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Role of interplay between IL-4 and IFN-γ in the in regulating M1 macrophage polarization induced by Nattectin

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ABSTRACT

Recently our group described that Nattectin, a C-type lectin of the venom of *Thalassophryne nattereri* shows a potent pro-inflammatory capacity. Here, we demonstrated that Nattectin is able to induce M1 macrophage marker iNOS, and up-regulate the expression of MHC class II, CD80, CD86 and CD40 molecules. The increase in MHC class II and CD49a integrin expression with MMP-9 production and endocytic capacity depend on lectin function of Nattectin. Moreover, the polarization of peritoneal and bone marrow-derived macrophages induced by Nattectin to M1 profile is dependent on Th1 cytokines (IL-12 and IFN-γ), and negatively regulated by Th2 cytokines (IL-4, IL-10 and IL-13). Also we reveal that IL-4 play a dual role in this polarization: a regular action of IL-4 was seen in the negative regulation of the CD40 expression, but an unexpected positive regulation was seen in the expression of CCR7 and MHC class II. Finally, our *in vivo* studies showed that the influx of neutrophils and small peritoneal macrophage - F4/80^{low}MHCII^{hi} induced by Nattectin is totally dependent on IL-4 and IFN-γ cytokines. Furthermore, the induction of IL-6 release is negatively regulated by IL-4 and positively regulated by IL-12 and IFN-γ. Together, the results allowed us to expand the knowledge about the regulation of macrophage activation, as well as confirmed the ability of Nattectin, a fish C-type lectin, as an important immunomodulatory agent.

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1. Introduction

Macrophages are important innate immune cells that play a critical role in sensing pathogens, directly or indirectly, and integrating this information to regulate the adaptive immune response. Heterogeneity and plasticity are hallmarks of cells belonging to the monocyte–macrophage lineage. On the basis of Th1/Th2 polarization concepts [1], phenotypically polarized macrophages are now generally termed pro-inflammatory M1 or classically activated, and anti-inflammatory M2 or alternatively activated [2,3].

The existence of multiple subsets of macrophages raises the question of whether they are functionally specialized to promote distinct immune responses. M1 macrophages, whose prototypical activating stimuli are IFN-γ and LPS, exhibit potent microbicidal properties and promote strong IL-12-mediated T helper 1 responses [4]. In contrast, M2 macrophages support T helper 2-associated effector functions and may play a role in resolution of inflammation through endocytic clearance and trophic factor synthesis [3]. However, the M2 designation has rapidly expanded to include other types of macrophages: M2a, induced by IL-4 or IL-13; M2b, induced by immune complexes and agonists of TLRs or IL-1 receptors; and M2c, induced by IL-10 and glucocorticoid hormones [5]. While the classification of the M1 and M2 macrophage functions has provided an important tool for understanding the regulation of the inflammatory process, at present the molecular mechanisms that govern M1/M2 polarization remain incompletely understood.

Cytokines and growth factors have been implicated in the reprogramming of M1 and M2 macrophages. Activation of macrophages by either Th1 (IFN-γ) or Th2 (IL-4 and IL-13) cytokines leads to dramatic changes in their physiology, including alterations in the expression of surface proteins as co-stimulatory molecules and the production of cytokines and pro-inflammatory mediators that influence T cell differentiation. Thus, adjuvants that modulate macrophage function, enhancing macrophages survival, inducing the expression of co-stimulatory molecules, and controlling the release of pro-inflammatory cytokines may be particularly effective in inducing T cell expansion.

Soluble C-type lectins, including molecules with specificity for galactose, have been identified in several teleost fish [6]. Recently our group

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described that Nattectin, a C-type lectin of the venom of *Thalassophryne nattereri* [7] shows a potent pro-inflammatory capacity, acting inducing a Th1 response through the control of recruitment and activation of monocytes/macrophages, licensing these cells to differentiate into cells exhibiting typical DC functions. These macrophage-derived DCs exhibit functional attributes: augmented Ag presentation capacity with avid phagocytosis; enhanced migration to the T cell–rich area of lymph nodes, through its effect on production and release of active MMP-9; high T cell co-stimulatory molecules expression, and enhanced capacity to activate T cells by the production of IFN-γ through the high-level of bioactive IL-12 p70 secretion [8].

Considering the role of macrophages in homeostasis as well as in host defense and in view of the possibility to obtain natural adjuvants able to modulate the immune system, specifically macrophage functions, the investigations presented here were undertaken to assess whether Nattectin modulates the process of macrophage activation and M1/M2 polarization, and to evaluate the role of T helper derived cytokines in the regulation of this process using IL-4, IL-12, and IFN-γ-deficient mice.

2. Materials and methods

2.1. Animals

Female BALB/c and male C57BL/6 mice were obtained from a colony at the Butantan Institute. IL-$4^{-/-}$, IL-$12^{-/-}$ and IFN-$\gamma^{-/-}$ male mice on C57BL/6 background were purchased from Institute of Biomedical Sciences at São Paulo University. All mice, 6 to 8 weeks old, were kept under pathogen-free conditions and given water and food *ad libitum*. They were housed under controlled temperature, humidity and lighting conditions. All the procedures involving mice were in accordance with the guidelines provided by Brazilian College of Animal Experimentation and approved by Animal Ethics Committee of Butantan Institute (634/09) and Institute of Biomedical Sciences (106/91-2).

2.2. Reagents and Abs

Nattectin obtained by Reversed-phase HPLC (Amersham Biosciences) from *T. nattereri* venom (IBAMA 16221–1) was confirmed by MALDI-TOF mass spectrometry (Amersham Biosciences, Buckinghamshire, U.K.) according to Lopes-Ferreira and coworkers [7]. Endotoxin Detoxi-GelTM (Pierce Chemical Co.) was used according to the manufacturer's instructions to remove >99% of the contaminating LPS in the Nattectin solution (resulting in a total dose <0.8 pg LPS), which was measured by Limulus Amoebocyte Lysate (Lonza Walkersville Inc., Walkersville, MD). Dulbecco´s Modified eagle medium (DMEM), and fluorescein 40,000 MW dextran were purchased from Invitrogen (Carlsbad, CA). IL-10 and IL-13 were obtained from eBiosciences (San Diego, CA). Total iNOS/NOS II (ABN26) anti-RELM-α (AB3365P, *resistin-like molecule* α) were purchased from Millipore (Concord Road, Billerica, MA) and β actin and horseradish peroxidase-labeled anti-rabbit IgG were purchased from Santa Cruz (Santa Cruz, California). ELISA kits for quantitative measurement of murine cytokines and MMPs were purchased from BD Pharmingen (San Jose, CA). FITC-, PE-, PE-Cy5-, PerCP-Cy5.5- or biotin-conjugated mAbs used to detect the expression of CD11c (HL3), Ly-6C (AL-21), TLR2 (203325), CD14 (Sa2.8), CD18 (C71/16), CD29 (eBioHMb1-1), CD40 (3/23), CD49b (Hma2), CD80 (16-10A1), CD86 (GL-1), I-A/I-E (M5/114.15.2), CD11b (M1/70), CCR7 (4B12), CD44 (IM7), purified F4/80R (6F12), purified CD16 (275003), purified CD49a (Ha31/8), purified CD49e (HM25-1), as well as isotype-matched control mAbs were obtained from BD Pharmingen, eBiosciences and R&D Systems (Minneapolis, MN). Donkey anti-rat IgG PE conjugated (polyclonal) and anti-rat IgG FITC conjugated (polyclonal) were purchased from eBiosciences. Mouse anti-Armenian and Syrian hamster IgG cocktail FITC conjugated (G70-204, G94-56) was obtained from BD Pharmingen.

