

Exploring the mechanisms behind the impairment of bone marrow neutrophil and eosinophil adhesion and chemotaxis by Staphylococcal Enterotoxin B

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Article

Keywords: Adhesion, Chemotaxis, Interferon- γ , VCAM-1, ICAM-1, Calcium

Posted Date: October 13th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11066366/v1>

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Additional Declarations: No competing interests reported.

Abstract

Objective and design: *Staphylococcus aureus* produces a range of Staphylococcal enterotoxins, causing a variety of clinical manifestations including inflammation and sepsis. We investigated the ability of Staphylococcal enterotoxin type B (SEB) to impair bone marrow (BM) neutrophil and eosinophil adhesion and chemotaxis, and potential involvement of IFN- γ and MHC class II activation in such responses. **Methods:** Bone marrow (BM) cells obtained from male BALB/C mice were incubated or not with SEB (1–30 ng/mL; 0.5 to 4 hours) and then activated with either CXCL8 (neutrophil chemoattractant) or CCL11 (eosinophil chemoattractant). We evaluated adhesion to VCAM-1-coated plates and chemotaxis by measuring myeloperoxidase (MPO) and eosinoperoxidase (EPO) activities. Intracellular Ca²⁺ levels, cell adhesion molecule expression, and cytokine/chemokine levels were also evaluated. **Results:** SEB significantly inhibited both adhesion and chemotaxis of neutrophils and eosinophils, which was associated with reduced intracellular Ca²⁺ levels. MHC class II blockade fully reversed SEB-mediated inhibition of neutrophil and eosinophil adhesion and chemotaxis. SEB also significantly increased IFN- γ production, and IFN- γ preincubation reduced cell adhesion and chemotaxis, which was reversed by MHC class II blockade in a similar fashion to SEB. Additionally, SEB significantly increased SDF1- α (CXCL12) and IL-10 levels, while decreasing G-CSF and TNF- α levels in both cell types. **Conclusion:** SEB impairs neutrophil and eosinophil function through an IFN- γ /MHC class II-dependent mechanism, which may explain the impairment of BM granulocyte egress into the circulation and from the circulation to tissues.

Introduction

Bone marrow (BM) granulocyte mobilization is a critical step for adequate infection resolution through the innate immune response to restrict pathogen proliferation. Consequently, inefficient BM neutrophil mobilization is observed at later stages of several infections, becoming a critical factor in restricting pathogen proliferation and disease control [1–3]. In the same context, eosinopenia represents an important biomarker for sepsis diagnostic [4]. Neutrophil and eosinophil adhesion and chemotaxis take place through α 4-integrin VLA-4 (α 4 β 1-integrin), MAC-1 (CD11b/CD18), and the integrin lymphocyte antigen function-1 (LFA-1 α ; CD11a/CD18), which connects in the endothelium with vascular cell adhesion molecule 1 (VCAM-1) [5] or intracellular adhesion molecule 1 (ICAM-1) or both [6–7].

Staphylococcal enterotoxin B (SEB) is a potent immunostimulatory protein regarded as one of the most potent toxins synthesized in high concentrations by *S. aureus* and involved in several events during infections induced by this bacterium [8]. SEB is a member of a family of structurally similar, heat-stable proteins, ranging from 25 to 30 kDa in size. [9] The literature has provided evidence that SEB exerts a critical role in *S. aureus* infections and inflammation, which is reduced by treatment with monoclonal antibodies against staphylococcal enterotoxins [10, 11]. For instance, SEB-induced acute lung injury and impaired lung function were characterized by extensive granulocyte accumulation with consequent release of different proinflammatory mediators, including interferon- γ (IFN- γ) [12, 14]. SEB exhibits superantigen activity by binding to class II major histocompatibility complex molecules (MHC class II) and specific V β regions of the T-cell receptors (TCRs), triggering the systemic release of different

inflammatory cytokines [15], leading to toxic shock syndrome, which can potentially progress to multiorgan failure and human death [10]. Our previous studies with Staphylococcal enterotoxin type A revealed that treatment with anti-MHC antibody reversed the impaired adhesion and chemotaxis of BM neutrophils exposed to Staphylococcal enterotoxin type A [16]. SEB interacts monovalently with the MHC class II α -chain in T cells, which differs from other Staphylococcal enterotoxins such as type A, which displays bivalent binding in both the α -chain and zinc-dependent high-affinity β -chain. This indicates that Staphylococcal enterotoxins can induce distinct immune responses in intensity, cellular targets, and cytokine production, depending on the predominant type at infection sites [17, 18].

Neutrophils and eosinophils express MHC class II after IFN- γ exposure [19, 20]. IFN- γ secreted by natural killer T cells during both innate and adaptive immune responses during bacterial infections [21] upregulates adhesion molecules on endothelial cells, intensifying leukocyte recruitment [22]. Moreover, elevated levels of IFN- γ can limit the hematopoiesis process, resulting in leukopenia, a condition often clinically observed following the resolution of severe infections [23, 24]. In mouse abdominal sepsis models, IFN- γ blockade reduced the production of the anti-inflammatory cytokine IL-10 by neutrophils, indicating that IFN- γ acts to prevent excessive inflammation [25]. Additionally, SEB produces high levels of IFN- γ in activated T cells [26]. Therefore, the recognized impairment of neutrophil [27] and eosinophil [28] migration induced by IFN- γ motivated us to investigate whether MHC class II and IFN- γ mediate the cell impairment after SEB exposure. Here, we have used neutrophils and eosinophils harvested from mouse BM to explore the effects of SEB on CXCL8-activated neutrophil and CCL11-activated eosinophil adhesion and chemotaxis and evaluate whether MHC class II and IFN- γ release mediate such effects. The contribution of cytokines and chemokines known to regulate BM granulocyte trafficking and/or their functional activity, such as Stromal Cell-Derived Factor-1 (SDF1- α ; CXCL12), Granulocyte Colony-Stimulating Factor (G-CSF), Tumor Necrosis Factor (TNF- α), and interleukin-10 (IL-10), was also evaluated in SEB-treated samples.

Materials and Methods

Animals

Male BALB/c mice, 4 weeks old, were obtained from the Central Animal House Services of the State University of Campinas (UNICAMP). They were housed (three per cage) at the Animal House Services of the Faculty of Medicine of Jundiaí (FMJ, Jundiaí, São Paulo), under a 12-hour light/dark cycle, in temperature-controlled rooms with free access to food and water.

