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Montivipera bornmuelleri venom has immunomodulatory effects mainly up-regulating pro-inflammatory cytokines in the spleens of mice

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ABSTRACT

Beside their toxicity, snake venom components possess several pharmacological effects and have been used to design many drugs. Recently, the cytotoxic, antibacterial, vasorelaxant, pro- and anti-coagulant as well as inflammatory activities of *Montivipera bornmuelleri* venom have been described *in vitro*. However, the *in vivo* effects of this Lebanese snake venom on the immune system has not been established yet. Here, we investigate the immunomodulatory effects of *M. bornmuelleri* venom on the murine splenic levels of TNF-α, IFN-γ, IL-4, IL-10, IL-1ß and IL-17 at 6 and 24 h post treatment. Different doses of the venom (1 mg/kg, 2 mg/kg, 4 mg/kg and 6 mg/kg) were injected intraperitoneally in BALB/c mice. Using the logit method, LD_{50} of *M. bornmuelleri* was proved to be 1.92 mg/kg in our experimental conditions. This study also shows that 1 mg/kg and 2 mg/kg of *M. bornmuelleri* venom are able to modulate the levels of cytokines in the spleen of mice, as assessed by ELISA. In fact, this snake's venom up-regulates TNF-α, IFN-γ, IL-1ß and IL-17 with a trend in decreasing IL-4 and IL-10. Therefore, by favoring Th1 and Th17 over Th2 and Treg responses, *M. bornmuelleri* venom might have important clinical implication especially in the field of cancer immunotherapy.

1. Introduction

Snake venom studies trace back to Aristotle (384–322 BCE); however, they were not experimentally used until the 18th century (by Felice Fontana) [1]. Snake venoms contain a mixture of mostly proteins and peptides that constitute 90–95% of the venom's dry weight including enzymes, non-enzymatic peptides and toxins. Although being dangerous, snakes have always been associated with healing; the true and authentic symbol of medicine is the Rod of Asclepius, illustrating a staff with a snake coiled around it. In fact, venom components form a pool of pharmaceutical products involved in many medical practices and drug discovery. For example, Captopril is the first drug based on a peptide from the pit viper *Bothrops jararaca* and is used to treat hypertension and some forms of congestive heart failure [2,3]. In addition, snakes venom are capable of modulating the immune system especially at the level of cytokines production. A member of the *Elapidae* family exhibited anti-arthritic and anti-inflammatory activities in arthritic rats mainly through reducing serum levels of the pro-inflammatory cytokines IL-1β, IL-17 and TNF-α [4]. However, most immunomodulatory studies focused on the venom of the *Viperidae* family especially the subfamily of *Crotalinae* with its both genera *Bothrops* and *Crotalus*. Intraperitoneal injection of *Bothrops atrox* and *Bothrops erythromelas* venoms in mice resulted in an inflammatory reaction characterized by the up-regulation of many serum and splenic cytokines such TNF-α, IL-6, IL-10, IL-12p70 as well as IFN-γ [5,6]. Conversely, *Crotalus* envenomations failed to generate a significant inflammatory reaction at the site of bite and induced an anti-inflammatory status in a mouse experimental model of colitis [7]. The distinct effects of Bothrops and Crotalus venoms on the inflammatory response might be attributed to the inability of *Crotallus durissus terrificus* to induce the expression of COX-2 and therefore the synthesis of the prostanoids PGD_2 and PGE_2 [8].

Montivipera bornmuelleri is a venomous snake belonging to the family of *Viperidae* and endemic to high-altitude Lebanese mountains [9]. A proteomic analysis of the venom of *M. bornmuelleri* showed that it contains 65 protein compounds corresponding to enzymatic protein families such as serine proteases, phospholipases A2 and metalloproteases III [10]. The crude venom of *M. bornmuelleri* possesses

