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REGULATORY PARAMETERS OF THE LUNG IMMUNE RESPONSE DURING THE EARLY PHASE OF EXPERIMENTAL TRICHINELLOSIS

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Highlights:

- Regulatory parameters emerge during the early lung immune response.
- This regulation could be linked with the passage of NBL through the lung.
- NBL can modulate the activation of lung macrophages *in vitro*.
- NBL excretory-secretory products and surface molecules can trigger this activation.

Abstract

Parasitic infection caused by *Trichinella spiralis* provokes an early stimulation of the mucosal immune system which causes an allergic inflammatory response in the lungs. The present work was intended to characterize the kinetics of emergence of regulatory parameters in Wistar rat lungs during this early inflammatory response, between days 0 and 13 p.i. The presence of regulatory cells such as regulatory T cells (Tregs) and alternatively activated macrophages (AAM) was analyzed in lung cell suspensions. Moreover, a regulatory cytokine (TGF-β) was studied in lung tissue extracts. Considering that newborn larvae (NBL) travel along the pulmonary microvasculature, the ability of this parasite stage to modulate the activation of lung macrophages was evaluated. For this purpose, lung macrophages from non-infected or infected rats (day 6 p.i.) were cultured with live or dead NBL. Arginase activity (characteristic of AAM) and nitric oxide (NO produced by iNOS, characteristic of classical activated macrophages) were measured after 48 hours. Our results revealed a significant increase in the percentage of Tregs on days 6 and 13 p.i., arginase activity on day 13 p.i. and TGF-β levels on days 6 and 13 p.i. Lung macrophages from non-infected rats cultured with live NBL showed a significant increase in arginase activity and NO levels. Live and dead NBL induced a significant increase in arginase activity in lung macrophages from infected rats. Only live NBL significantly increased NO levels in these macrophages. The present work demonstrates for the first time, the emergence of regulatory parameters in the early lung immune response during *T. spiralis* infection. The immumodulatory properties exerted by NBL during its passage through this organ could be the cause of such regulation. Moreover, we have shown the ability of NBL to activate macrophages from the lung parenchyma by the classical and alternative pathways.

Abbreviations: regulatory T cells (Tregs), alternatively activated macrophages (AAM), newborn larvae (NBL), muscle larvae (ML), live NBL (NBL_l), dead NBL (NBL_d), nitric oxide (NO), iNOS (inducible nitric oxide synthase).

Keywords: *Trichinella spiralis*; newborn larvae; lung; regulatory T cells; alternatively activated macrophages; TGF-β.

Introduction

Helminth infections are often associated with polarized Th2 responses and with the presence of regulatory immune cells and mediators such as regulatory T cells (Tregs, CD4+CD25+Foxp3+), alternatively activated macrophages (AAM, characterized by the expression of arginase-1), regulatory B cells, IL-10 and TGF-β. Helminths are considered masters of immunomodulation, they downregulate the effector immune response and generate a regulatory environment. During infection, the immune system is exposed to multiple helminth molecules present either at the surface of the parasites and/or in their excretory-secretory products, capable of modulating the immune response. It was proposed that this immune regulation could be beneficial to both host and parasite (Allen and Maizels, 2011; Aranzamendi *et al*., 2013a).

Trichinella spiralis causes a chronic infection in which muscle larvae (ML) survive for years encapsulated in the host skeletal muscles (Gottstein *et al*., 2009). For the establishment of this infection, regulation of the host immune response is necessary. It is known, by *in vivo* and *in vitro* experiments, that *T. spiralis* stages cause the expansion of regulatory immune cells accompanied by elevated production of regulatory cytokines (reviewed in Aranzamendi *et al*., 2013a).

T. spiralis newborn larvae (NBL) migrate, via the circulatory system, through several organs, including the lung, to reach skeletal muscle (Despommier *et al*., 2005). Recent results from our

laboratory have demonstrated an allergic inflammatory response in the lung parenchyma, with development of bronchus-associated lymphoid tissue hyperplasia before and during NBL passage through this organ. This inflammation is characterized by qualitative and quantitative cellular changes and by the local production of cytokines, chemokines and antibodies (Venturiello *et al*., 2007; Gentilini *et al*., 2011; Falduto *et al*., 2015).