Ethical approval

and compliance with guidelines

Animal experimentation and experimental procedures were approved by the Ethics Committee in Animal Use (CEUA-FMJ; Protocol No. 492/2012). All methods involving animals were performed in accordance with the relevant institutional, national, and international guidelines and regulations for the care and use

of laboratory animals. This study followed the recommendations of the Brazilian guide for the production, maintenance, or use of animals in teaching or scientific research activities/CONCEA [29] and the PREPARE Guidelines [30]. The animal studies reported here followed the ARRIVE guidelines [31]

Bone marrow (BM) granulocyte isolation and in vitro incubation with SEB or IFN- γ

Animals were anesthetized with isoflurane at a concentration exceeding 5%, and cervical dislocation was performed to confirm euthanasia. Granulocyte isolation from mouse BM was performed following the procedures outlined in a previous study [16]. Following harvesting BM, the cells were transferred to 100 mm $\times$ 20 mm culture dishes and incubated for 30 minutes at 37°C in a 5% CO₂ atmosphere. Subsequently, supernatants from BM culture were collected and washed twice with 2 mL of Iscove's modified Dulbecco's culture medium, and then centrifuged at 500 g for 10 minutes at 4°C. The resulting non-adherent granulocyte population (70%) was resuspended in 2.5 mL of culture medium. Total and differential cell counts were performed using a Neubauer chamber and Diff-Quick staining, respectively. The final cell suspension, consisting of about 70% granulocytes, was adjusted to a final concentration of either 4×10^6 cells/mL or 8×10^6 cells/mL.

In vitro incubation BM granulocytes with SEB or IFN- γ

BM granulocytes were incubated with SEB (1–30 ng/mL), IFN- γ (10 ng/mL), or sterile culture medium (control group) at 37°C, 5% CO₂ for time periods of 0.5 to 4 h. In separate assays, cells were pretreated for 30 min with MHC class II blocking antibody (2 μ g/mL) before the addition of SEB or IFN- γ . Neutrophils were identified through the measurement of myeloperoxidase (MPO) activity, whereas eosinophils were identified by eosinoperoxidase (EPO) activity [16].

Mouse BM neutrophil adhesion assays

Following incubation with SEB, IFN- γ , or culture medium (Control), BM neutrophil suspensions (4×10^6 cells/mL; 50 μ L) or BM eosinophil suspensions (8×10^6 cells/mL; 50 μ L) were transferred to 96-well plates pre-coated with recombinant mouse VCAM-1 (2.5 μ g/mL) [16]. The neutrophils were then stimulated with CXCL8 (300 ng/mL) and eosinophils with CCL11 (300 ng/mL) for 30 min. Neutrophil adhesion was quantified by measuring MPO activity in the adherent cells. Shortly, 25 μ L of hexadecyltrimethylammonium bromide (HTBA; 0.5%) in 50 mM of potassium phosphate buffer, pH 6.0, was added to each well, followed by 200 μ L of o-dianisidine solution (o-dianisidine: 0.167 mg/mL; hydrogen peroxide: 0.0005% in 50 mM of phosphate buffer, pH 6.0). The absorbance at 460 nm was immediately determined using a Synergy H1 Hybrid microplate reader (Biotek, USA). Neutrophil adhesion was quantified by correlating absorbance readings to the known number of neutrophils initially added per well [16].

Eosinophil adhesion was calculated by the measurement of eosinophil peroxidase (EPO) activity of adherent cells. To address this, EPO substrate (50 μ L: 1 mM H₂O₂, 1 mM o-phenylenediamine, and 0.1%

Triton X-100 in Tris buffer, pH 8.0) was added to each well. After 30 min of incubation at room temperature, 25 μL of H_2SO_4 (4 M) was added to each well to stop the reaction, and absorbance was determined at 490 nm with a microplate reader (Biotek, USA). Eosinophil adherence was calculated considering the optical density (OD) obtained in the EPO assay divided by the number of eosinophils added in each well.

Mouse BM neutrophil chemotaxis assays

Chemotaxis experiments were conducted using a 96-well chemotaxis chamber [32]. Following treatment or not with SEB, cell suspensions (4×10^6 cells/mL when measuring MPO or 8×10^6 cells/mL when measuring EPO) were placed directly on the upper surface of the filter. The lower wells of the chamber were filled with 29 μL of CXCL8 (300 ng/mL) or CCL11 (300 ng/mL) as chemoattractants. A polycarbonate membrane with 5 μm pores was placed over the microplate, and 25 μL of treated cell suspension was added to each filter site. The chemotaxis chamber was incubated at 37°C, 5% CO_2 , for 1 h. Afterward, non-migrating cells remaining on the upper surface of the membrane were gently removed, and the chamber was centrifuged at 200 g for 5 min at 20°C. The filter was then discarded, and neutrophils that had migrated to the lower wells were quantified by measuring MPO activity, while eosinophils were quantified by EPO activity, as previously described. Results were expressed as the percentage of migrating neutrophils or eosinophils relative to a standard curve of varying concentrations in the original cell suspension [16, 33].

Measurements of intracellular Ca levels in SEB-treated mouse BM cells

Intracellular Ca^{2+} levels were assayed using the fluorogenic calcium-sensitive dye FluoForte [34, 35]. In summary, cells (1×10^6 cells/mL) were treated with SEB (30 ng/mL) in a medium supplemented with 3 μM FluoForte for 45 minutes at room temperature, shielded from light. After this incubation period, the cell suspension was centrifuged at 400 g for 10 minutes, and aliquots of 300 μL were transferred into 96-well plates (Synergy H1 Hybrid Reader, BioTek, USA) equipped with a stirring mechanism. Measurement of total intracellular Ca^{2+} levels was performed by maintaining the extracellular Ca^{2+} concentration at 1 mM with CaCl_2 . CXCL8 (300 ng/mL) or CCL11 (300 ng/mL) were then added to stimulate activation, and FluoForte fluorescence was monitored continuously using excitation and emission wavelengths of 339 nm and 500 nm, respectively. The external influx of Ca^{2+} was calculated by the subtraction of the total Ca^{2+} levels from the internal storage mobilization. The intracellular calcium levels were calculated by using a general formula: $[\text{Ca}^{2+}]_i = K_d \times (F - F_{\min}) / (F_{\max} - F)$, where K_d = (389) is the FluoForte dissociation constant, as established in earlier studies [35]. The $F_{\max}$ value was obtained with Triton X-100 (0.1%) in the presence of saturating calcium (1 mM CaCl_2), whereas $F_{\min}$ value was obtained with the calcium chelator EGTA (10 mM) plus Tris (20 mM; pH = 8).

Measurement of cytokines and chemokines in BM cell supernatant after SEB treatment

Commercial ELISA kits were used to quantify IFN- γ , SDF1- α (CXCL12), G-CSF, TNF- α , and IL-10 levels in BM cell supernatants after SEB incubation, according to the instructions provided by the manufacturer (Mouse Duo Set ELISA Development System, R&D Systems, Minneapolis, MN, USA).