2.3. Generation of bone marrow-derived macrophages (BMDM)

Bone marrow-derived macrophages isolated from BALB/c or C57BL/6 mice were obtained according to Falk and coworkers [9] and Muller and coworkers [10]. Initially, bone marrow was flushed from the femurs and tibias of mice, and cells (2×10^6/mL) were cultured in Petri dishes in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 2% horse serum, 1% vitamin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, 1% penicillin/streptomycin-glutamine, and 30% L929 cell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF) under a humidified atmosphere of 5% CO_2 at 37 ° C. Cells were fed on day 2 and half of the old medium was replaced on day 6. After culture for 7 days, the homogeneous population of macrophages was determined by flow cytometry (97–99% of $CD11b^{high}F4/80^{int\text{-}high}CD14^{high}Ly6C^{low}$).

2.4. Stimulation of macrophages with Nattectin

After BMDM generation, cells were stimulated with and without Nattectin (10 μg/mL) in Petri dishes (2×10^6 cells/mL) for 24 h according to Saraiva and coworkers [8]. To evaluate whether Nattectin effects were dependent on the carbohydrate-binding activity, D-galactose at 10 mM were added to the culture medium, with sucrose at the same concentration serving as a control. Finally, to evaluate the influence of Th1/Th2 cytokines in macrophage polarization induced by Nattectin, cells were stimulated for 24 h with Nattectin (10 μg/mL) plus recombinant murine IL-10 or IL-13, both at 5 ng/mL.

2.5. Protein lysate preparation and western blot analysis

Whole-cell extracts were prepared from BMDM cultured cell as follows: the culture medium was drained off the cells, and the adherent cells were washed twice with ice-cold PBS. The cells were lysed with ice-cold Tris buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 2,5% β-mercaptoethanol) containing protease inhibitor cocktail (P1860, Sigma-Aldrich, Spruce St. Louis, MO). The cell suspension was transferred into a centrifuge tube, left on ice, and vortexed for 5 min to lyse the cells. The lysate was then centrifuged at 12,000 g in a pre-cooled centrifuge for 20 min. The supernatant was collected and the concentration of protein in extracts was determined by using Bradford [11] assay. Protein samples (100 μg aliquots) were mixed with sample buffer (100 mM Tris [pH 6.8], 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 50 mL/mL β-mercaptoethanol), resolved by 12% SDS- polyacrylamide gel (PAGE) and electroblotted onto Hybond-C nitrocellulose membranes, using a semi-dry Bio-Rad apparatus. Ponceau staining of electroblotted membrane was used to visually monitor protein transfer. The blot was placed in blocking solution (5% nonfat dried milk in TBST) for 1 h at room temperature on a shaking table. Total iNOS/NOS II (1:1000), RELMα (1:1000) and β actin (1:2000) were respectively detected with a secondary peroxidase-conjugated anti-rabbit IgG polyclonal antibody. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging software and represented in the relative intensities.

2.6. Flow cytometry

Following different treatments and stimulation of macrophages with Nattectin, cells (1×10^6 cells/mL) were incubated with 10% mouse serum for 20 min at 4 °C to block unspecific binding. Subsequently, cells were stained in the dark at 4 °C for 30 min with FITC-, PE-,

PE-Cy5-, PerCP-Cy5.5- or biotin-conjugated mAbs for CD11c (HL3), Ly-6C (AL-21), TLR2 (203325), CD14 (Sa2.8), CD18 (C71/16), CD19 (1D3), CD29 (eBioHMb1-1), CD40 (3/23), CD49b (Hma2), CD80 (16-10A1), CD86 (GL-1), I-A/I-E (M5/114.15.2), CD11b (M1/70), CCR7 (4B12), CD44 (IM7), purified F4/80R (6F12), purified CD16 (275003), purified CD49a (Ha31/8) and purified CD49e (HM25-1). Appropriate isotype controls were used in all cases. After staining, the cells were fixed with 2% w/v formaldehyde analyzed immediately on a FACScalibur flow cytometer using CellQuest 3.1 software (Becton Dickinson, San Jose, CA). For flow cytometric analysis, typical forward and side scatter gate was set to exclude dead cells and aggregates. Results were expressed as median of fluorescence intensity (MFI) ± SEM.

2.7. Modulation of endocytosis of macrophages by Nattectin

BMDM were washed, resuspended in complete medium, and stimulated at 4 °C for 30 min with Nattectin (10 μg/mL) treated with and without D-galactose (10 nM). After washing, macrophages (2 x 10^6/mL) were incubated at 37 °C with 0.1 mg/mL of FITC-dextran (0.431-mm, Sigma-Aldrich, St Louis, MO) for 30 min. Uptake was stopped by adding cold PBS containing 1% FCS and 0.01% NaN3. Cells were then washed three times and analyzed by flow cytometry (FACSCalibur, BD Pharmingen, San Diego, CA). The markers for the monovariant histogram (unpublished data) were set based on the negative staining control, which was provided by incubating BMDM with FITC-dextran at 4 °C. Results of phagocytosis of FITC-dextran were expressed as median of fluorescence intensity (MFI) ± SEM. Experiments were repeated twice.

2.8. Quantitation of MMPs by ELISA

Cell-free supernatants from unstimulated or Nattectin-stimulated macrophages were harvested after 2 or 24 h culture. MMP-2 and MMP-9 in the supernatants were quantified using ELISA kits (BD Pharmingen) according to the manufacturer's instructions. The minimal detection levels for MMP-2 were 1.5 ng/mL and for MMP-9 was 1.25 ng/mL.