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antimicrobial activities against Gram positive and Gram negative bacteria, with the most significant effect on *Staphylococcus aureus* and *Morganella morganii,* as well as against the fungus *Candida albicans* [10]. *M. bornmuelleri* venom also affects the hemostatic system since it has been shown to have pro- and anti-coagulant activities on human plasma at different concentrations [11]. Vipers venom are known to reduce blood pressure; this is also true for *M. bornmuelleri* venom. In fact, the venom displays vasorelaxant effects by acting synergistically on different pathways. It can possibly act on endothelial cells inducing the release of the vasoactive mediator NO, reducing Ca^{2+} influx through voltage dependent calcium channels and inhibiting angiotensin I induced vaso-constriction [12]. Furthermore, it was suggested that *M. bornmuelleri* venom lacks direct lytic factors since it induces indirect hemolysis in the presence of PLA2, which is able to hydrolyze lecithin [11]. To further characterize the role of PLA2, it was purified and its biological activity was tested showing that it exhibits strong anti-bacterial, hemolytic, anti-coagulant and pro-inflammatory activities [13,14].

Despite the extensive studies of the Viperidae venom's biological activities, the immunomodulatory effect of the *M. bornmuelleri* venom has not been previously tested *in vivo*. Therefore, we investigate here the effect of the intraperitoneal injection of several *M. bornmuelleri* venom doses on the levels of various cytokines (TNF-α, IFN-γ, IL-4, IL-10, IL-1ß and IL-17) in spleen tissue of BALB/c mice, which will help to further evaluate the potential use of the venom in immunotherapy.

2. Materials and methods

2.1. Chemicals

Bovine Serum Albumin (BSA), NP-40, Phosphate Buffer Saline (PBS), Sodium Chloride (NaCl), Sodium Dodecyl Sulftae (SDS) and Sodium Deoxycholate were obtained from Sigma-Aldrich chemie, Steinheim, Germany. Tris-Hydrochloride (Tris-HCl) and tween-20 were obtained from Bio Basic Inc., Ontario, Canada.

2.2. Venom

Venom was supplied by Dr. Riad Sadek (American university of Beirut) in its lypholised form and stored at −20 °C. Venom was dissolved in PBS prior to the experiment and filtered through 0.2 µm sterile syringe filters.

2.3. Mice handling

Eight to ten weeks old female BALB/c mice, procured from the University of Balamand animal house, were fed a standard diet and kept at 25 °C in 12 h day/night cycle. They were handled according to the Guide for Care and Use of Laboratory Animals of the Faculty of Sciences.

Mice were placed in groups and injected with different doses of venom: 1 mg/kg, 2 mg/kg, 4 mg/kg and 6 mg/kg. Control animals were injected with either PBS (negative control) or fed a normal diet (naïve). Mice were sacrificed by neck dislocation at time of death or at 6 and 24 h following the injections. Spleens were removed, weighed and kept in eppendorf tubes at −80 °C. All experimental procedures were carried out with ethics committee approval from the University of Balamand and with strict adherence to the ethical guidelines for the study of experimental pain in conscious animals [15].

2.4. Determination of the median lethal dose

According to the world health organization (WHO), the median lethal dose (LD_{50}) is the amount of venom causing the death of 50% of treated animals. Most of the mice injected with 4 mg/kg and 6 mg/kg of venom died by 24 h, with death occurring mainly between 2 and 4 h post injection. These two doses were thus considered as toxic and not of relative significance to the rest of the results. Six out of 10 of the mice injected with 2 mg/kg of venom were dead by 24 h, whereas, mice injected with 1 mg/kg of venom remained all viable except for 1. Plotting the natural logarithm of the proportion of mice dead for each injection versus their corresponding logarithmic concentration generates a linear curve used to determine the toxicity of a certain compound on living organisms.

2.5. Tissue preparation

Frozen samples were homogenized in 1.5 ml RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS pH = 7.6) supplemented with Protease inhibitors at 4 °C. The homogenate was incubated on ice for 30 min and then centrifuged at 10 000*g* for 30 min at 4 °C. Following centrifugation, the supernatants were transferred to labeled eppendorfs and stored at −80 °C for cytokine measurement.

