institutional guidelines.

Cell preparation and cell culture conditions

Lymphocytes were isolated from axillary and inguinal lymph nodes of Balb/c mice. Isolated lymph nodes were cut into small pieces, rinsed twice with PBS, and minced with forceps and scalpels. The suspensions were passed through a 100 µm stainless steel mesh to obtain a single-cell suspension, and erythrocytes were lysed at room temperature using ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2-EDTA). The cells were washed and resuspended in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) and incubated at 37°C in 5% CO_2 with appropriate humidity.

Cell proliferation analyses by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

Cells at the concentration of 1×10^5 cells/100 µl were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Sigma-Aldrich, Munich, Germany) with 1, 10, 10^2, 10^3, 10^4 ng/µl of either MPL or SEB, or both MPL and SEB (MPL + SEB), and incubated at 37°C in the 5% CO_2 with appropriate humidity. Stimulation of mice lymphocytes was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. After incubation, an aliquot of 100 µl of MTT reagent (0.5 mg/ml final concentration) was added to each well and incubated for another 4 h. Then the optical density was measured at 540 nm using a UV microplate reader (Tecan Austria GmbH, Austria). The stimulation Index (SI) was calculated according to the following formula:

$$SI = \frac{\text{OD } (test)}{\text{OD } (negetive\ control)}$$

where OD – optical density.

Phytohemoagglutinin (PHA = 1 ng/µl) was used as the positive control and sterile PBS as negative control. Each assay was repeated three times [17].

In vitro apoptosis assay

A lower dose than the reported LD_{50} for enterotoxin type B [18] was used to activate lymphocytes [19]. Induced apoptosis caused by SEB, MPL, or both of them (MPL + SEB) was examined by staining with Hoechst 33258 (Wako, Osaka, Japan) [20]; a total of 1000 lymphocytes were randomly observed under the high power field (×400 magnification) to evaluate the number of apoptotic cells under different conditions; the ratio of apoptotic cells per 1000 lymphocytes was calculated [21]. The lymphocytes exposed to different concentrations of SEB, MPL, or SEB + MPL were cultured for 72 h; then the lymphocyte smears was prepared, fixed in 10% formalin, and stained with Hoechst 33258. Finally, the ratios of apoptotic cells were determined using a florescent microscope (Figure 1). In the next step, the optimal concentrations of SEB, MPL, and MPL + SEB for generating apoptosis were determined by kinetic curve, and then this optimal concentration was further studied. The procedure was repeated three times (Figures 2, 3).

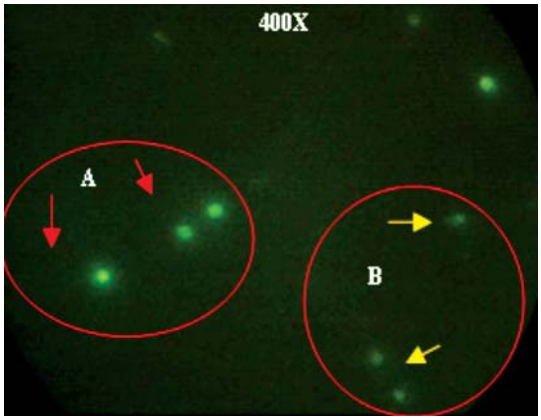

Figure 1. A) Lymphocyte cells are completely stained with Hoechst 33258, magnification of 400×. They demonstrate that the chromosomes have not been damaged (the red arrow indicates cells that are not undergoing apoptosis); **B**) Lymphocyte cells that are not completely stained with Hochest33258, magnification of 400×. They are colorless, demon-strating that chromosomes have been damaged (yellow arrow indicating cells undergoing apoptosis)

Enzyme-linked immunosorbent assay (ELISA) for measuring cytokines

Mice splenocytes were isolated by the same methods used for isolation of lymphocytes from lymph nodes. Cells at the concentration of 1×10^5 cells/100 µl were cultured with MPL, SEB, or MPL + SEB at a concentration of 100 ng/ml. After three days of incubation, the supernatants were collected, and IFN-γ and IL-4 were measured by ELISA kits (R&D, Minneapolis, MN) according to manufacturer's instructions.