The research reported here was aimed at characterization of the kinetics of emergence of regulatory parameters in *T. spiralis* infected Wistar rats, during the early lung inflammatory response, between days 0 and 13 p.i. In particular, the presence of Tregs, AAM and TGF-β were evaluated. Moreover, the ability of NBL to modulate the activation of lung macrophages from non-infected and infected rats was studied by *in vitro* assays.

1. Materials and methods

2.1. Animals and infection

Two-month-old female Wistar rats were orally inoculated with 2000 ML per rat through a gastric canula. ML were obtained from muscle tissue of previously infected Swiss mice by an artificial digestion method (Nöckler and Kapel, 2007). Throughout all the experiments, animals were provided with water and food *ad libitum*, exposed to 12-h light–dark cycles and room temperature was kept at 21 ± 1 °C. All experimental protocols were approved by the Institutional Review Board of the Instituto de Estudios de la Inmunidad Humoral (IDEHU) and conducted in compliance with the guidelines established by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

2.2. Preparation of lung cell suspensions

To obtain lung cell suspensions, rats were anaesthetized, bled by cardiac puncture, then perfused with 40 ml of sterile PBS (pH 7.4) containing 10 IU/ml heparin into the heart right ventricle until lungs turned white. Lungs were removed, cut into small pieces, and digested in RPMI medium (Gibco, NY, USA) containing collagenase A (0.5 mg/ml, Roche Diagnostics, Mannheim, Germany), DNase (0.1 mg/ml, Roche Diagnostics), penicillin (100 IU/ml, Gibco), and streptomycin (100 μg/ml, Gibco) for 40 min at 37 °C with occasional shaking. Tissues were homogenized and cell suspensions were filtered through a nylon mesh to remove tissue debris. The remaining erythrocytes were lysed using ammonium chloride buffer solution. Cell suspensions were suspended and washed twice with PBS plus EDTA 5 mM and 3% fetal calf serum (Natocor, Córdoba, Argentina). Lung cells were counted using a hemocytometer and Trypan blue dye (Gibco). Cell viability was invariably higher than 95%. Lung cell suspensions contained between 84–87% leukocytes and 13–16% non-leukocyte lung cells. After counting, cells were re-suspended in RPMI medium containing 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5% fetal calf serum.

2.3. Collection of lung tissue extracts

Lung tissue extracts were obtained from non-infected and infected rats (1, 2, 3, 6 and 13 p.i.) using the Perfext method (Villavedra *et al*., 1997) with slight modifications. Briefly, rats were bled and perfused with PBS plus heparin (5,000 IU/ml) as described. The perfused organs were cut into small pieces, placed in an extraction solution (2 μl of solution/mg of tissue) containing 90 mM CHAPS (Research Organics, OH, USA) in PBS and protease inhibitors (Roche Diagnostics), and stored at -70 °C until used. After thawing, the extraction was performed overnight at 4 °C using a rotary agitator.

After homogenization, the extract was centrifuged at 9000xg for 10 min, supernatants were collected, filtered through a 0.22 µm filter (Millipore Co, MA, USA), aliquoted and stored at -70 °C until used.

2.4. Collection of newborn larvae

NBL were obtained as described previously (Dennis *et al*., 1970). Briefly, adult worms were recovered from the intestine of Wistar rats infected orally with 7000 ML 5-6 days earlier. Following recovery, adult worms were cultured in RPMI containing 100 µg/ml streptomycin, 100 IU/ml penicillin, and 5% fetal calf serum) at 37 °C in an atmosphere of 5% CO_2. NBL were collected after 2 h; larvae were alive and in good condition as judged by their motility; four washes in culture media were performed before use. Some NBL were killed by freezing at -20 °C for 7 days.