2.6. Cytokine measurement

Quantitative measurement of the levels of cytokines was performed using Mini Enzyme-Linked Immunosorbant Assay (ELISA) Development Kits (Peprotech). 96-well plates were set up according to the manufacturer's instructions and read using an ELISA plate reader at 405 nm with 650 nm as the correction wavelength. Concentrations of the cytokines TNF-α, IFN-γ, IL-4, IL-10, IL-1ß and IL-17 were estimated using standard curves established with the appropriate recombinant cytokines. The results were expressed as pg/ml and then pg/g of tissue.

2.7. Statistical analysis

Differences among groups were analyzed using GraphPad Prism 6.00 software (GraphPad Software Inc., San Diego USA) by one-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test. Results were expressed as means ± SEM (n = 4–3 animals/group). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Determination of LD50 of Montivipera bornmuelleri venom

Mice were injected once with the following venom concentrations: 1 mg/kg, 2 mg/kg and 4 mg/kg and the LD50 was estimated. Our results show that the intraperitoneal LD_{50} of *M. bornmuelleri* venom is 1.92 mg/kg is in our experimental conditions (Fig. 1). This finding therefore explains the observed lethality of the 2 mg/kg dose on almost half of the injected mice.

3.2. The effect of M. bornmuelleri venom on the levels of IFN-γ in the spleen of mice

Intraperitoneal injections of mice with 1 mg/kg of venom failed to induce IFN-γ up-regulation 6 h post-treatment (Fig. 2A). However, 24 h following the treatment an increase in IFN-γ levels, though insignificant, was observed (Fig. 2B). Furthermore, intraperitoneal injections of mice with 2 mg/kg was able to induce a significant increase in IFN-γ levels as compared to the control ($p < 0.01$).

3.3. The effect of M. bornmuelleri venom on the levels of TNF-α in the spleen of mice

The dose of 1 mg/kg of *M. bornmuelleri* venom was able to significantly induce the production of TNF-α as compared to the control groups ($p < 0.01$) at 6 h (Fig. 3A) but not 24 h post-treatment (Fig. 3B). However, injecting 2 mg/kg of venom caused a significant

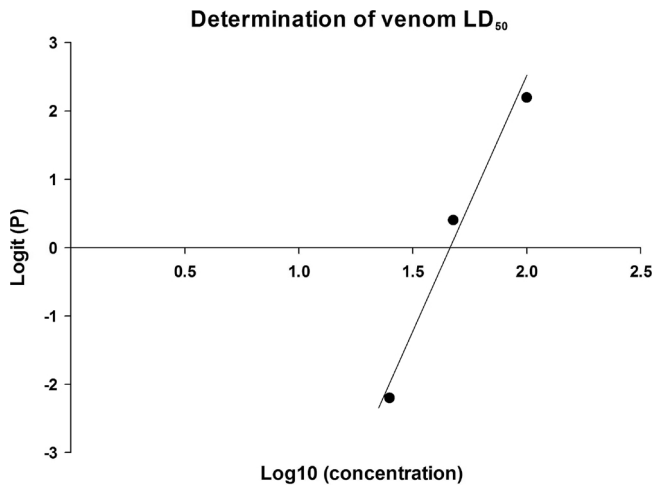

Fig. 1. Determination of the LD_{50} of M. bornmuelleri following the administration of increasing doses of the venom (1 mg/kg, 2 mg/kg and 4 mg/kg). The graph represents the natural logarithm of the percent mortality of mice versus the corresponding logarithms of venom concentration.