Statistical analysis

All experiments were performed four times, and the mean ± SD was calculated. Statistical analyses were performed using the two-tailed Mann Whitney nonparametric test and a p value of $p < 0.05$ was considered as statistically significant. All statistical analyses were conducted with SPSS 13.0 software (SPSS Inc., Chicago, IL).

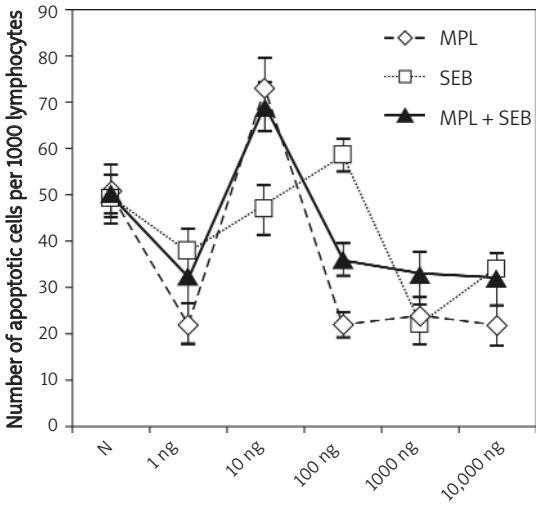

Figure 2. Kinetic curve of apoptosis evaluation in mouse lymphocyte cells after exposure to varying concentrations (10, 10^2, 10^3, 10^4 ng/ml) of SEB, MPL, or SEB + MPL. We found that concentrations of 100, 10, and 10 ng/ml of SEB, MPL, and SEB + MPL, respectively, were the optimal concentrations for induction of apoptosis

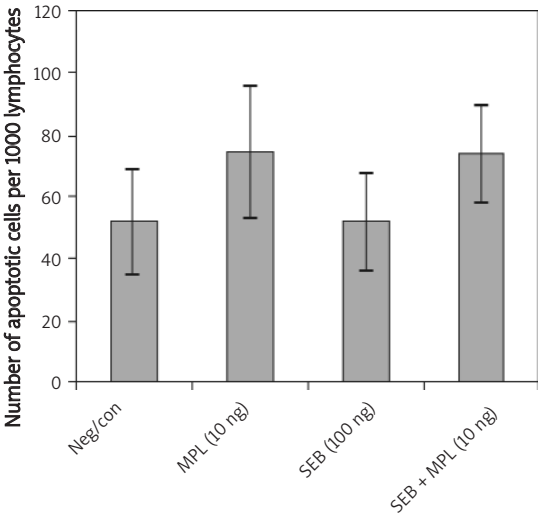

Figure 3. Evaluation of apoptosis caused by optimal concentrations of SEB (100 ng/ml), MPL (10 ng/ml) and SEB + MPL (10 ng/ml) (as determined by kinetic curve) in mouse lymphocytes. The mean ± SD of triplicate determinations are shown. No significant defferences were found by Mann-Whitney nonparametric test ($p < 0.05$) different in comparison to the negative control (PBS)

Results

Cell proliferation assay (MTT assay)

Lymphocytes from Balb/c mice were cultured in RPMI-1640 containing graded concentrations of the following mitogens for 48 h; MPL, SEB, or both of them (MPL + SEB). Then the lymphocytic proliferations were examined by MTT assay. Results are shown in Figure 4. Only the concentration of 10^2 ng/ml showed a significant difference between MPL alone and MPL + SEB. No further significant differences were observed. Therefore, we set the concentration at 10^2 ng/µl for proper activation of lymphocytes. With regard to toxin LD50 and other details, the optimal concentration was 10^2 ng/µl, and its activation ability in comparison with other concentrations was significantly better [22].