2.5. Study of Tregs in lung

An immunomagnetic selection of T cells from lung cell suspensions was performed. The lung cell suspension was magnetically labeled with rat Pan T Cell MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and loaded onto a MS column (Miltenyi Biotec GmbH) which was placed in a magnetic field (Miltenyi Biotec GmbH). Magnetically retained cells were eluted from the column with cold PBS plus EDTA 2 mM and 0.5% fetal calf serum.

Lung T cells ($3x10^5$) from non-infected and infected rats (days 6 and 13 p.i.) were triple-stained with Alexa Fluor 647 anti-rat CD4 (BioLegend, CA, USA), Alexa Fluor 488 anti-rat CD25 (BioLegend) and PE anti-mouse/rat/human Foxp3 (BioLegend), following manufacturer's instructions. Samples were processed on a FACSAria II flow cytometer (BD Biosciences, MA, USA); Cyflogic

software version 1.2.1 was used for data analysis. Results were expressed as the percentage of Tregs within the CD4+ cell population.

2.6. TGF-β assay

The mouse/rat/porcine/canine TGF-β1 Quantikine® commercial ELISA kit (R&D Systems, MN, USA) was used to determine concentrations of activated TGF-β in lung tissue extracts from non-infected and infected rats (days 1, 2, 3, 6 and 13 p.i.). Manufacturer's protocol was followed step by step and the reaction was measured at 450 nm.

2.7. Presence of AAM in lung

Lung cell suspensions from non-infected and infected rats (days 3, 6, 9 and 13 p.i.) were analyzed. Macrophages were purified based on their selective adherence to plastic surfaces. Lung cell suspensions, re-suspended in RPMI (containing 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5 % fetal calf serum), were incubated in 75 cm^2 cell culture flasks at 37 °C in 5% CO_2. After 2 h, non-adherent cells were removed and adherent cells were obtained by incubation with cold PBS in an ice bath. This procedure yielded ≥85% macrophages as determined by Giemsa stain. Approximately, one-hundred thousand cells were lysed with 50 μl 0.1% Triton X-100 containing protease inhibitor cocktail (Roche Diagnostics). This mixture was stirred for 30 min at room temperature. Arginase activity and protein concentration were measured in these cell lysates (see 2.9.).

2.8. *In vitro* activation of lung macrophages by newborn larvae

For macrophage isolation, $2x10^6$ lung cells (per well) from non-infected or infected rats (day 6 p.i.) re-suspended in RPMI (containing 100 µg/ml streptomycin, 100 IU/ml penicillin, and 5% fetal calf serum) were incubated in 24-wells plates at 37 °C in 5% CO_2. After 2 h, non-adherent cells were removed. Adherent cells were cultured in triplicate with 500 live (NBL_l) or dead (NBL_d) NBL in a final volume of 700 µl. Wells with cells only were used as controls. After 48 h, supernatants were collected and cells were lysed with 400 µl 0.1% Triton X-100 containing a protease inhibitor cocktail (Roche Diagnostics). Arginase activity and production of nitric oxide (NO) were measured in cell lysates and culture supernatants respectively (see 2.9. and 2.10.).

2.9. Determination of arginase activity

Arginase activity was measured in cell lysates as described previously (Corraliza *et al*., 1994). Briefly, 25 µl lysed cells were combined with 25 µl of 10 mM $MnCl_2$ and 50 mM Tris-HCl and the enzyme activated by heating for 10 min at 56 °C. Arginine hydrolysis was initiated by adding 50 µl 0.5 M L-arginine, pH 9.7, at 37 °C for 60 min. The reaction was stopped with 400 µl H_2SO_4 (96%), H_3PO_4 (85%) and H_2O (1/3/7, v/v/v). Urea concentration was measured at 540 nm after the addition of 25 µl α-isonitrosopropiophenone (dissolved in ethanol 100%), followed by heating at 95 °C for 45 min. A calibration curve was prepared with increasing amounts of urea between 1.8 and 30 µg; 400 µl of acid mixture and 25 µl of α-isonitrosopropiophenone were added to 100 µl urea solution. Results were expressed as µg of urea per µg of protein present in cell lysates.