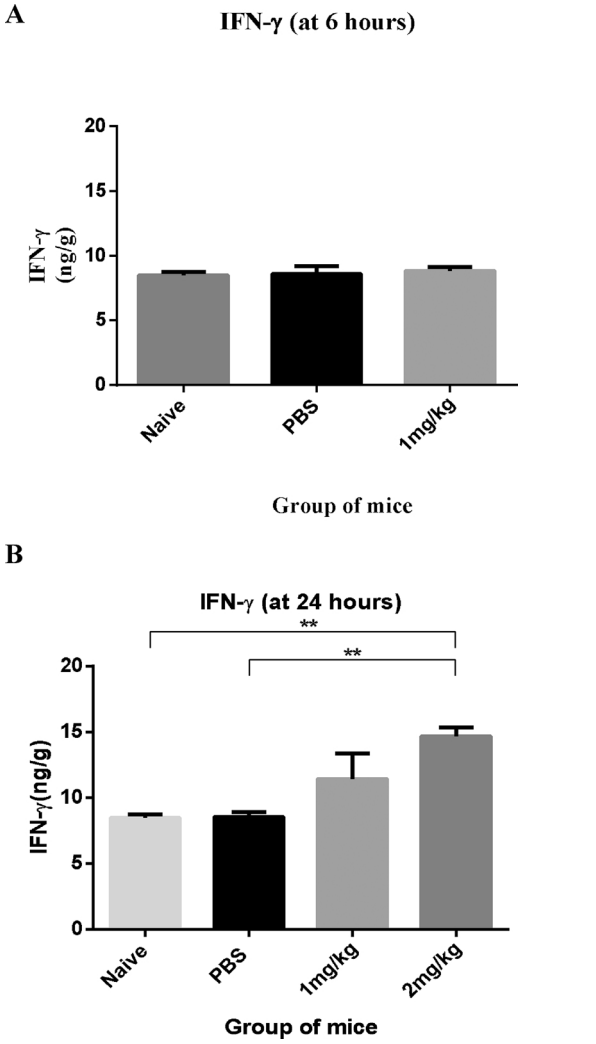

Fig. 2. Concentration of IFN-γ in the spleens of mice at: (A) 6 h and (B) 24 h following venom injection. Cytokines were quantified using by Enzyme-Linked Immunosorbant Assay (ELISA) according to the manufacturer's instructions. Each bar represents the Mean ± SEM for n = 4 per group. ** $p < 0.01$.

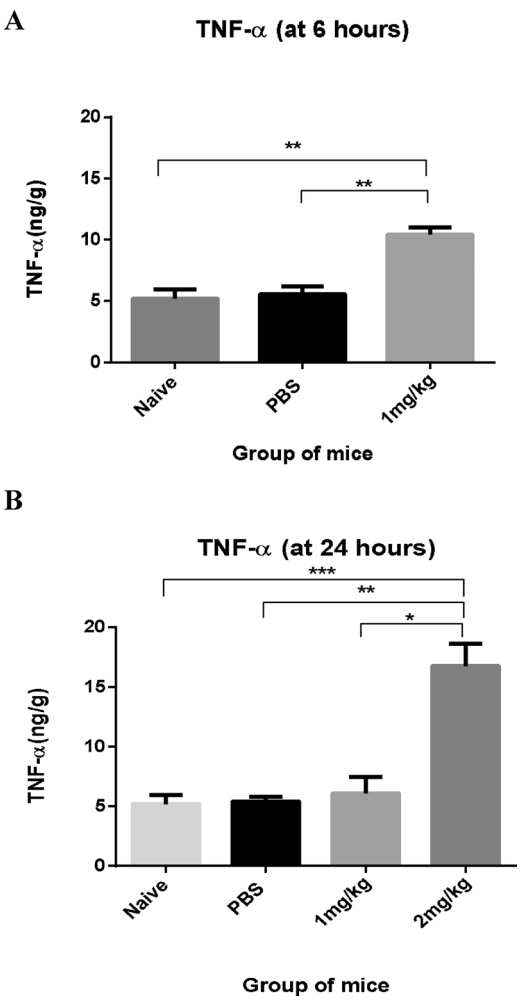

Fig. 3. Concentration of TNF-α in the spleens of mice at (A) 6 h and (B) 24 h following venom injection. Cytokines were quantified using Enzyme-Linked Immunosorbant Assay (ELISA) according to the manufacturer's instructions. Each bar represents the Mean ± SEM for n = 3 per group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

increase in TNF-α at 24 h post-treatment as compared to the control ($p < 0.01$) and to the 1 mg/kg dose ($p < 0.05$).