2.9. Nattectin-induced peritonitis

In order to evaluate the immune response induced by Nattectin, different groups of mice on the C57BL/6 background (n = 5) were intraperitoneally (i.p.) injected with 10 μg of Nattectin diluted in 500 μL of sterile saline 0.9%. Control animals were injected with the same volume of saline. At time points indicated (6 or 24 h) after injection, animals were killed by CO_2 inhalation and peritoneal cells were obtained by peritoneal lavage with 5 mL of sterile PBS/EDTA 10 mM. For the recovery of cellular contents, lavage fluid was centrifuged at 378 g for 10 min and the cell pellet was resuspended in cold PBS/BSA 0.1% for leukocyte cell counts. For the analysis of cytokine levels in the peritoneum, recovered lavage fluid was stored at −20 °C until analyzed by ELISA. Using a hemocytometer and cytocentrifuge, slides were prepared, air dried, fixed in methanol, and stained (Wright-Giemsa, Scientific Products, Chicago, IL). For differential cell counts, 300 leukocytes were enumerated and identified as mononuclear cells or polymorphonuclear neutrophils, based on staining and morphologic characteristics using a light microscope (Nikon Eclipse E200). Representative photomicrographs of each group were acquired digitally using an Axio Imager A1 microscope (Carl Zeiss, Germany) with an AxioCam ICc1 digital camera (Carl Zeiss).

2.10. Statistical analysis

All values were expressed as mean ± SEM. Statistical differences between experimental groups were detected after analysis of variance (One-way ANOVA) followed by Bonferroni test. A value of $p < 0.05$ was considered statistically significant. The SPSS statistical package (Release 8.0, Standard Version, 1997) was employed.

3. Results

3.1. Modulation of macrophage endocytosis and antigen presentation-related molecules by Nattectin

To study the function of Nattectin in the process of macrophage activation, we evaluated the expression of co-stimulatory molecules. Here, the use of L929 supernatant containing M-CSF, excellent for BMDM morphologic differentiation was able to generate cells characterized by high or intermediate expression of F4/80 and high expression of CD11b (Fig. 1A, left). Given that Nattectin possesses affinity for galactose/N-acetylgalactosamine-terminated glycans, we also examined whether this activity is required for Nattectin to induce macrophage activation. Thus, $CD11b^{pos}F4/80^{pos}$ macrophages were stimulated for 24 h with Nattectin alone or with D-galactose-treated Nattectin. Next, further investigations were conducted using M1/M2 markers to support the macrophage phenotypical shift in BMDM stimulated with Nattectin. The inducible nitric oxide synthase (iNOS) protein was detected by western blot in 24 h pos-stimulated Nattectin cells (Fig. 1A, right). Notably, the lack of a detectable change in expression of RELM-α protein levels (10 kDa) suggests the M1 polarization induced by Nattectin.

As depicted in Fig. 1B, Nattectin induced an increase in co-stimulatory molecules (CD40, CD80, CD86) expression as well as in MHC class II expression compared with control group. However, the blockade of carbohydrate recognition domain (CRD) of Nattectin by pre-incubation with carbohydrate did not alter its ability to induce the expression of co-stimulatory molecules CD80 and CD86. Interestingly, D-galactose-treated Nattectin increased the expression of CD40, but induced a decrease in MHC class II expression. These results show that the ability of Nattectin to prepare macrophages to MHC-II-dependent Ag presentation is dependent of its lectin properties.

- Dextran is a representative antigen of endocytosis and to a lesser extent, pinocytosis that is taken by antigen-presenting cells (APCs) primarily via specific mannose receptor [12]. Here, macrophages stimulated with D-galactose-treated Nattectin were evaluated for endocytic activity followed by incubation of macrophages with FITC-dextran particles. According to Fig. 1C, Nattectin induced an increase in ability of macrophages to endocyte FITC-dextran particles, and this effect was reversed in the culture of macrophages stimulated with D-galactose-treated Nattectin, indicating that Nattectin binding to specific saccharide ligand on the surface of macrophages is necessary for the observed effects.

3.2. Effect of Nattectin on macrophage adhesion and migration capacity

Binding of the RGD-containing ECM proteins to integrins transmits environmental cues to cells, which activates intracellular signaling and induces cytoskeletal changes, as well as an up-regulates metalloproteinases [13–15].

As depicted in Fig. 2A, Nattectin induces an increased expression of CD49e and CD49a α-chain integrins, the latter being partially down regulated after stimulation with D-galactose-treated Nattectin. On the other hand, pre-treatment of Nattectin with its specific ligand induced an up-regulation in the expression of CD29 β1-integrin and CD49b α2-integrin, which was not observed in macrophages stimulated with the lectin alone. Furthermore, we also observed that the expression of CD18 β2-integrin was not altered by Nattectin stimulation.

CD44 is a single pass transmembrane glycoprotein involved in cell-cell interactions, cell adhesion and, such as integrins, CD44 also participate in cell migration [16,17]. In Fig. 2B, we observed an up-regulation of CD44 expression induced by Nattectin, which was not altered by stimulation with D-galactose-treated Nattectin, indicating