Expression of CD11b (MAC-1), CD11a (LFA1- α) and α 4-integrin (VLA4) in BM granulocytes after SEB treatment

BM cells (100 μ L of 3×10^6 cells/mL) were incubated with SEB or culture medium (used as a control) for 2 hours. After this period, the samples were further incubated for 20 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11b (MAC-1) or CD11a (LFA1- α) and phycoerythrin (PE)-conjugated anti-mouse VLA-4 antibodies. Flow cytometric analysis was carried out using a Becton-Dickinson FACSCalibur cytometer (San Jose, CA) and CellQuest Pro Software. Fluorescence signals for MAC-1, LFA1- α , and VLA4 were detected in the FL-2 and FL-1 channels, respectively. Events used in the analysis were gated based on regions enriched in granulocytes, as identified on side scatter (SSC) vs. forward scatter (FSC) dot plots. A total of 10,000 events were collected for each sample. Before fluorescence data acquisition, cells incubated with FITC-labeled isotype controls (rat IgG2a for LFA1- α and IgG2b for MAC-1) were analyzed to account for non-specific binding in the FL-1 and FL-2 channels. Surface expression levels of MAC-1, LFA1- α , and VLA4 were quantified based on the mean fluorescence intensity (MFI) values from histogram plots.

Materials

SEB, and ethylene glycol-bis (β -aminoethyl ether)-N, N, N', N' tetraacetic acid (EGTA) were acquired from Sigma Aldrich Co (St. Louis, MO, USA). Iscove's modified Dulbecco's medium was purchased from Life Technologies (New York, USA). IFN- γ was purchased from Boehringer Ingelheim (Berkshire, UK). ELISA kits for mouse IFN- γ , SDF1- α (CXCL12), G-CSF, TNF- α , IL-10, VCAM-1, ICAM-1, anti-mouse CD11b (MAC-1), anti-mouse CD11a (LFA 1- α), anti-mouse VLA4 and CXCL8 were obtained from BD Biosciences Pharmingen (San Jose, CA, USA). Fluoroforte was obtained from Enzo Life Sciences International (New York, USA). Antibody anti-MHC class II was obtained from Abcam (Cambridge, UK).

Statistical analysis

Data are presented as mean values $\pm$ SEM and were analyzed using analysis of variance (ANOVA) for multiple comparisons, followed by Bonferroni post-test, utilizing a statistical analysis software package (GraphPad software, version 5.00; San Diego, USA). $P < 0.05$ was considered significant.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

SEB impairs neutrophil and eosinophil adhesion to VCAM-1

Neutrophil stimulation with CXCL8 (300 ng/mL) markedly increased adhesion to VCAM-1, which was inhibited by preincubation with SEB (3–30 ng/mL). The inhibition by SEB was detected at 1 and 2 h but not at 4 h of preincubation (Fig. 1A).

Eosinophil stimulation with CCL11 also significantly enhanced the adhesion to VCAM-1 in comparison with unstimulated cells (Fig. 1B). SEB preincubation (1, 3 and 10 ng/mL) significantly reduced CCL11-induced cell adhesion in all doses and time-points studied compared with CCL11-induced adhesion in SEB-unstimulated cells (Fig. 1B).

Spontaneous neutrophil and eosinophil adhesions (independent of CXCL8 or CCL11 activation) were not influenced by SEB at any concentration or time employed (data not shown).

SEB impairs mouse BM neutrophil and eosinophil chemotaxis

Neutrophil stimulation with CXCL8 (300 ng/mL) significantly increased cell chemotaxis in comparison with unstimulated cells ($P < 0.05$). Pre-incubation of neutrophils (2 h) with SEB at 30 ng/mL significantly ($P < 0.05$) inhibited CXCL8-driven chemotaxis compared with the control group (Fig. 2A). Next, we performed a time-course response with SEB at 10 ng/mL. We observed an inhibitory response at 4 h (Fig. 2A). Spontaneous neutrophil chemotaxis (in the absence of CXCL8) was not significantly altered by SEB at any concentrations and time-points tested (data not shown).

Eosinophil stimulation with CCL11 also significantly enhanced chemotaxis responses in comparison with unstimulated cells. SEB preincubation (1 and 3 ng/mL) significantly reduced CCL11-induced cell chemotaxis. In a time-course assay, SEB (10 ng/mL) significantly reduced CCL11-induced chemotaxis at 0.5 h and 1 h (Fig. 2B).

Spontaneous neutrophil and eosinophil chemotaxis (independent of CXCL8 or CCL11 activation) were not influenced by SEB at any concentration or time employed (data not shown).

SEB reduces intracellular Ca^{2+} levels

In CXCL8 (300 ng/mL) or CCL11 (300 ng/mL)-stimulated cells, intracellular Ca^{2+} levels in BM cells were significantly higher ($P < 0.05$) than basal levels, which was significantly reduced by preincubation (30

min) with SEB (30 ng/mL; Table 1). In non-stimulated cells, SEB did not significantly affect the basal Ca²⁺ levels.

Table 1

Intracellular Ca²⁺ mobilization in bone marrow (BM) granulocytes after *in vitro* incubation with Staphylococcal enterotoxin B (SEB).

Intracellular Ca ²⁺ levels (nM)			
	Basal	CXCL8	CCL11
Control	198.5 ± 18.8	551.0 ± 44.0*	333.5 ± 38.3
SEB	188.4 ± 6.00	387.0 ± 59.0*#	210.6 ± 20.0#
BM cells after <i>in vitro</i> incubation with SEB (30 ng/mL) were suspended in a medium supplied with Fluoforte (3 µM, 45 min). Afterward, cells were stimulated with CXCL8 (300 ng/mL) or CCL11 (300 ng/mL). Results are mean values ± SEM from 8 mice for each group. * P < 0.05 compared with basal values. #P < 0.05 compared with respective CXCL8 or CCL11 values.			

Blockade of MHC class II restores SEB-mediated inhibition of neutrophil and eosinophil adhesion and chemotaxis

BM cell incubation with an anti-MHC class II blocking antibody (2µg/mL, 30 min) nearly reversed the inhibition by SEB (30 ng/mL) on both neutrophil and eosinophil adhesion in VCAM-1-coated plates (Fig. 3A, B). Additionally, the anti-MHC class II blocking antibody reversed the CXCL8/CCL11-induced chemotaxis after SEB incubation (Fig. 4A, B).

In the absence of SEB (unstimulated cells; Control group), incubation with the anti-MHC class II antibody did not affect the adhesion and chemotaxis responses.