In vitro apoptosis assay

Using Hoechst 33258 staining, MPL, SEB, and MPL + SEB were analyzed for their ability to induce apoptosis of lymphocytes *in vitro*. Typical examples are shown in supplementary Figure 4. As shown in Figure 2, the results obtained suggested that concentrations of 10 to 10^2 ng/µl of MPL, SEB, and SEB + MPL seemed optimal for stimulating apoptosis, although SEB alone required a higher concentration. However, no statistically significant differences were observed. Therefore, we concluded that concentrations of 10^2 ng/µl of MPL, SEB, and MPL + SEB seems to be appropriate levels for activation of lymphocytes and prevention of tumor cell growth without considerable adverse side effects on the lymphocytes.

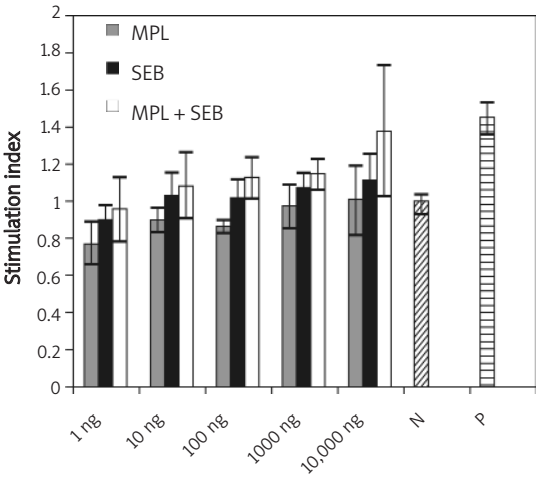

Figure 4. The proliferation rates of mouse lymphocyte cells (MTT Assay) after 48 h exposure to MPL, SEB and SEB + MPL expressed as Stimulation Index (SI). The mean ± SD of triplicate determinations are shown. Statistical analysis was performed using the two tailed Mann Whitney nonparametric test, and a p value of $p < 0.05$ was considered as a statistically significant difference. PBS was used as the negative control (N), and phytohemoagglutinin (PHA) at 1 ng/µl was used as the positive control (P)

Level of interferon-γ and interleukin-4 after the monophosphoryl lipid A and *Staphylococcal enterotoxin* B injection

Splenocytes were challenged, and their ability to produce interferon-γ (IFN-γ) and IL-4 were evaluated. Results are shown in Figure 3. Although no significant differences in the IL-4 producing ability were observed, SEB and SEB + MPL showed significant higher levels of IFN-γ producing ability ($p < 0.01$). These results suggest that the MPL + SEB at 10^2 ng/µl level can be a good candidate for tumor therapy because the combination can induce IFN-γ by utilizing the response of immune system.

Discussion

Because the pathways of murine T-cell activation are similar to those of human T-cells, experimental results in mice can be also generalized to humans [23]. *Staphylococcal enterotoxin* B binds quickly and strongly to the surface of the external membrane of lymphocyte cells and is recognized as a strong polyclonal mitogen for T-cells. *Staphylococcal enterotoxin* B binds strongly to the V_β receptors of T-cells and remains there in spite of several washings, leading to increasing T-cell proliferation [24].

Other bacterial components, including peptidoglycan, lipotecoic acid and lipopolysacharide (LPS), are recognized by toll-like receptors (TLR). The healthy immune system reacts against these components. Dalpke and Heeg showed that superantigens synergies with TLR, including LPS, that TLR cooperate with endotoxin in T-cell activation, and that the superantigen and toll-like receptor pathways regulate each other [25].

Dalpke and Heeg also believe that SEB and LPS have synergistic effects and that their combined application leads to more stimulation of T-cells and greater production of cytokines [25]. For this reason, we used the MTT assay in order to evaluate the proliferation or suppressive effects of above-mentioned components. This test is based on a reaction between the MTT reagent and the dehydrogenase existing in mitochondria of cells that lead to formation of Formosan crystals.

The kinetics of above-mentioned components was examined in mouse lymphocyte cells in order to determine the non-lethal concentrations of toxin and monophosphoryl lipid A for normal cells. A greater proliferation of the cells obviously leads to the formation of more crystals and an increase in the stimulation index.