2.10. NO assay

NO production was measured indirectly by assaying nitrites in culture supernatants (see 2.7.) using the Griess reaction. Supernatants were mixed with an equal volume of Griess reagent (Britania, Buenos Aires, Argentina). The absorbance was measured at 540 nm and nitrite concentration was calculated by using a $NaNO_2$ calibration curve between 3.91 and 250 μM.

2.11. Statistical analysis

Data were analyzed using the one-way ANOVA test, a $p<0.05$ was considered significant. The GraphPad Prism 6 software was used.

3. Results

3.1. Study of lung regulatory parameters

The study of Tregs in lung cell suspensions was carried out at two time-points p.i. important in the *T. spiralis* life cycle. Day 6 p.i., is the time of highest passage of NBL through the lung, and day 13 p.i., when the migratory phase is ending (Harley and Gallicchio, 1971). Flow cytometry results are shown in Fig. 1. A significant increase in the percentage of Tregs was found on day 6 p.i., compared with non-infected rats (6.03 ± 0.31% vs. 4.2 ± 0.26%, $p<0.01$). On day 13 p.i., the percentage of Tregs was significantly higher than that found on day 6 p.i. (7.82 ± 0.44% vs. 6.03 ± 0.31%, $p<0.01$).

To determine whether a change occurs in lung AAM during the early phase of *T. spiralis* infection, arginase activity was measured in rat lung macrophages from days 3, 6, 9 and 13 p.i. (Fig. 2). A significant increase in arginase activity was observed only on day 13 p.i., compared with non-infected rats (0.34 ± 0.05 μg urea/μg protein vs. 0.21 ± 0.03 μg urea/μg protein, $p<0.05$).

To evaluate the effect of *T. spiralis* infection in the production of regulatory cytokines by lung cells, TGF-β concentrations were measured in rat lung tissue extracts from days 1, 2, 3, 6 and 13 p.i. (Fig. 3). TGF-β levels were significantly higher in infected rats compared with non-infected rats on days 6 (11,426 ± 1374 pg/ml vs. 5085 ± 1953 pg/ml, $p<0.05$) and 13 p.i. (13,276 ± 1517 vs. 5085 ± 1953 pg/ml, $p<0.01$).

3.2. Activation of lung macrophages by newborn larvae

An *in vitro* assay was used to study the ability of NBL to modulate the activation of lung macrophages. NBL_d were used to analyze the effect of parasite surface molecules, while NBL_l were used to analyze the combined effect with metabolic products. Lung macrophages from non-infected and infected rats (day 6 p.i.) were cultured with these larvae.

The appropriate amount of NBL was determined by performing a preliminary assay. One hundred, 500 and 1000 NBL_l per well were tested. The highest values of arginase activity and NO production were obtained working with 500 and 1000 NBL_l. Considering these results; it was decided to use 500 NBL in subsequent assays.

When working with lung macrophages from non-infected rats, culture with NBL_l resulted in an increase in arginase activity (Fig. 4a, 1.34 ± 0.13 vs. 0.89 ± 0.07 μg urea/μg protein, $p<0.05$) and NO production (Fig. 4c, 66.8 ± 11.13 vs. 21.08 ± 6.81 μM, $p<0.01$), significantly different from control wells (cells without NBL). Incubation with NBL_d induced a non-significant increase in arginase activity (1.09 ± 0.04 vs. 0.89 ± 0.07 μg urea/μg protein) and NO production (45.91 ± 7.48 vs. 21.08 ± 6.81 μM).

Regarding lung macrophages from infected rats (day 6 p.i.), a significant increase ($p<0.05$) was observed in arginase activity (Fig. 4b) when cells were cultured with NBL_l (1.26 ± 0.15 μg urea/μg protein) or NBL_d (1.26 ± 0.16 μg urea/μg protein) compared with control wells (0.68 ± 0.026 μg

urea/μg protein). Furthermore, culture with NBL_l resulted in an increase in NO production (Fig. 4d), significantly different from control wells (58.45 ± 15.75 vs. 12.47 ± 1.23 μM, $p<0.05$). The increase in NO production induced by NBL_d was not significant (34.43 ± 6.12 vs. 12.47 ± 1.23 μM), as observed in lung macrophages from non-infected rats.