3.4. The effect of M. bornmuelleri venom on the levels of IL-1β in the spleen of mice

Intraperitoneal injection of 1 mg/kg of *M. bornmuelleri* venom caused a significant increase in the levels of IL-1β as compared to the control at 6 h ($p < 0.001$) (Fig. 4A) and to a lower extent at 24 h post-treatment (Fig. 4B). Also, the 2 mg/kg dose significantly increased splenic IL-1β levels at 24 h post injection but to a lesser extent than dose 1 mg/kg ($p < 0.05$).

3.5. The effect of M. bornmuelleri venom on the levels of IL-4in the spleen of mice

Intraperitoneal injection of 1 mg/kg of venom had no significant effect on the level of IL-4 in the spleen of mice (Fig. 5A). However, both doses (1 mg/kg and 2 mg/kg) caused a significant increase in the levels of IL-4 to a comparable extent as compared to the control group at 24 h post treatment ($p < 0.01$) (Fig. 5B).

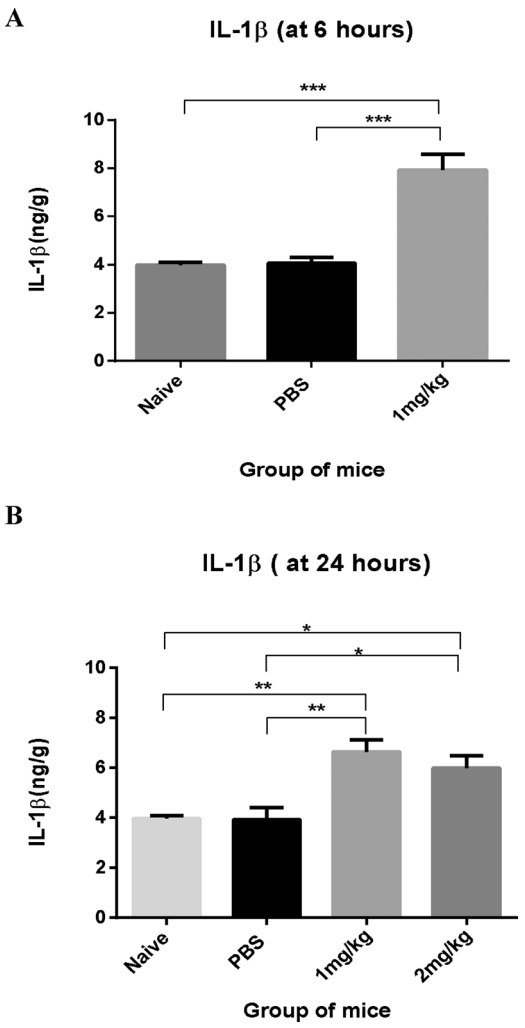

Fig. 4. Concentration of IL-1β in the spleens of mice at: (A) 6 h and (B) 24 h following venom injection. Cytokines were quantified using Enzyme-Linked Immunosorbant Assay (ELISA) according to the manufacturer's instructions. Each bar represents the Mean ± SEM (n = 4 per group). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