Incubation with IFN-γ reduces BM neutrophil and eosinophil adhesion and chemotaxis

BM cell incubation with SEB (10 ng/mL) significantly increased IFN-γ levels by about 2.4-fold (P < 0.05) compared with the control group (Fig. 5A). Therefore, we next investigated whether cell incubation with INF-γ mimics the inhibitory effects of SEB on CXCL8- and CCL11-stimulated adhesion and chemotaxis. INF-γ (10 ng/mL) significantly decreased (P < 0.05) CXCL8- (Fig. 5A) or CCL11-induced (Fig. 5B) adhesion on VCAM- 1-coated plates, which was fully restored by the anti-MHC class II antibody. Similarly, INF-γ significantly reduced CXCL8-induced neutrophil chemotaxis (Fig. 5D) and CCL11-induced eosinophil chemotaxis (Fig. 5E). Pretreatment with anti-MHC class II was also effective in restoring the reduced cell chemotaxis by INF-γ.

CD11a (LF1-α), CD11b (MAC-1) and α4-integrin (VLA4) expressions in neutrophils after SEB incubation

CD11a (LF1-α), CD11b (MAC-1), and α4-integrin (VLA4) expressions in BM granulocytes were evaluated by flow cytometry after incubation with SEB (10 ng/mL). Expressions of CD11a (LF1-α), CD11b (MAC-1) and α4-integrin (VLA4) remained unaffected after SEB incubation when compared with the control group (Fig. 6A, B, C).

Other cytokine and chemokine levels in SEB-treated BM cell supernatants

BM cell incubation with SEB (10 ng/mL) significantly increased the levels of SDF1-α (CXCL12) and IL-10 (P < 0.05). In contrast, G-CSF and TNF-α levels were reduced by incubation with SEB (P < 0.05) compared with the control group (Table 2).

Table 2 Effect of Staphylococcal enterotoxin B (SEB) on levels of cytokines/chemokines in bone marrow (BM) cells		
Cytokine/Chemokine	SEB, fold change vs. control	p value
G-CSF	0.34 ± 0.06	***p < 0.0003
SDF1-α (CXCL12)	3.11 ± 0.27	****p < 0.0001
TNF-α	0.19 ± 0.05	*** p < 0.0006
IL-10	1.84 ± 0.11	*** p < 0.0001
Mouse BM supernatants were obtained 2 h after <i>in vitro</i> incubation with SEB (10 ng/mL) or Iscove's modified Dulbecco's medium (Control). Cytokine/chemokine levels are expressed as fold change relative to the SEB-untreated group (control), which was normalized to 1. Results are mean values ± S.E.M from 5 mice for each group. *P < 0.05 compared with the control group.		

Discussion

It is widely recognized that Staphylococcal enterotoxins reproduce many symptoms and signs of *S. aureus* infection and sepsis in humans and animals [10], and SEB released by *S. aureus* also acts as a potent activator of the immune system [26]. Our current research revealed that SEB significantly decreases CXCL-8-induced neutrophil adhesion and chemotaxis and CCL11-induced eosinophil adhesion and chemotaxis, which was accompanied by decreased intracellular Ca2 + levels. Moreover, SEB increased significantly IFN-γ levels, and IFN-γ itself inhibited neutrophil and eosinophil adhesion and migration, thus mimicking SEB responses. Interestingly, prior cell incubation with anti-MHC class II fully restored the inhibitory effect of both SEB and IFN-γ on neutrophil and eosinophil adhesion and chemotaxis, indicating that SEB via the release of IFN-γ acts to activate MHC class II ultimately resulting in cell activity inhibition.

Previous studies show that SEB exhibits superantigen activity by binding to MHC class II [15], triggering the systemic release of different cytokines, including IFN-γ [26]. MHC class II can be expressed in

neutrophils and eosinophils after IFN- γ exposure [19, 20]. Therefore, we initially looked at the role of MHC class II in mediating the inhibition of cell adhesion and chemotaxis by SEB. Our results showed that pretreatment of CXCL-8-activated neutrophils or CCL11-activated eosinophils with anti-MHC class II antibody fully restored the SEB-mediated inhibition of cell adhesion in VCAM-1-coated plates and chemotaxis, indicating that MHC class II activation may be a key mechanism by which SEB impairs neutrophil and eosinophil functions. Despite MHC class II molecules having been traditionally associated with antigen-presenting cells [36], studies have documented that neutrophils and eosinophils express MHC class II and acquire properties of antigen-presenting cells in response to selected cytokines such as IFN- γ [19, 20]. In our study, increased levels of IFN- γ were observed in SEB-treated cells. Among the many chemokines and cytokines released under Staphylococcal enterotoxin exposure, IFN- γ has been reported to participate in innate and acquired immune responses in the course of bacterial infections [21]. IFN- γ is often produced by natural killer T cells in response to pathogens, such as mycobacteria and viruses [37–39], and elevated levels of this cytokine can inhibit hematopoiesis, resulting in leukopenia, which is clinically observed following severe infections [37]. These studies led us to hypothesize that IFN- γ , through MHC class II activation, may mediate the suppressive effects of SEB in BM granulocytes. Accordingly, our findings demonstrated that incubation of neutrophils or eosinophils with IFN- γ itself reduced adhesion and chemotaxis in CXCL8- or CCL11-stimulated cells in a similar fashion to SEB. Furthermore, the inhibitory activity of IFN- γ on both cell adhesion and chemotaxis was restored by MHC class II blockade, strongly suggesting that SEB-mediated inhibition of BM granulocyte activity is mediated by IFN- γ release that in turn activates MHC class II.

Neutrophil and eosinophil adhesion to endothelium-bound integrins is a key step in mobilizing these cells to inflammatory tissues. Neutrophils express MAC-1 (CD11b/CD18) and LFA-1 α (CD11a/CD18), which interact with ICAM-1 and VCAM-1, respectively, while eosinophils interact with VLA4 [40]. Our findings show that SEB failed to modify the expression of LFA-1 α , MAC-1, or VLA4 in eosinophils, excluding the possibility that inhibitory effects of SEB on BM granulocyte activity reflect down-regulation of these integrins.

The cytokine G-CSF is a central mediator of BM granulocyte mobilization to circulating blood, whereas the chemokine SDF1- α (CXCL-12), via CXCR4 signaling, is a critical determinant of BM granulocyte retention [41, 42]. In the present study, increased SDF1- α (CXCL-12) levels and reduced G-CSF levels in BM cells following SEB exposure suggest that, in addition to impairing neutrophil and eosinophil functional activity, this enterotoxin may also impair granulocyte egress from the BM to the peripheral blood by modulating G-CSF and SDF1- α production.