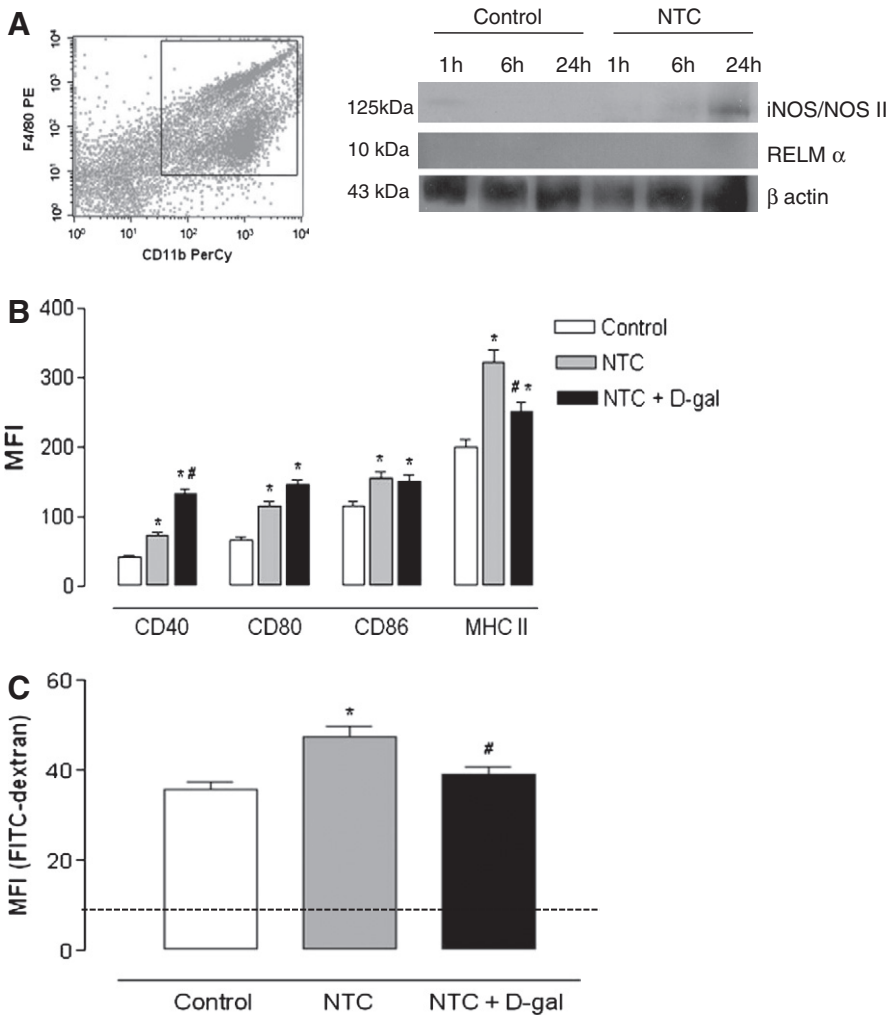

Fig. 1. Nattectin induces the activation of bone marrow-derived macrophages. BMDM were stimulated for 24 h with Nattectin (10 μg/mL) previously incubated or not with D-galactose at 10 nM for 1 h. After that, CD11b^{pos}F4/80^{pos} macrophages (A, left panel) were used for collection of total cell lysate for immunoblot to detect iNOS/NOS II and RELM-α (A, right panel). (*B*) Macrophages were incubated for 30 min with monoclonal antibodies anti-CD40 PE, anti-CD80 PE, anti-CD86 PE, anti-MHC class II PE and analyzed by flow cytometry. The bars represent the expression of molecules in terms of mean fluorescence intensity (MFI) ± SD. In Fig. 1*C*, BMDM stimulated for 24 h with Nattectin (10 μg/mL) previously treated or not with D-galactose at 10 nM for 1 h at 37 °C were incubated with FITC-dextran particles, and 30 min later, the fluorescent dextran capture was determined by flow cytometry. The dashed line represents cells incubated with FITC-dextran particles at 4 °C (negative control). *$p<0.05$ compared with control, # $p<0.05$ compared with Nattectin (NTC) + D-gal.

that the expression of this molecule does not depend on Nattectin binding to glycosylated structures expressed in macrophages.

Considering that macrophages are cells able to migrate to sites of infection or injury, and that this ability is dependent not only by integrins, but also matrix metalloproteinases (MMP) action, we evaluated the capacity of Nattectin to induce MMP-2 and MMP-9 production by macrophages. Both MMP-2 and MMP-9 are especially important in cell migration, because they cleave collagen IV, a major component of basement membranes. As shown in Fig. 2C Nattectin induced the production of MMP-2 and MMP-9 in supernatant from macrophage culture stimulated for 2 h. We also found a positive dependence of Nattectin binding to carbohydrate in MMP-9 production, but this effect was not observed for MMP-2.

3.3. Positive regulation of IFN-γ and IL-12 in Nattectin–mediated macrophage M1 polarization

Cytokines have been implicated in the reprogramming of M1 and M2 macrophages. M1 macrophages are induced in the presence of microbial agents or Th1 cytokines such as IFN-γ or IL-12, whereas the activation of alternatively activated macrophages (M2) depends on Th2 cytokines such as IL-4 or IL-13. APC maturation has tree phenotypic hallmarks including up-regulation of certain surface marker expression, induction of pro-inflammatory cytokines, and acquisition of functional CCR7. Thus, we evaluated the influence of these cytokines in the expression of markers for macrophage activation (CD40), antigen presentation (MHC class II) and migration (CCR7) induced by Nattectin, using CD11b^{pos}F4/80^{pos} macrophages from C57BL/6 *WT*, or IL-4-, IL-12- and IFN-γ-deficient mice.

The up-regulation of CD40 (clear gray bar, Fig. 3A), MHC class II (clear gray bar, Fig. 3B) and CCR7 (clear gray bar, Fig. 3C) expression induced by Nattectin in macrophages from WT mice was prevented in macrophages from IFN-γ (black bars) and IL-12 KO mice (gray bars), indicating that the expression of these molecules is strongly positively regulated by Th1 cytokines. In IL-4 KO mice, the Nattectin-stimulated macrophages presented an augmented increase in CD40 expression and a lower expression of MHC class II and CCR7 compared to macrophages from WT mice, indicating a negative regulation of IL-4 in CD40 expression and positive for MHC class II and CCR7.

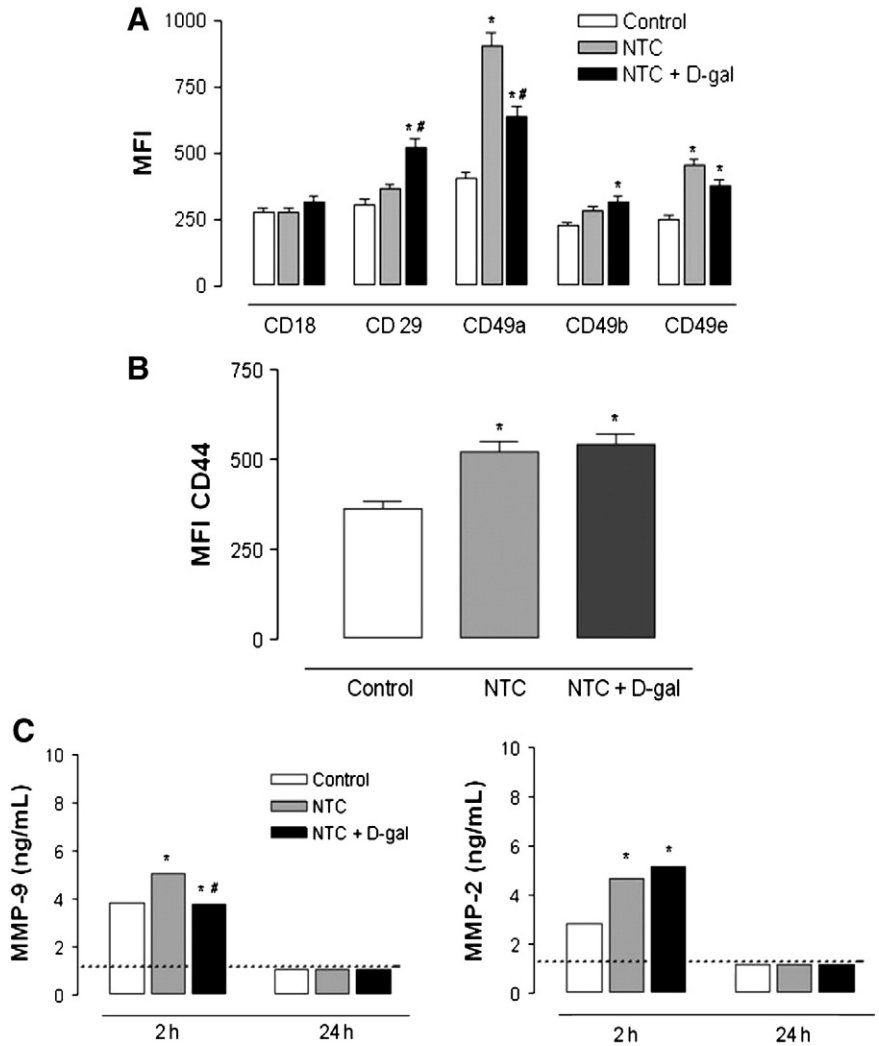

Fig. 2. Nattectin induces the up regulation of adhesion molecules. BMDM were stimulated for 24 h with Nattectin (10 μg/mL) previously incubated or not with D-galactose at 10 nM for 1 h and after washing the culture was incubated for 30 min with anti-CD18 PE, anti-CD29 PE, anti-CD49a FITC, anti-CD49b FITC, anti-CD49e FITC monoclonal antibodies (A), and (B) biotin-conjugated anti-CD44 and analyzed by flow cytometry. The bars represent the expression of molecules in terms of mean fluorescence intensity (MFI) ± SD. The supernatants of 2 h or 24 h culture were assayed for MMP-2 and MMP-9 by ELISA (C). Dashed line represent the limit of detection for MMP-2 (1.5 ng/mL) and MMP-9 (1.25 ng/mL). *$p<0.05$ compared with control, # $p<0.05$ compared with NTC.