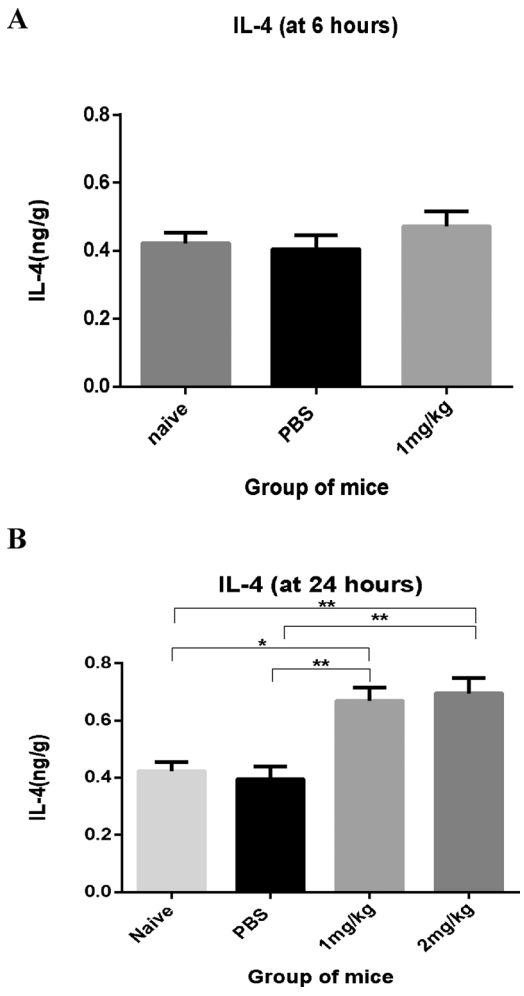

Fig. 5. Concentration of IL-4 in the spleens of mice at (A) 6 h and (B) 24 h following venom injection. Cytokines were quantified by Enzyme-Linked Immunosorbant Assay (ELISA) according to the manufacturer's instructions. Each bar represents the Mean ± SEM (n = 4 per group). * $p < 0.05$ and ** $p < 0.01$.

3.6. The effect of M. bornmuelleri venom on the levels of IL-10 in the spleen of mice

A decreasing trend of IL-10 levels can be observed 6 h after injecting the mice with 1 mg/kg *M. bornmuelleri* venom, as compared to the control (Fig. 6A). A similar trend is depicted 24 h following treatment with a slightly further decrease in IL-10 levels for dose 2 mg/kg as compared to the decrease noted for dose 1 mg/kg of venom (Fig. 6B).

3.7. The effect of M. bornmuelleri venom on the levels of IL-17 in the spleen of mice

Injecting mice with 25 µg of venom caused a significant increase in the splenic levels of IL-17 as compared to the control groups at 6 h ($p < 0.01$) (Fig. 7A) and at 24 h post-injection ($p < 0.05$) (Fig. 7B). Furthermore, a more significant increment in IL-17 levels at 24 h was detected for dose 2 mg/kg when compared to the control ($p < 0.01$).

4. Discussion

Many studies focused on identifying, characterizing, and purifying proteins within *M. bornmuelleri* crude venom, while other studies highlighted the biological properties of the venom *in vitro* [11–14]. However, the *in vivo* effects of this Lebanese snake venom is poorly known. In the literature, it has been shown that snake venom components can induce pro-inflammatory cytokines secretion and mediate tissue damage. In accordance, PLA2 isolated from the venom of *M. bornmuelleri* exhibited pro-inflammatory activities *in vitro* [13]. This could be explained by the fact that this venom component can act directly on leukocytes, macrophages and endothelial cells to stimulate cytokine production. In line with these findings, we observed in this work an inflammatory status in mice injected with *M. bornmuelleri* venom.

In this study, we show that the *M. bornmuelleri* venom (2 mg/kg) can increase the IFN-γ levels in the spleen of mice 24 h post-treatment suggesting that this venom might exacerbate or even induce some autoimmune and chronic inflammatory conditions and can as well inhibit Th2 cells recruitment and cytokine release. On the other hand, IFN-γ is known to play a key role in cancer immunosurveillance in spontaneous lymphomas and lung adenocarcinomas [16] as well as in an MCA-induced squamous cell carcinomas model [17]. Therefore, the *M. bornmuelleri* venom might have important potential in cancer immunotherapy.