During infections, a precise equilibrium between pro- and anti-inflammatory cytokines is essential for effective pathogen clearance and to prevent excessive tissue-damaging inflammation. TNF- α promotes a rapid increase in circulating neutrophil and eosinophil counts in humans [43, 20] by enhancing endothelial neutrophil adhesiveness through MAC-1 upregulation and eosinophil adhesion through VLA4 [44, 20]. In contrast, high levels of IL-10 under bacterial infections [40] inhibit the production of pro-inflammatory cytokines and impair granulocyte recruitment [44, 45]. Our findings suggest that SEB-

induced impairment of BM granulocyte activity may be partially explained by increased IL-10 production in association with reduced TNF- α levels. Moreover, the TNF- α /IL-10 balance may explain the failure of SEB to affect granulocyte CD11b/CD11a and VLA4 expression.

A limitation of the present study consists of using a mixed BM granulocyte population. Because obtaining circulating granulocytes from mice is methodologically difficult due to the small size of the animal, we proceeded to harvest the mouse BM to obtain enough neutrophils and eosinophils for the *in vitro* assays; the procedure yielded a mixed cell population, consisting of 70% mature granulocytes, mostly neutrophils [8–10% eosinophils], with the remaining 30% comprising myeloblasts, immature myelocytes, and mononuclear cells. The adhesion and chemotaxis assays were assessed by measuring MPO and EPO activity, selective markers for neutrophils and eosinophils, respectively, thus ensuring that the responses could in fact be credited to neutrophils and eosinophils. On the other hand, our data with measurements of intracellular Ca²⁺ and cytokine levels could not be linked exclusively to granulocytes; therefore, the possibility that non-granulocyte contaminating cells account for the subsequent impairment of granulocyte function can not be excluded.

In conclusion, our findings demonstrate that SEB impairs neutrophil and eosinophil adhesion and chemotaxis through an IFN- γ /MHC class II-dependent mechanism, which may contribute to the impaired granulocyte recruitment during *S. aureus* infection. SEB also modulates the production of key cytokines/chemokines (G-CSF, TNF- α , IL-10 and SDF1- α) involved in the egress of BM mature granulocytes into circulation. Our findings provide new mechanistic insights into the immunomodulatory effects of SEB on innate immune responses. Although the underlying mechanism of SEB was primarily characterized in murine BM neutrophils and eosinophils, our data offers a mechanistic insight for future studies investigating immune dysregulation during human *S. aureus* infections.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics Approval

Animal experimentation and experimental procedures were approved by the Ethics Committee in Animal Use (CEUA-FMJ; Protocol No. 492/2012). This study followed the recommendations of the Brazilian guide for the production, maintenance, or use of animals in teaching or scientific research activities/CONCEA and the PREPARE Guidelines. The animal studies reported here followed the ARRIVE guidelines.

Funding

Ivani A. Desouza received research support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support (Grant N^o: 2017/25867-8).

Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Verônica Santos-Ramos, Ana Paula Ferreira-Duarte, Aneslize S Pinheiro-Torres, Gabriel Anhê, and Ivani A Desouza. The first draft of the manuscript was written by Ivani A Desouza and Edson Antunes, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

Acknowledgement

Ivani A. Desouza thanks Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support (Grant No: 2017/25867-8).

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

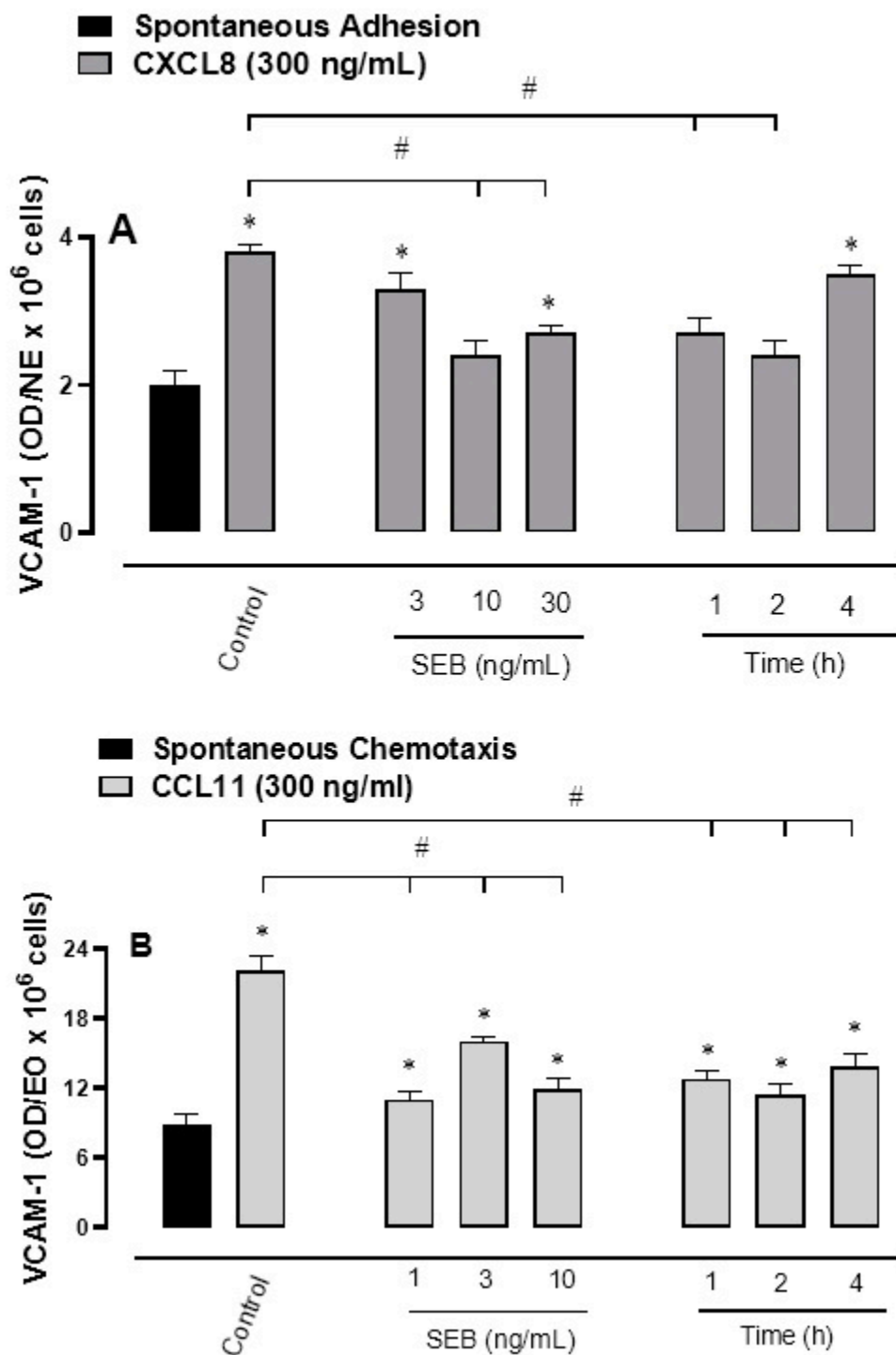
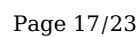