It has been clarified in this study that the combination of SEB and MPL can cause more stimulation and proliferation of mouse lymphocyte

cells than does any of the individual components or the negative control. Because it has been clarified that MPL increases the expression of gene MHC-II molecules and that MHC-II functions as a specific receptor for SEB, it is therefore expected that MPL will also increase the proliferation of mouse lymphocyte cells. On the other hand, MPL has been shown to be a suitable adjuvant that probably leads to a more sustained effect of SEB [26]. Researchers consider the factors of dosage and time as two important players in the stimulation and proliferation of lymphocyte cells. A higher SEB dosage leads naturally to more stimulation and proliferation, but previous reports have indicated that the maximum stimulation occurs within the first 24 h of exposure [23, 24]. However, we believe that more exposure over a longer time leads to more stimulation and that the best results for stimulation can be obtained in 72 h. Because SEB binds strongly to lymphocyte cells and cannot easily be separated from them, the longer exposure leads to binding of SEB to more cells and the stimulation of even more. It seems that a longer exposure of lymphocyte cells to MPL leads to the expression of more MHC-II molecules and the subsequent binding of MHC-II molecules to SEB, ultimately resulting in a greater proliferation of lymphocyte cells. This argument is supported by the fact that tumor cells in their natural state impose suppressive effects on the expression of MHC-II molecules [27, 28].

Kenneth *et al*. used a high dose of SEB (50 µg/ml) that probably led to an initial saturation of all the cells by SEB. Their dose of SEB is higher than the LD_{50}. Our current research had used lower concentrations than the LD_{50} because we intended to use these concentrations for *in vivo* experiments [23]. In our research, we clarified that concentrations between 10 and 10^2 ng/µl of combined SEB + MPL, compared with MPL and SEB individually, have a greater stimulating effect at a significantly different level (Figure 4). However, concentrations between 10^3 and 10^4 ng/µl, compared with MPL and SEB individually, have no statistically significant different effect despite increased stimulation and proliferation of mouse lymphocyte cells. The study of Tapan *et al.* confirms our result [22]. For *in vivo* studies, they used 10 ng/µl. In our research, the toxicity of different concentrations of SEB and MPL and the magnitude of their apoptosis effect on mouse lymphocyte cells were evaluated. It was clarified that the concentrations of 10 and 10^2 ng/µl had only stimulating effects and are able to stimulate T-cells *in vivo* (Figures 2, 3). Because of this greater activity of T-cells, the magnitude of apoptosis and necrosis might increase in tumor cells, but these concentrations had no apoptotic effect on lymphocyte cells. In other development, Weber et al have shown that a concentration of 10^3 ng/µl SEB is able to cause apoptosis of invasive cells [29]. A possible explanation for this difference in comparison with our study may involve the type of lymphocyte cells (spleen) that we used or the conditions of each experiment. In our investigation, the use of MPL in addition to SEB appeared to lead to a more sustained effect of SEB on stimulating T-cells. Increased expression of MHC-II molecules by using lower dose of SEB was increase proliferation and stimulation of T-cells [26]. A synergistic effect between SEB and MPL could explain the effectiveness of using a lower dose of SEB for stimulating T-cells than did Anne *et al*. [29]. Additionally, we believe that *in vivo* condition can involve many factors that influence the reactions between lymphocyte cells, SEB, and MPL and that the system must be studied further in more detail.

In considering the apoptotic effects of SEB and MPL, it must be pointed out that it is necessary to use a specified concentration of SEB and MPL that has the minimal apoptoic effect combined with the maximal stimulating effect on lymphocyte cells. This need arises because the apoptosis of lymphocyte cells, in addition to disrupting the treatment of cancer, can also result in chronic inflammatory diseases including arthritis, Kawasaki disease, psoriasis, and atopic dermatitis. Superantigens can also cause such dangerous diseases, and, if they are used in an uncontrolled manner during treatment, can have serious side effects. Because superantigens enhance Th1 and produce unlimited cytokines like IFN-γ, they may be involved in inducing diseases related to the immune system [30].