This tendency of *M. bornmuelleri* venom is further elaborated by its ability to up-regulate the levels of the pro-inflammatory cytokines TNF-α and IL-1ß in the spleen of mice at 6 h (1 mg/kg) and 24 h (1 mg/kg and 2 mg/kg) post-treatment. This is in line with previous studies using the *Bothrops asper* and *Bothrops jararaca* venoms [18]. The parallel modulation of these "alarm cytokines" in mice treated with the venom

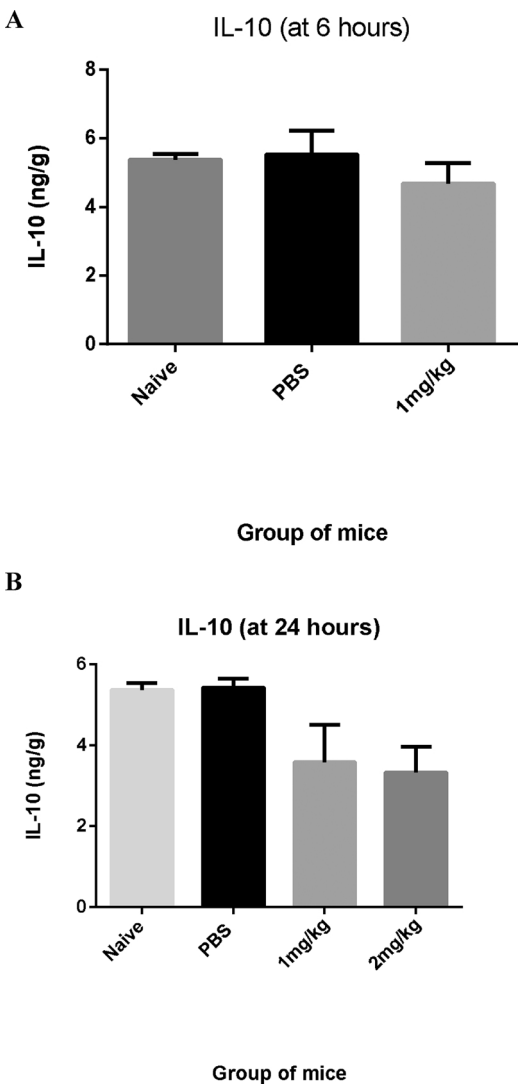

Fig. 6. Concentration of IL-10 in the spleens of mice at: (A) 6 h and (B) 24 h following venom injection. Cytokines were quantified by Enzyme-Linked Immunosorbant Assay (ELISA) according to the manufacturer's instructions. Each bar represents the Mean ± SEM (n = 4 per group).

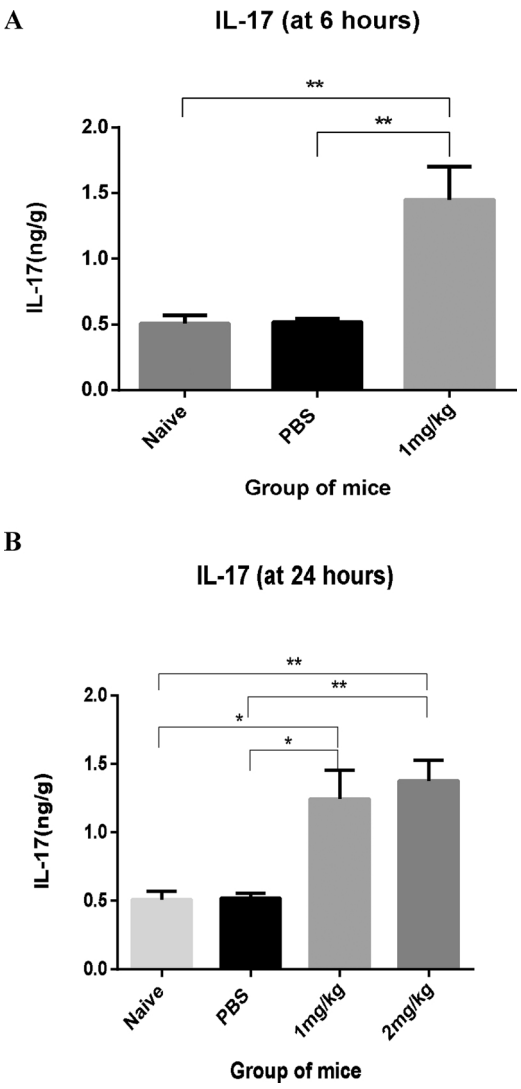

Fig. 7. Concentration of IL-17 in the spleens of mice at: (A) 6 h and (B) 24 h following venom injection. Cytokines were quantified by Enzyme-Linked Immunosorbant Assay (ELISA) according to the manufacturer's instructions. Each bar represents the Mean ± SEM (n = 4 per group). * $p < 0.05$ and ** $p < 0.01$.