Figure 1

Staphylococcal enterotoxin B (SEB) inhibits CXCL8-induced neutrophil (Panel A) and CCL11-induced eosinophil (Panel B) adhesion. Bone marrow-derived neutrophils and eosinophils were incubated with SEB at different concentrations (1, 3, 10, and 30 ng/mL) and time periods (0.5 to 4 h), after which cells were placed on plates coated with vascular cell adhesion molecule-1 (VCAM-1) under CXCL8 (300 ng/mL; Panel A) or CCL11 (300 ng/mL; Panel B) stimulation. Neutrophil and eosinophil adhesion was

Figure 2



Staphylococcal enterotoxin B (SEB) inhibits CXCL8-induced BM neutrophil (Panel A) and CCL11-induced eosinophil (Panel B) chemotaxis. Bone marrow neutrophils or eosinophils were incubated with SEB at different concentrations (1, 3, 10, and 30 ng/mL) and time periods (0.5 and 4 h), after which they were placed in a chemotaxis chamber to migrate in the presence of CXCL8 (300 ng/mL; Panel A) or CCL11 (300 ng/mL; Panel B). Neutrophil and eosinophil chemotaxis were assessed by quantifying myeloperoxidase (MPO) or eosinoperoxidase (EPO) activity, respectively. Data are mean values $\pm$ S.E.M from five mice per group. * $P < 0.05$ versus spontaneous chemotaxis (absence of CXCL8 or CCL11); # $P < 0.05$ versus control (untreated cells).

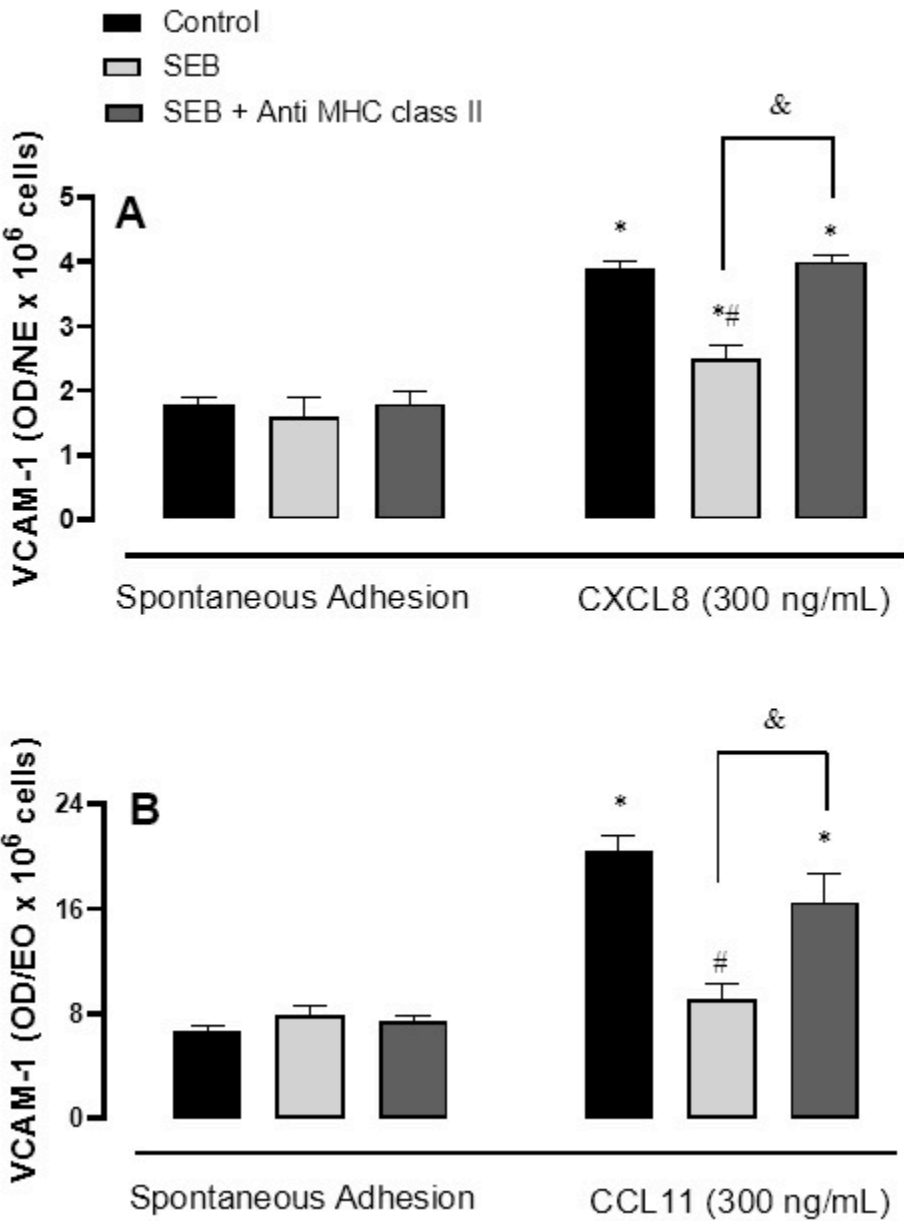


Figure 3

MHC class II blocking antibody reverses the inhibitory effect of SEB in neutrophil (Panel A) or eosinophil (Panel B) adhesion. Bone marrow neutrophils were pre-incubated with an anti-MHC class II antibody (2 $\mu\text{g/mL}$) for 30 minutes, followed by incubation with SEB (10 ng/mL for 2 h for neutrophil and 3 ng/mL for eosinophil adhesion assays). Mouse BM neutrophil adhesion (Panel A) was assessed in response to CXCL8 stimulation (300 ng/mL), and eosinophil adhesion (Panel B) in response to CCL11 stimulation (300 ng/mL) using VCAM-1-coated plates. Both neutrophil and eosinophil adhesion were quantified by MPO and EPO measurements, respectively. Data are presented as mean values $\pm$ S.E.M from five mice per group. * $P < 0.05$ versus spontaneous activities (absence of CXCL8 or CCL11); # $P < 0.05$ versus control (SEB-untreated cells); & $P < 0.05$ versus presence of anti-MHC class II antibody