The results obtained from measuring the cytokines IFN-γ and IL-4 showed that amounts of

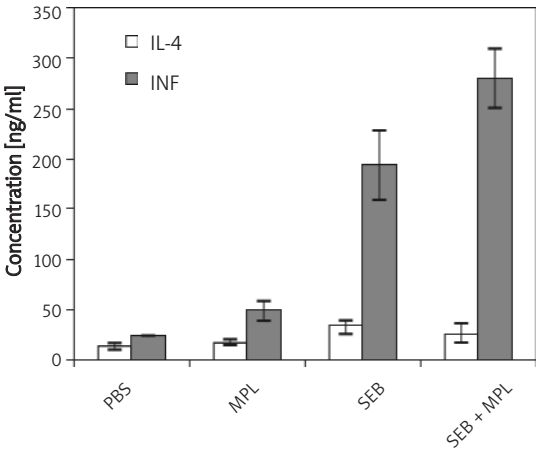

Figure 5. IFN-γ and IL-4 production by splenocytes. Splenocytes were cultured *in vitro* with SEB, MPL, or SEB + MPL. After 3 days culture, the supernatants were harvested, and IFN-γ and IL-4 releases were measured by ELISA. The mean ± SD of triplicate determinations are shown (n = 5 mice per group). There was a significant difference by Mann-Whitney nonparametric test ($p < 0.01$) from the negative control (PBS)

IFN-γ produced compared with IL-4 produced and the negative control are significantly different ($p < 0.01$) (Figure 5). Furthermore, the amounts of IFN-γ produced by SEB+MPL group compared with SEB and MPL individually are also significantly different ($p < 0.01$) (Figure 5).

This increase of IFN-γ compared with IL-4 indicates that the response of immune system is directed to Th1, thus enhancing the antitumor activity.

Because MPL causes the increased expression of MHC-II molecules on the surface of T-cells, it was expected that the intravenous injection of SEB + MPL would be more effective than the individual injection of each of them. Our measured immunologic factors have verified this fact. Because the intravenous injection of SEB leads to its binding to its receptors on the surface of T cells and causes both T cell stimulation and proliferation, MPL, which increases the expression of these receptors leads to a more intensified and sustained immune response. Tappan *et al*. obtained similar results when studying the synergistic effects of *Staphylococcal enterotoxin* type A with protein A (SEA + PA).

SEA alone, as well as SEB, is not able to stimulate the immune system; it always has to be used as a factor in combination with other components and/or antibodies [22, 31]. However, in our current study, the effects of SEB together with MPL have indicated that SEB individually and combined with MPL has a better antitumoral effect than SEA + PA. It has been determined that PA is effective in stimulating the humeral immune system [22], but that MPL is effective in stimulating cellular immunity as well as the humeral immune system; therefore, its antitumor activity is greater than that of PA.

Shimizu *et al*. showed that V_β of T-cells was a specific receptor for the SEB superantigen and that binding of SEB increases V_β proliferation and the antitumor activity of T-cells. Imani Fooladi *et al*. showed that necrosis was induced in mice fibrosarcoma after intravenous injection of type B *Staphylococcal enterotoxin* [31]. In consideration of the above-mentioned findings, our results after IV injection of SEB were to be expected. Although MPL has been used for vaccine design, it seems possible that a SEB + MPL conjugate vaccine may be used in the future in order to prevent the formation of tumors [32].

In conclusion, the effects of SEB as a mitogenic agent have been a subject of many studies in recent years, but the effects of MPL and the combined effects of SEB + MPL have not been clarified. MPL appears to be a suitable adjuvant. It causes the increased expression of MHC-II molecules (specific receptor for SEB) on the surface of T-cells and therefore can influence the triggering effects of *Staphylococcal enterotoxin* type B to make these effects longer in duration and more sustained. It is necessary to do further studies in order to understand the antitumor effects of this combination and their implications for vaccine design.

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