3.4. IL-10 and IL-13 inhibits Nattectin-mediated macrophage M1 polarization

To confirm the macrophage M1 polarization induced by Nattectin, recombinant Th2 cytokines were added to the macrophage culture derived from *WT* or cytokines-deficient mice. The results showed in Fig. 4A and B indicate that the stimulation of *WT*-derived macrophages with Nattectin in the presence of IL-10 or IL-13 (white bars) induced a significant decrease in the expression of CD40 and MHC class II, which was similar to the expression of *WT*-derived macrophages without stimulation (control). Also, the addition of IL-10 or IL-13 to the culture of *KO*-derived macrophages with Nattectin did not alter the low level of expression of these molecules (Fig. 4A and B).

In contrast to the decreased expression of CD40 and MHC class II observed in *WT*-derived macrophages stimulated by Nattectin in the presence of IL-10 or IL-13, the treatment of *WT*-derived macrophages with Nattectin plus IL-10 had no effect in CCR7 expression, being partially reduced only by the addition of IL-13. Further, IL-12 *KO*-derived macrophages stimulated with Nattectin in the presence of IL-13 and IFN-γ *KO*-derived macrophages stimulated with Nattectin in the presence of IL-10 showed augmented expression of CCR7 (Fig. 4C). Together these results confirm the positive regulation of IL-12 and IFN-γ and negative control of IL-10 and IL-13 in macrophage M1 polarization induced by Nattectin and also reveal that IL-4 play a dual role in this polarization: a regular action of IL-4 was seen in the negative regulation of the CD40 expression, but an unexpected positive regulation was seen in the expression of CCR7 and MHC class II.

3.5. The influx of cells induced by Nattectin is positively regulated by IL-4 and IFN-γ

We next assessed the *in vivo* significance of these *in vitro* findings using a murine model of peritonitis in *WT*, IL-4, IL-12, and IFN-γ-deficient mice. Our results showed in Fig. 5A demonstrated that Nattectin induced an intense influx of leukocytes in peritoneal cavity of C57BL/6 *WT* mice. The influx of cells induced by Nattectin was mainly characterized by a neutrophil recruitment at 6 h, which was replaced by macrophages in 24 h. The recruitment of neutrophils elicited by Nattectin in C57BL/6 *WT* was completely abolished in IL-4 or IFN-γ-deficient mice, and also macrophage recruitment was positively regulated by both cytokines. The recruitment of neutrophils at 6 h and macrophages at 24 h was not modulated in IL-12-deficient mice injected with Nattectin.

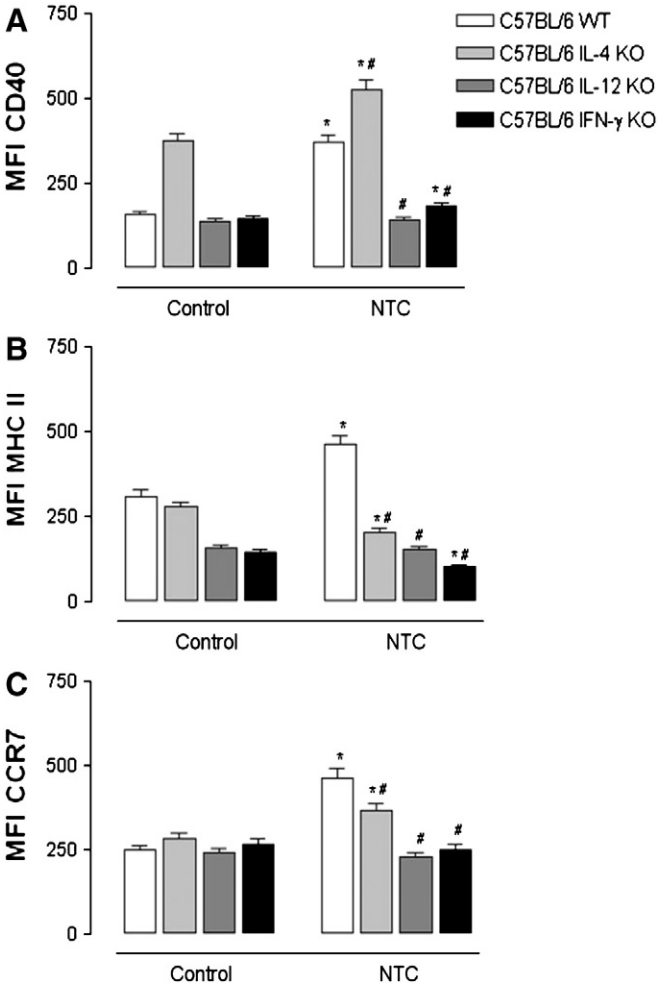

Fig. 3. Positive regulation of IFN-γ and IL-12 in Nattectin-mediated M1 polarization. BMDM from C57BL/6 *WT* mice or deficient in IL-4, IL-12 or IFN-γ were stimulated with Nattectin (10 μg/mL) for 24 h. After that, macrophages were incubated for 30 min with monoclonal antibodies anti-CD40 PE, anti-MHC class II PE, anti-CCR7 PE-Cy5 and analyzed by flow cytometry. The bars represent the expression of (A) CD40, (B) MHC class II, and (C) CCR7 in terms of mean fluorescence intensity (MFI) ± SD. *$p<0.05$ compared with control; # $p<0.05$ compared with Nattectin-injected C57BL/6 *WT* mice.