4. Discussion

Little is known about immunological changes in the lung during the first days of *T. spiralis* infection. The results presented in this study show increases in regulatory cells (Tregs and AAM) and TGF-β during the development of the lung inflammatory response (Venturiello *et al*., 2007; Gentilini *et al*., 2011). The appearance of these regulatory parameters takes place simultaneously with the Th1 to Th2 response switch (Gentilini *et al*., 2011).

It is known that helminth infections induce an expansion of Tregs in both number and function; this extends parasite survival and reduces host pathology (Boer *et. al*, 2015; Maizels *et al*., 2014). Several authors have shown *in vivo* and *in vitro* that *T. spiralis* is capable of inducing an expansion of Tregs (Beiting *et al*., 2007; Ilic *et al*., 2008; Aranzamendi *et al*., 2012; Cho *et al*., 2012; Aranzamendi *et al*., 2013b). To our knowledge, this is the first report of Tregs in lung tissue during the early phase of *T. spiralis* infection. This study shows an increase in lung Tregs as early as day 6 p.i. This population was also studied on day 13 p.i., when the migratory phase is ending in the murine model, and the percentage was even higher.

AAM have been identified as regulatory cells, having the ability to counteract pro-inflammatory and cellular immune effector mechanisms (Martinez and Gordon, 2014). This alternatively activated phenotype is induced during the Th2 immune responses to several helminth parasites (Rodríguez-Sosa *et al*., 2002; Nair *et al*., 2003; Herbert *et al*., 2004; Donnelly *et al*., 2005). It has been reported that

excretory-secretory products from different stages of *T. spiralis* can modulate macrophage function, *in vivo* and *in vitro*, towards the alternative phenotype (Du *et al*., 2011; Bai *et al*., 2012). Our study shows an increase in arginase activity in lung macrophages when the *T. spiralis* lung migratory phase is ending (day 13 p.i.). This late increase in lung AAM could be associated with repairing the damage caused by inflammation and NBL migration. This is in line with Kreider *et al*. (2007) who reported that AAM play an essential role in wound healing during helminth infections. However, Dzik *et al*. (2004) obtained different results while working with guinea pigs alveolar macrophages as they found a significant increase in arginase activity only on day 6 p.i. This discrepancy could be due to different types of lung macrophages. Cell composition and activation of bronchoalveolar lavage are not representative of the entire lung (Pabst *et al*., 2008; Siracusa *et al*., 2008) and alveolar macrophages should not be considered as representative of the lung macrophage population (Lambrecht, 2006; Hussell and Bell, 2014).

Considering the immunosuppressive properties of TGF-β and that it can be secreted by Tregs and AAM, we studied TGF-β concentrations in lung tissue extracts. A significant increase in TGF-β levels was found on days 6 and 13 p.i. This correlates with Tregs and AAM results presented here and lung IL-10 levels, published recently (Gentilini *et al*., 2011).

It is noteworthy that these increases in regulatory parameters occur after the initiation of migration of NBL through the lung (day 6 p.i.), suggesting that NBL immunomodulatory properties could induce this regulation of the lung immune response. The immunomodulatory properties of NBL have already been demonstrated by other authors (Faubert, 1976; Ilic *et al*., 2008; Ilic *et al*., 2011). Taking into account this hypothesis, we analyzed the ability of NBL to modulate the activation of lung macrophages *in vitro*. Our results showed that NBL are capable of inducing the alternative activation pathway in lung macrophages. Remarkably, this parasite stage also boosted classical activation in these macrophages. Our results are in line with Ilic *et al*. (2011), whose work demonstrated that NBL soluble

antigen induces polarization towards a mixed Th1/Th2 profile. On the other hand, Bai *et al*. (2012) showed that NBL and adult worm culture supernatant can direct macrophages towards the alternative pathway by inducing arginase-1 expression and inhibiting inducible NO synthase and proinflammatory cytokine production. NBl_l and NBL_d were used to study the effect of parasite metabolic products and surface molecules respectively. The activation of lung macrophages from non-infected and infected rats (day 6 p.i.) was triggered by metabolic products from NBL_l. Parasite surface molecules (NBL_d) only were capable of increasing arginase activity in lung macrophages from infected rats (day 6 p.i.), reaching the same values as those generated by metabolic products (NBL_l). This is probably due to variances in macrophage sensitization and activation caused by infection.