reveals their synergetic and important actions in initiating and promoting inflammatory responses. In addition to their important functions during inflammation especially through the induction of chemokines secretion by endothelial cells and the release of neutrophils into the blood [19], TNF-α and IL-1ß are implicated in anti-tumor responses. Dondossola et al. [20] recently proved that genetically engineered tumor cells that produce TNF-α, inhibits the growth of primary and metastatic tumors in three different murine models, following systemic administration. On the other hand, although IL-1ß is known to induce anti-tumor responses at low local doses by activating specific immune responses [21], it participates in the generation of malignant cells and in the invasiveness of already established tumor cells[30].

The up-regulation of IL-4 levels 24 h following the injection of mice with 1 mg/kg of the venom highly correlates with the insignificant changes in the levels of TNF-α and IFN-γ, which usually antagonizes the IL-4 effects on the immune system and vice versa [22]. This was not the case with 2 mg/kg of the venom which was able to up-regulate the levels of IFN-γ in the spleen of mice inducing therefore the production of TNF-α and IL-1ß 24 h post-treatment. Those results favor the pro-inflammatory tendency and the antitumor potentials of the venom especially that IL-4 can exhibit antitumor effects by infiltrating eosinophils and macrophages (which are major sources of TNF-α and IL-1ß) into the tumor [23].

This pro-inflammatory tendency is further supported by its ability to cause a decreasing trend of IL-10 levels especially 24 h post-treatment with 50 µg of venom. Since IL-10 is known to suppress many inflammatory mediators [24], its decreased levels observed in this work correlate very well with the positive modulation of IFN-γ, IL-1ß and TNF-α by the venom. Consequently, the *M. bornmuelleri* venom might be associated with hypersensitivity reactions and autoimmune conditions [25] as well as with cancer immunotherapy.

The beneficial role of the venom in immunotherapy might also be associated with its ability to up-regulate IL-17 [26] which, as in the case of other pro-inflammatory cytokines (mainly IL-1β and TNF-α), its levels were significantly increased at 6 h post-treatment with 1 mg/kg of the venom. This up-regulation was sustained at 24 h post-treatment when the 2 mg/kg dose had similar effect. Such pattern of IL-17 correlates very well with the decreased levels (though not highly significant at both time points) of IL-10 which usually inhibits IL-17-producing macrophages and T cells [27]. However, this up-regulation of IL-17 seems to be regulated 24 h post-treatment by the increased levels of IL-4 which is known to exhibit a therapeutic role in Th17-mediated inflammatory diseases by silencing IL23/Th17 responses [28].

Many stimuli, such as snakes venoms, are able to induce

immunomodulatory responses; hence, it is necessary to check if the response could be potentially beneficial. Further experiments can thus be targeted towards revealing the effect of *M. bornmuelleri* venom in mice models of inflammatory diseases or in cancer models. This being said, many studies may be conducted in order to purify the component that triggers this immunomodulatory response. Once known, this component may present a promiscuous therapeutic potential in immunotherapies.

5. Conclusion

The present study shows that *Montivipera bornmuelleri* venom is able to significantly modulate the immune system towards a Th1/Th17 pro-inflammatory response, rather than to a Th2/Treg anti-inflammatory one. This shift seems to be mediated and sustained by the up-regulation of the pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ and the down-regulation of the anti-inflammatory cytokine IL-10 and, on the other hand, to be regulated by the ability of the venom to induce IL-4 production. These results, together with the finding that the *M. bornmuelleri* venom is selectively cytotoxic on the human derived keratinocytes cancer cell lines (low-grade malignant II4) as compared to the non-tumorigenic HaCaT cell line [29] demonstrate that this Lebanese viper venom is worthy for further investigations in the field of immunotherapy.

Conflict of interest

No conflict of interest about this work.

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