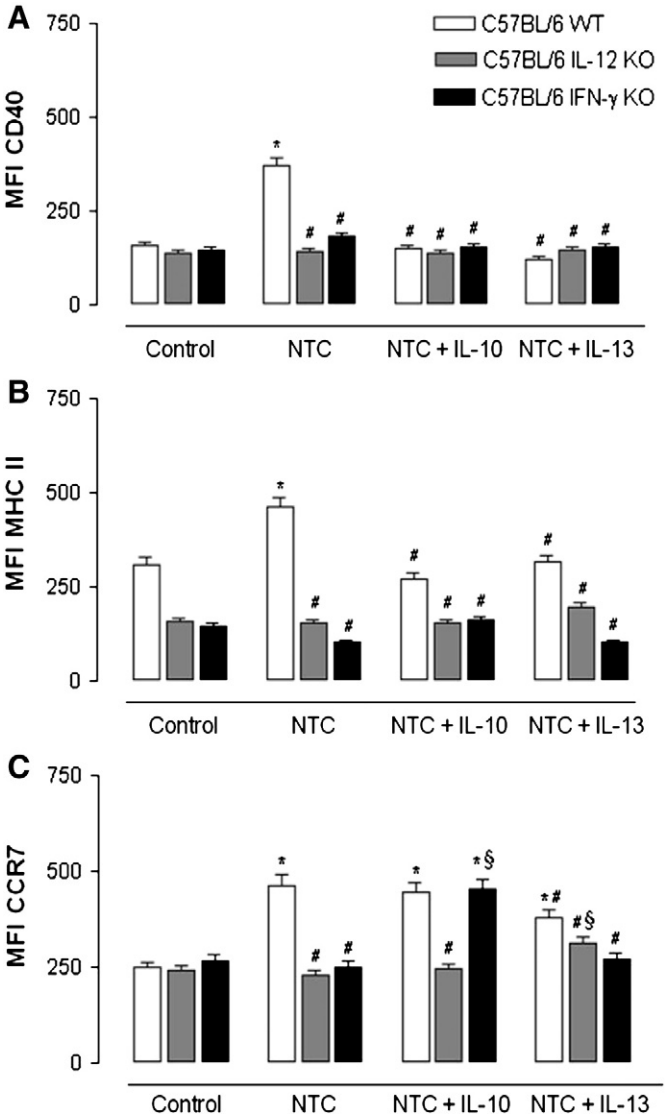

Fig. 4. IL-10 and IL-13 inhibit Nattectin-mediated macrophage M1 polarization. BMDM from C57BL/6 *WT* mice and deficient in IL-12 and IFN-γ were stimulated with Nattectin (10 μg/mL) for 24 h in the absence or presence of recombinant cytokines IL-10 or IL-13, each at a concentration of 5 ng/ml. After that, CD11b^{pos}F4/80^{pos}CD14^{pos}Ly6C^{low} macrophages were incubated for 30 min with monoclonal antibodies anti-CD40 PE, anti-MHC class II PE, anti-CCR7 PE-Cy5 and analyzed by flow cytometry. The bars represent the expression of (A) CD40, (B) MHC class II and (C) CCR7 in terms of mean fluorescence intensity (MFI) ± SD. *$p<0.05$ compared with control; # $p<0.05$ compared with Nattectin-injected C57BL/6 *WT* mice; § $p<0.05$ compared with Nattectin-injected Nattectin-injected C57BL/6 IL-12 *KO* or IFN-γ *KO* mice.

Utilizing a high-dimensional digital FACS with 11-color combination, Ghosn et al. [18] recently demonstrated that peritoneal macrophages comprises two distinct sub-populations, namely large (LPM) and small peritoneal macrophage (SPM); these sub-populations have phenotypes and functions distinct from monocytes and DC. In the present study, these sub-populations were identified using a 4-color flow cytometry according to Cassado and coworkers [19]. In dot plot of Fig. 5B we observed that C57BL/6 *WT* mice injected only with saline showed predominance of LPM (91.09% ± 1.82 — F4/80^{hi}MHC class II^{neg}) and a low proportion of SPM (5.99% ± 0.04 — F4/80^{low}MHC class II^{hi}). Nattectin promoted a decrease in the number of LPMs (78.50% ± 0.63) and an intense recruitment of SPM (10.20% ± 0.12) and neutrophils at 6 h after stimulation. The composition of peritoneal cavity after 24 h of Nattectin stimulation was similar to control group (SPM: 3.88% ± 1.02 and LPM: 92.08% ± 0.82).

In IL-4-deficient mice, we observed a drastic reduction in the SPM influx triggered by Nattectin to the peritoneal cavity at 6 h (2.5% ± 0.05) and 24 h (1.8% ± 0.09) after injection compared with *KO* mice injected with saline (7.5% ± 0.10). Also, light micrograph of peritoneal cells in Fig. 5C confirms a predominant influx of LPM at 6 h in both IL-4 and IFN-γ *KO* mice injected with Nattectin, indicating that IL-4 and IFN-γ positively regulate the recent influx of SPM induced by Nattectin. In addition, we also observed a replacement of neutrophils by eosinophils in IFN-γ-deficient mice 6 h after Nattectin injection. Finally, the influx of LPM at 24 h was not altered by the functional absence of both cytokines.

Among the several soluble mediators released in peritonitis, the results depicted in Fig. 6 indicate that 1 h after i.p. injection of Nattectin, only IL-6 was released in the peritoneal cavity of C57BL/6 *WT* mice compared to control group. However, IL-4-deficient mice showed a more pronounced production of IL-6, indicating that its release is negatively regulated by IL-4. In contrast, the absence of IFN-γ or IL-12 abolished the IL-6 production induced by Nattectin, showing a positive regulation. These data suggest that *in vivo* IL-6-producing macrophage M1 polarization induced by Nattectin is positively regulated by Th1 cytokines as IFN-γ and IL-12.