5. Conclusion

The present work demonstrates the occurrence of regulatory parameters (Tregs, AAM and TGF-β) during the early lung immune response against *T. spiralis*. This regulation could have occurred due to the immumodulatory properties exerted by NBL during its passage through this organ. The ability of NBL to activate lung macrophages *in vitro*, by the classical and alternative pathways, is shown for the first time. This effect is triggered by parasite metabolic products and/or surface molecules.

Conflict of interest statement

The authors have no conflicts of interest to report.

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Figure captions

Fig. 1. Regulatory T cells in lung cell suspensions. (A) Percentages of regulatory T cells within the CD4+ T cell population. Lung cells suspensions from non-infected and infected rats (days 6 and 13 p.i.) were analyzed (n=5 rats/day). Results are expressed as the mean ± S.E.M. Data were analyzed using one-way ANOVA, asterisks indicate significant differences (* p<0.01, ** p<0.0001). (B) Representative dot plots of CD25 versus Foxp3 expression, percentages of CD25+Foxp3+ previously gated on CD4+ T cells are shown.

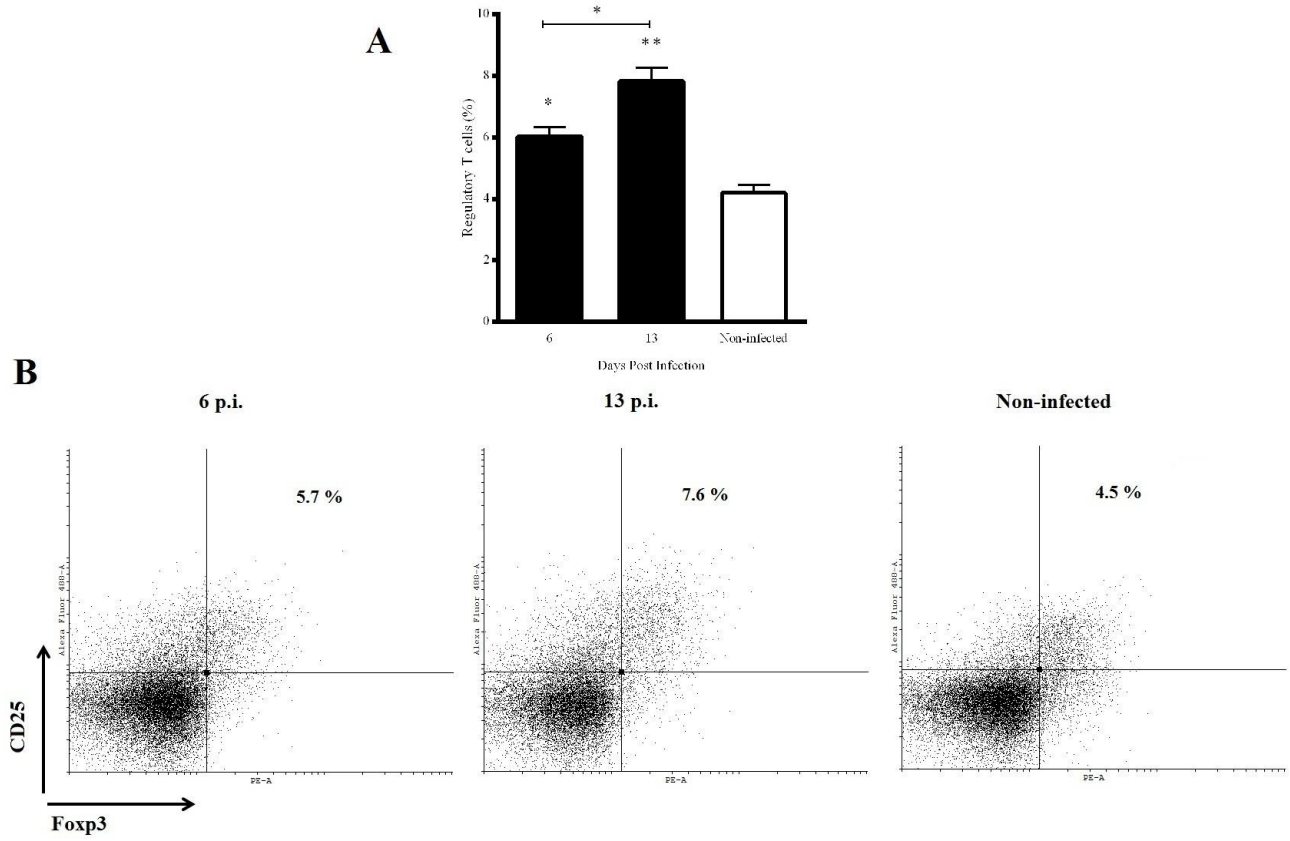

Fig. 2. Alternatively activated macrophages in lung cell suspensions. Arginase activity (µg urea/µg protein) in lung macrophages. Lung cells suspensions from non-infected and infected rats (days 3, 6, 9 and 13 p.i.) were analyzed (n=5 rats/day). Results are expressed as the mean ± S.E.M. Data were analyzed using one-way ANOVA; asterisk indicates a significant differences between infected and non-infected rats (* $p<0.05$).

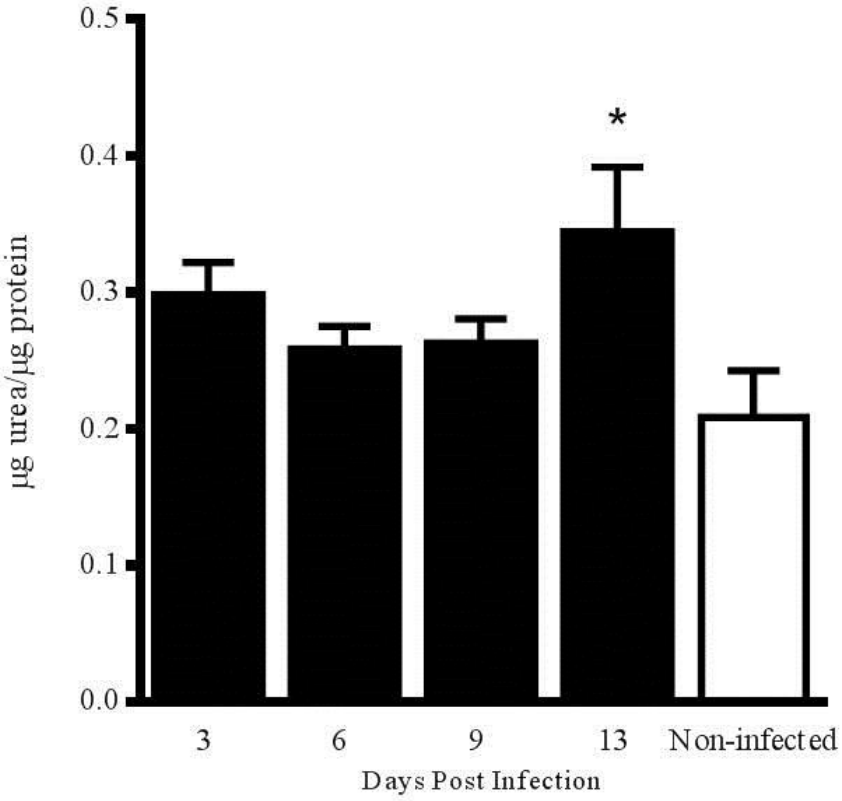

Fig. 3. TGF-β in lung tissue extracts. TGF-β concentrations (pg/ml) in lung tissue extracts from non-infected and infected rats (days 1, 2, 3, 6 and 13 p.i.; n=5 rats/day). Results are expressed as the mean ± S.E.M. Data were analyzed using one-way ANOVA; asterisk indicates significant differences between infected and non-infected rats (* $p<0.05$, ** $p<0.01$).