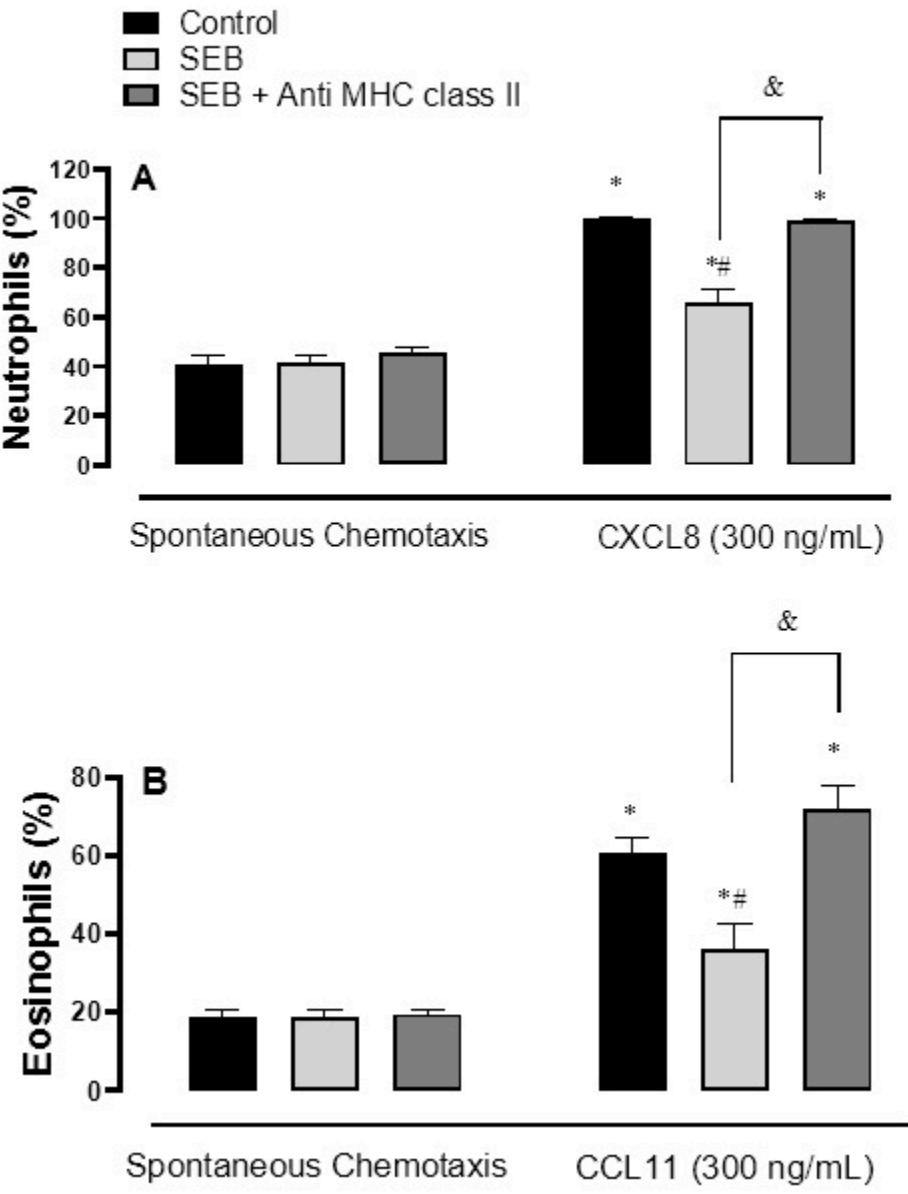


Figure 4

MHC class II blocking antibody reverses the inhibitory effect of SEB in neutrophil (Panel A) or eosinophil (Panel B) chemotaxis. Bone marrow neutrophils or eosinophils were pre-incubated with an anti-MHC class II antibody (2 µg/mL) for 30 minutes, followed by incubation with SEB (10 ng/mL for 2 h for neutrophil and 3 ng/mL for eosinophil chemotaxis assays). Mouse BM neutrophil chemotaxis (Panel A) was assessed in response to CXCL8 stimulation (300 ng/mL), and eosinophil chemotaxis (Panel B) in response to CCL11 stimulation (300 ng/mL) using a 96-well chemotaxis chamber. Both neutrophil and eosinophil chemotaxis were quantified by MPO and EPO measurements, respectively. Data are presented as mean values ± S.E.M from five mice per group. *P<0.05 versus spontaneous activities (absence of CXCL8 or CCL11); #P<0.05 versus control (untreated cells); &P<0.05 versus the presence of anti-MHC class II antibody.