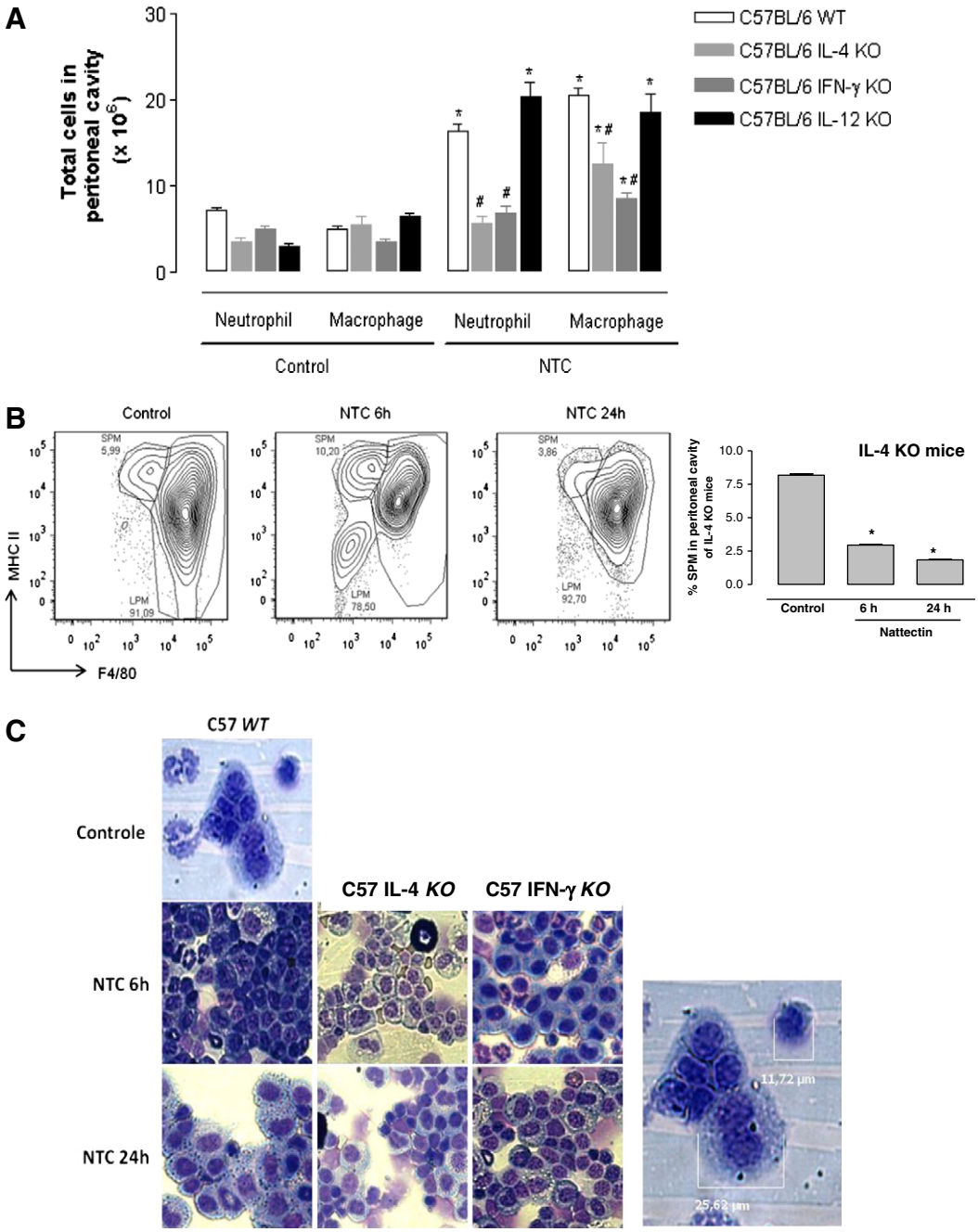

Fig. 5. The influx of cells induced by Nattectin is positively regulated by IL-4 and IFN-γ. (A) C57BL/6 *WT*, IL-4 *KO*, IFN-γ *KO* or IL-12 *KO* mice were i.p. injected with Nattectin (10 μg). After 6 or 24 h, animals were killed and peritoneal cavities were washed with PBS EDTA 10 mM for cell suspension collection. Cytocentrifuge stained slides were used for neutrophil (6 h) and macrophage (24 h) cell differentiation and count. (B) Peritoneal cells from control or Nattectin-injected C57BL/6 *WT* or IL-4 *KO* mice were harvested and stained with fluorochrome-labeled antibodies directed against F4/80, CD19, CD11c and IAb for flow cytometry analysis. Doublet cells were excluded according to forward scatter profiles (FSC-A and FSC-H). $CD19^{high}$ cells and $CD11c^{high}$ cells were also excluded. F4/80+ cells were selected, andF4/80 and IAb expression defined LPM ($F4/80^{high}IAb^{neg}$) and SPM ($F4/80^{low}IAb^{high}$). (C) Photomicrograph of macrophage subsets and cell morphology obtained from C57BL/6 *WT*, IL-4 *KO* or IFN-γ *KO* mice i.p. injected with Nattectin (10 μg). $^{*}p<0.05$ compared with control; # $p<0.05$ compared with Nattectin-injected C57BL/6 *WT* mice.

4. Discussion

Cytokines and growth factors have been implicated in the reprogramming of M1 and M2 macrophages. The response of macrophages to the endogenous danger signals is only one example of how different stimuli can induce the activation of macrophages in tissues [3]. The ability to redirect inappropriate macrophage activation, therefore, presents a novel and potentially highly effective therapeutic approach for treating macrophage-mediated diseases. Thus, several studies have been performed to look for drugs or components that somehow are able to modulate macrophage functions.

Lectins are one of the important pattern-recognition proteins (PRPs) that trigger a series of protective immune responses by interacting with conserved pathogen associated molecular patterns (PAMPs) molecules [20]. Fish have evolved natural immune modulators such as galectins and C-type lectins within tissues and cells associated with host defense functions against microbial pathogens. The venom of *T. nattereri* have been widely investigated in relation to

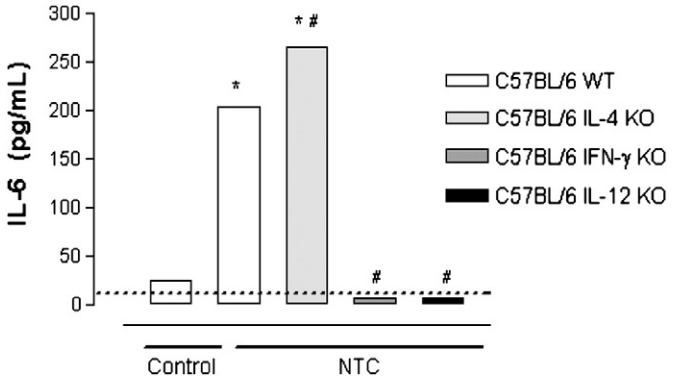

Fig. 6. The production of IL-6 induced by Nattectin is dependent on IFN-γ and IL-12. Quantification of IL-6 by ELISA in peritoneal fluids obtained from C57BL/6 *WT*, IL-4 *KO*, IFN-γ *KO* or IL-12 *KO* mice i.p. injected with Nattectin (10 μg). Dashed line represents the limit of detection for IL-6 (7.88 pg/mL). *$p<0.05$ compared with control; # $p<0.05$ compared with Nattectin-injected C57BL/6 *WT* mice.

different aspects [21–31] and recently, a 15 kDa monomeric toxin belonging to the family of C-type lectins denominated Nattectin was identified and characterized [7,8,32,33].

In the present study, we have shown that Nattectin can directly activate macrophages and induce a M1 polarization. The findings in this study demonstrate that Nattectin is able to induce M1 macrophage marker iNOS, and up-regulate the expression of MHC class II, CD80, CD86 and CD40 molecules. The increase in MHC class II expression with augmented expression of CD49a integrin, MMP-9 production and endocytic capacity depend on lectin function of Nattectin. A report of Ivory and Chadee [34] showed that stimulation of DC with Gal-lectin resulted in activation and maturation with an up-regulation of DC maturation markers CD80, CD86, CD40 and MHC class II. The Gal-lectin also induced DC production of IL-12 and stimulated T cell proliferation in an allogeneic mixed leukocyte reaction, indicating a Th1 response. In our study, Nattectin was able to induce macrophages to produce inflammatory mediators such as MMP-2 and MMP-9, without inducing the synthesis of TNF-α, IL-1β or IL-12p70 proteins (data not shown). Cytokines control the MMP production through negative or positive regulatory elements at the genetic level, and also influence the production of proteolytic enzymes that activate or inhibit MMPs [35–37]. Although inflammatory cytokines were not detected in the supernatant of macrophage stimulated with Nattectin, we can infer that a favorable microenvironment for the production of MMPs by macrophages was generated by this lectin.