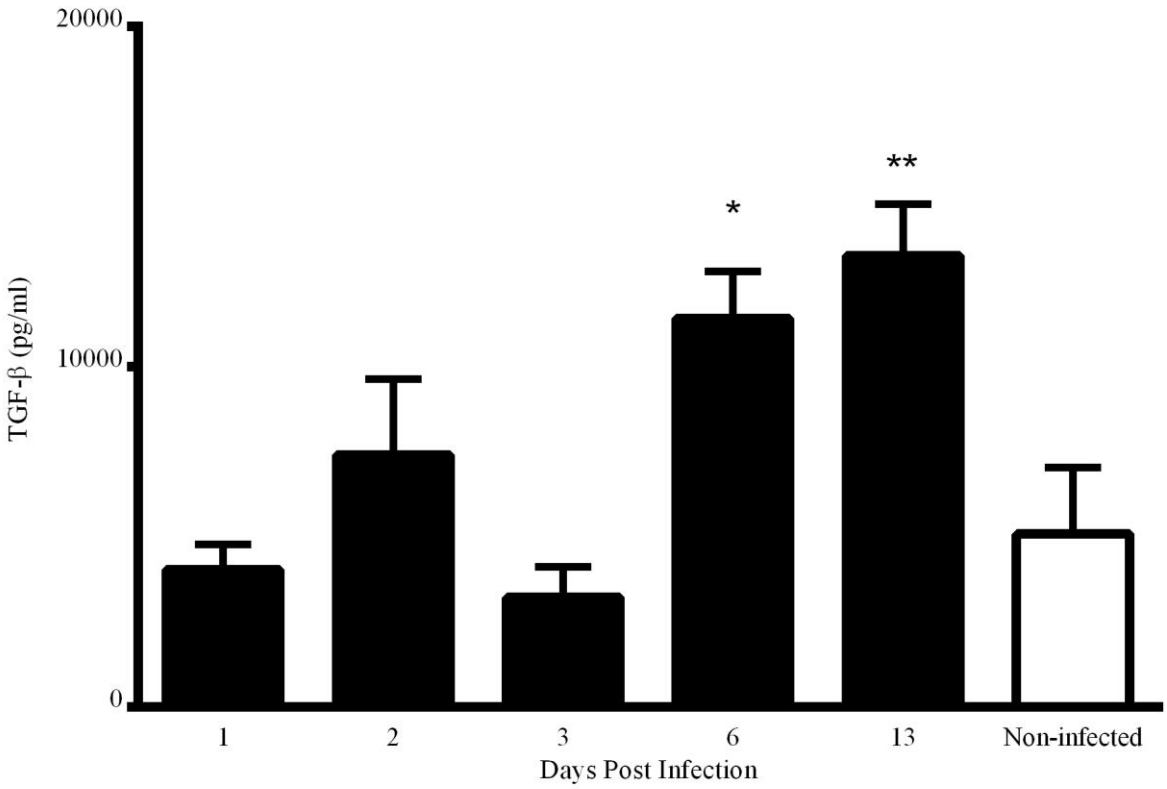

Fig. 4. Activation of lung macrophages by newborn larvae (NBL). Lung macrophages were cultured for 48 h with live (NBL_l) or dead NBL (NBL_d). Wells with cells only (-) were used as controls. Arginase activity (µg urea/µg protein) was measured in macrophages lysates from (A) non-infected and (B) infected rats (day 6 p.i.). NO production was assessed by measuring nitrites concentration (µM) in culture supernatants with lung macrophages from (C) non-infected and (D) infected rats (day 6 p.i.). Results are expressed as the mean ± S.E.M obtained from four independent experiments (n=1 rat/day/experiment). Data were analyzed using one-way ANOVA; asterisk indicates significant differences between cells cultured with NBL and control wells (* $p<0.05$).