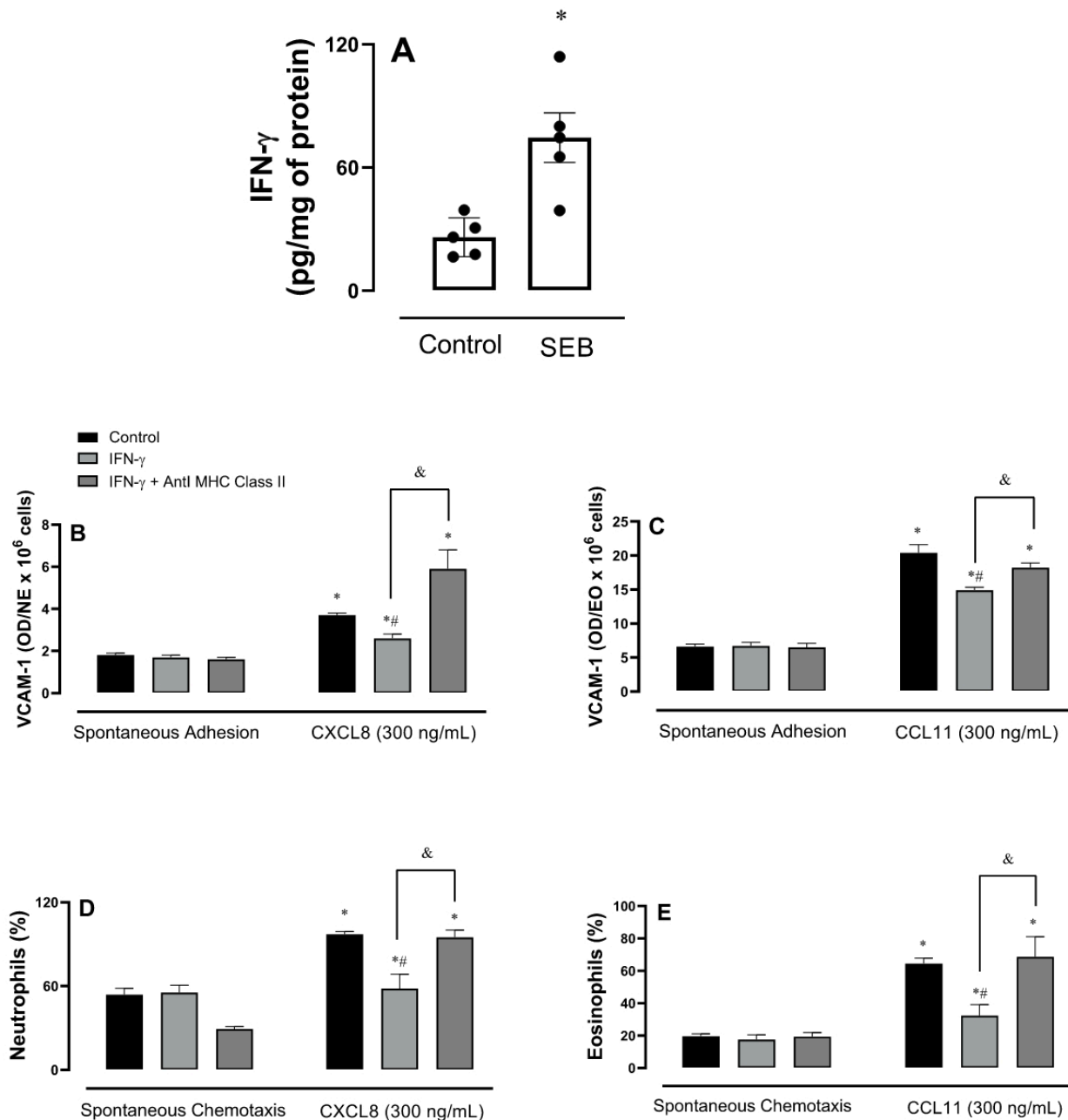


Figure 5

MHC Class II Mediates IFN- γ -mediated Inhibition of Bone Marrow Granulocyte Adhesion and Chemotaxis. IFN- γ levels were quantified in BM cells supernatant after SEB (30 ng/mL for 2 h) incubation (Panel A). BM granulocytes were pre-incubated with anti-MHC class II antibody (2 μ g/ml) for 30 minutes, followed by incubation with IFN- γ (10 ng/mL, 2 hours). Neutrophil adhesion (Panel B) and chemotaxis (Panel D) were assessed in response to CXCL8 stimulation (300 ng/mL). Eosinophil adhesion (Panel C) and chemotaxis (Panel E) were assessed in response to CCL11 stimulation (300

ng/mL). Both adhesion and chemotaxis were quantified by MPO or EPO measurements. Data are presented as mean values $\pm$ S.E.M from five mice per group. * $P < 0.05$ versus spontaneous activities (absence of CXCL8 or CCL11); # $P < 0.05$ versus control (untreated cells); & $P < 0.05$ versus anti-MHC class II antibody untreated cells.

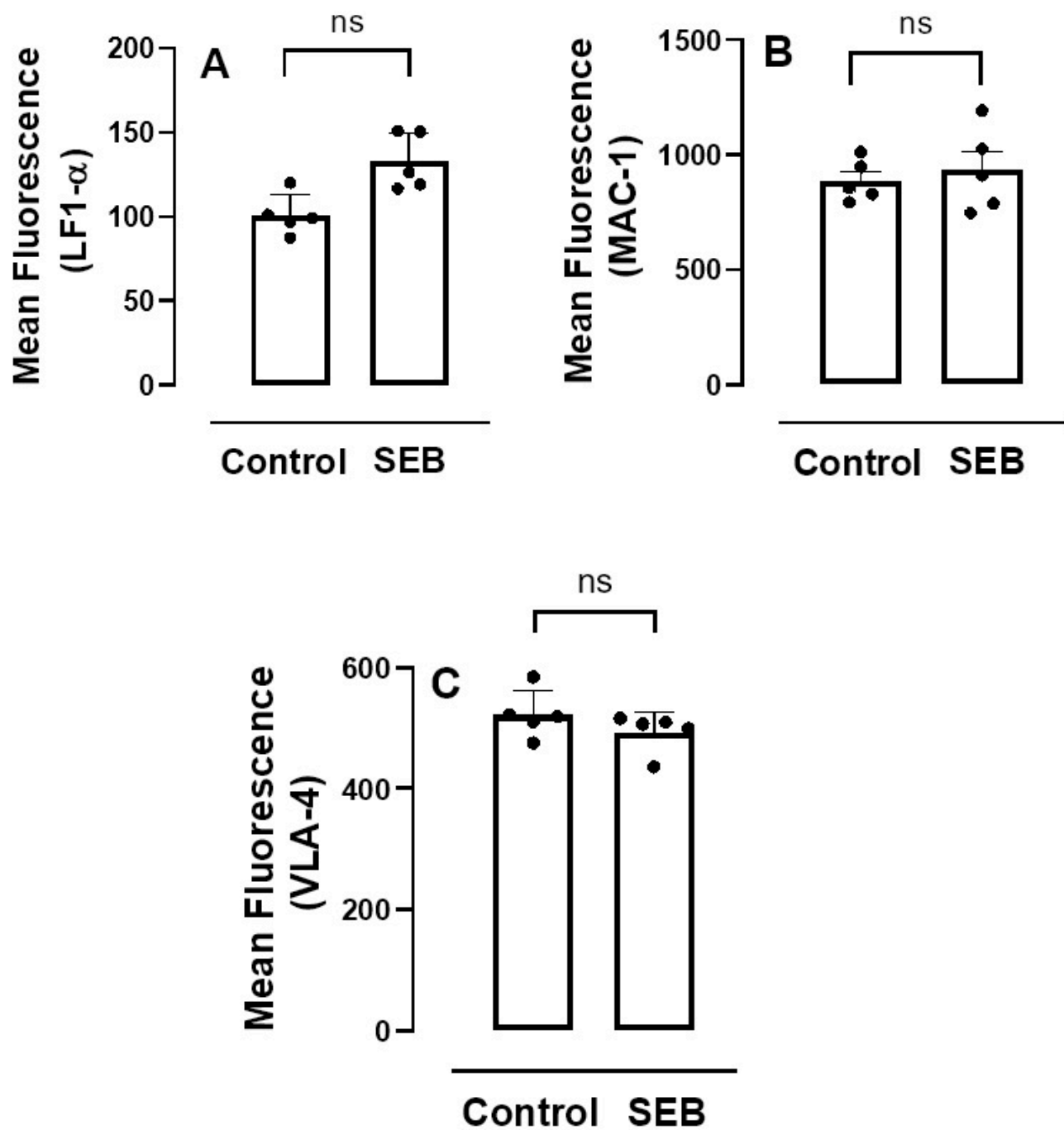


Figure 6

Staphylococcal enterotoxin B (SEB) does not affect the expression of CD11a (LFA1- α), CD11b (MAC-1) and α 4-integrin (VLA4) on bone marrow granulocytes. BM cells were incubated with SEB (10 ng/mL) or Iscove's modified Dulbecco's medium (Control). Cells were gated for CD11b (MAC-1; panel A), CD11a (LFA1- α ; panel B) or α 4-integrin (VLA4) population on a flow cytometer. Data are presented as mean values of mean fluorescence $\pm$ S.E.M from five mice per group.