In migrated cells, integrins are the main transmembranic receptors that provide dynamic interactions between actin cytoskeleton and ligants in the extracellular matrix. Beyond integrins, other receptors mediate this process, including multi-functional receptors as CD44 and sindecans. CD44 is a transmembrane glycoprotein for the glycosaminoglycan hyaluronan, one of the components of the extracellular matrix degraded during an inflammatory response [38–40]. Its membrane expression is increased with the polymerization of actin that occurs after the activation of leukocytes that also promotes a space alteration of the molecule with high accessibility to the action of pro-MMP-9 and its consequent activation and secretion [41,42]. Furthermore, expression of galectin-1 up-regulates a substantial subset of genes related to DC migration [43]. Our results that show Nattectin generating increase in the expression of α1 and α5 integrins, CD44 and MMP-9 production support the idea that Nattectin-activated macrophages acquire a competent molecular complex of migration that qualifies them to have access to tissues.

Cytokines (IL-4, IL-13 or IFN-γ) or microbial products trigger the *in vitro* differentiation of macrophage precursors in two polarized types [4,44], however, it is not fully understood how macrophages become polarized *in vivo* and the degree to which they retain plasticity or remain committed to a particular activation state. In this context, the next step in this study was the evaluation of the role of Th1 and Th2 cytokines in polarized activation of macrophages induced by Nattectin, using *in vivo* models. Sano and coworkers [45] demonstrated that galectin-3 induces migration of human monocytes and macrophages dependent on PTX-sensitive pathway *in vitro*. Recently, Saraiva and coworkers [8] demonstrated that Nattectin is able to induce an acute inflammatory response in murine peritoneal cavity with a massive influx of leukocytes, characterized by early influx of neutrophil (6 h) followed by macrophages in 24 h. Also, these authors demonstrated that Nattectin promotes a Th1 response with specific IgG2a production. Our studies here corroborate these results, but reveal that the influx of neutrophils and macrophages induced by Nattectin is totally dependent on IL-4 and IFN-γ cytokines action. Furthermore, the induction of IL-6 release is negatively regulated by IL-4 and positively regulated by IL-12 and IFN-γ.

It has been previously shown that IL-6 is needed for the development of protective T cells against intracellular parasite infections such as *M. avium* [46], *L. monocytogenes* [47] and *M. tuberculosis* [48]. In some of these works, IL-6 was shown to be necessary for the development of a Th1 response as assessed by the ability of the antigen-specific T cells to secrete IFN-γ [49,50]. In this context, we suggest that Nattectin antigen initiates a pro-inflammatory response and is able to induce the recruitment of macrophages, which depends on a microenvironment containing IL-4 and IFN-γ.

A recent study conducted by Ghosn and coworkers [18] identified two subpopulations of peritoneal macrophages named SPM (small peritoneal macrophage - F4/80^{low}MHCII^{hi}) and LPM (large peritoneal macrophage - F4/80^{hi}MHCII^{low}). SPM and LPM differ markedly in their *in vitro* and *in vivo* responses to inflammatory stimuli, and SPM rather than LPM or monocytes, have higher phagocytic activity and are the main source of IL-12, indicative of M1 macrophages [19]. In our studies, in contrast to C57BL/6 *WT* control mice that presented a predominance of LPM and a low proportion of SPM, we observed that Nattectin induced a change in the dynamics of peritoneal macrophages, recruiting numerous SPM and neutrophils at 6 h and decreased number of LPM. After 24 h, a LPM influx was observed in Nattectin-mice.

Mechanistically, stimulation with IL-4 leads to phosphorylation of Stat6 and IFN-γ induces Stat1 phosphorylation, suggesting different activities concerning macrophage function for IL-4 and IFN-γ. A novel and surprising finding of our studies was the striking positive regulation of IL-4 in the recent influx of SPM triggered by Nattectin, indicating that IL-4 is able to work as a coadjuvant with IFN-γ and IL-12 in the macrophage M1 polarization induced by Nattectin.

The inhibitory effect of IL-4 over the IFN-γ-mediated activation of the macrophage for the metabolism of reactive oxygen radicals was demonstrated by Appelberg et al. [51]. They showed that inhibitory activity may account for the anti-protective effects of IL-4 in the infections caused by pathogens to which ROI are important antimicrobial effector molecules. But, this inhibitory effect of IL-4 was not a general effect on macrophage functions since it did not affect the IFN-γ-mediated activation for the generation of nitrogen metabolites, mycobacteriostasis or anti-toxoplasma activity. In support of a role for differential regulation in the functional heterogeneity of macrophages some authors showed that the functional pattern displayed by macrophages treated with IL-4 depends on whether the macrophages were treated with IL-4 prior to or concurrently with the activating signal as IFN-γ. Thus, prior treatment with IL-4 results in elevated TNF-α and reduced IL-10 production upon LPS stimulation whereas the opposite result is obtained if IL-4 treatment is concurrent with LPS stimulation [52,53]. Our data presented here reinforce the view of progressive functional adaptation of macrophages during the course of the response; shift their function to changes in the tissue environment.

Together, the results obtained indicate that Nattectin is able to induce maturation of BMDM that have increased expression of co-stimulatory and MHC class II molecules, increased endocytic capacity and greater ability to migrate through extracellular matrix. Moreover, the phenotypic changes in peritoneal and bone marrow-derived macrophages induced by Nattectin are consistent with the classical M1 activation (iNOS expression), dependent on Th1 cytokines (IL-12 and IFN-γ), and negatively regulated by Th2 cytokines (IL-4, IL-10 and IL-13). Finally, these findings presented here allow us to extend the M1/M2 dichotomy, which is strongly influenced by the action of mutually inhibitory cytokines, since we have demonstrate that Nattectin generates a microenvironment where the co-participation of IL-4 and IFN-γ is decisive for the recruitment of SPM macrophages that are classically activated (*in vivo*) and for the induction of up-regulation of MHC class II and CCR7 in BMDM (*in vitro*). However, a negative regulatory role of IL-4 was observed in CD40 expression (*in vitro*) and IL-6 synthesis (*in vivo*). Thus, the results presented here allowed us to expand the knowledge about the regulation of macrophage activation and polarization, as well as confirmed the ability of Nattectin, a member of C-type lectin family, as an important immunomodulatory agent, thus opening the possibility of using synthetic agents derived from secretions of marine animals as immunomodulators, useful in vaccine development and for therapeutic application.

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