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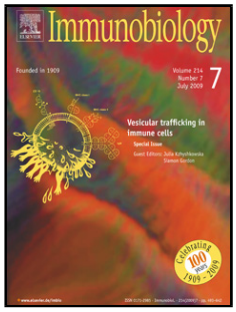

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Regulatory cells induced by acute toxoplasmosis prevent the development of allergic lung inflammation

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Keywords: *allergy, immune suppression, inflammation, lung, regulatory cells, Toxoplasma gondii.*

Abbreviations: *OVA, chicken egg white albumin; BAL, bronchoalveolar lavage; PAS, Periodic acid-Schiff; TLN, thoracic lymph nodes; FBS, fetal bovine serum; ConA, concanavalin A; CS, cell supernatant; H&E, hematoxylin and eosin stain. PBS, phosphate buffered saline, TLR, toll like receptor.*

Abstract

The increased prevalence of allergies in developed countries has been attributed to a reduction of some infections. Supporting epidemiological studies, we previously showed that both acute and chronic *T. gondii* infection can diminish allergic airway inflammation in BALB/c mice. The mechanisms involved when sensitization occurs during acute phase would be related to the strong Th1 response induced by the parasite. Here, we further investigated the mechanisms involved in *T. gondii* allergy protection in mice sensitized during acute *T. gondii* infection. Adoptive transference assays and *ex vivo* co-cultures experiments showed that not only thoracic lymph node cells from infected and sensitized mice but also from non-sensitized infected animals diminished both allergic lung inflammation and the proliferation of effector T cells from allergic mice. This ability was found to be contact- independent and correlated with high levels of $CD4^{+}FoxP3^{+}$ cells. IL-10 would not be involved in allergy suppression since IL-10-deficient mice behaved similar to wild type mice. Our results extend earlier work and show that, in addition to immune deviation, acute *T. gondii* infection can suppress allergic airway inflammation through immune suppression.

Introduction

Allergic asthma is characterized by lung inflammation, airway obstruction, excessive mucus production, airway hyperresponsiveness and increased allergen specific IgE production (Holgate et al., 2012). The pulmonary infiltrates consist mainly of eosinophils, basophils, mast cells, macrophages and activated Th2 cells. The mechanisms that cause allergic asthma are complex but overall they appear to result from an intrapulmonary allergen-driven Th2 response characterized by an increased production of type 2 cytokines (IL-4, IL-5, IL-9, and IL-13) secreted by $CD4^+$ T lymphocytes.

In last decades, the incidence of asthma and other allergic diseases has dramatically increased particularly in developed countries (Holgate et al., 2012). The reasons for the increased incidence of allergy are still not well understood. The pathogenesis of asthma reflects the influences of multiple risk factors including genetic susceptibility and environmental factors such as pollution, diet and a lack of some infectious stimuli arising from modern sanitary practices and the widespread use of antibiotics (Holgate et al., 2012; Strachan et al., 1989; Obihara et al., 2007). Certainly, these last three would be mainly responsible for the growing prevalence. Several epidemiological studies have reported an inverse correlation between asthma incidence and different chronic infections such as *Helicobacter pylori* (Blaser et al., 2008), hepatitis A and *Toxoplasma gondii* (Matricardi et al., 2000; Ellertsen et al., 2008; Fernandesa et al., 2010). These observations were supported by several experimental studies showing allergy modulation by diverse pathogens. Indeed, these models showed that not only chronic infections such as *T. gondii* or *Helicobacter pylory* (Fenoy et al., 2008; Wagner et al., 2009; Arnold et al., 2011), but

also acute infection protocols induced by bacterium (Mycobacterium), viruses (Adenovirus, Influenza) and helminths (*Heligmosomoides polygyrus*) have proved to modulate immune responses (Erb et al., 1998; Stämpfli et al 1998; Wohlleben et al., 2003; Kitagaki Ket al., 2006). The mechanisms involved include both Th1 cytokine induction by infectious agents, as was initially postulated for the mechanism behind the hygiene hypothesis, and the induction of regulatory cells.

Toxoplasma gondii is an obligate intracellular protozoan. Infection with the parasite, through stimulation of TLR2, TLR4, TLR-11, TLR12 and CCR5 receptors, induces a strong Th1-type immune response, particularly during the acute phase of infection (Melo et al., 2011; Koblansky et al., 2013). In turn, as a result of the response and in order to prevent the imbalance of the strong Th1 response resulting in immunopathology, anti-inflammatory cytokines including IL-10 and TGF-β that inhibit IFN-γ production and impair macrophage activation are produced (Gaddi et al., 2007). Moreover, studies using IL-10-deficient mice showed increased mortality during the acute phase of infection, confirming the important role of simultaneous induction of regulatory cytokines (Gaddi et al., 2007).

We and others (Fenoy et al., 2008; Wagner et al., 2009) provided experimental support for the epidemiological data (Matricardi et al., 2000; Ellertsen et al., 2008; Fernandesa et al., 2010) by showing that *T. gondii* infection can block the development of allergic airway inflammation in adult BALB/c mice. This effect operates during both acute and chronic phases of infection. When sensitizing during chronic infection, regulatory cells

in thoracic lymph nodes could ameliorate allergic lung inflammation (Fenoy et al., 2012). The high levels of IFN-γ that characterize the acute phase of *T. gondii* infection suggest that the mechanisms involved in allergy protection when sensitizing during this stage, are related to this strong Th1 response. Indeed, a shift to Th1 IgG isotypes was detected (Fenoy et al., 2008; Wagner et al., 2009). However, other regulatory mechanisms could also be participating. To further investigate the mechanisms involved in *T. gondii* allergy protection we extended our studies to mice sensitized during acute *T. gondii* infection.

Materials and Methods

Animals

BALB/c (H-2^d) mice were obtained from the animal facilities of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (UBA), Argentina and maintained in our animal SPF facilities for use throughout these experiments. IL-10 deficient mice (BALB/c) were bred and housed at the animal facilities of Instituto de Investigaciones Biotecnológicas (IIB), Universidad Nacional de San Martín (UNSAM), Buenos Aires, Argentina, and were kindly provided by J.E. Ugalde (IIB-UNSAM). Mice were used at the age of 6 to 8 weeks. All procedures requiring animals were performed in agreement with institutional guidelines and were approved by the Independent Ethics Committee for the Care and Use of Experimental Animals of National University of San Martin (C.I.C.U.A.E., IIB-UNSAM, 09/2011).

Infection, Sensitization and Exposure

The Beverley strain of *T. gondii* was used in this study. For infection, cysts were obtained from the brains of orally infected C3H/HeN mice and maintained by monthly passage. For the experimental model BALB/c mice were orally infected with 25 cysts. One week later, sensitization was achieved by two i.p. injections of 0.2 ml PBS containing 20 μg of chicken egg white albumin (OVA) (grade V, Sigma-Aldrich) and 2mg of alum hydroxide (Sigma-Aldrich) one week apart. One week later, mice were exposed to aerosols of allergen (3% (w/v)) OVA in PBS for 20 min on 3 consecutive days (TO group). Aerosol exposure was performed within individual compartments of a mouse pie chamber using a nebulizer

(SAN-UP, Argentina, OVA solution flux 0.33 ml/min in air flux of 6–8 L/min). Mice were analyzed 48 h after the last exposure. Negative controls include *T. gondii* infected (T) and non-infected (naïve).

Pathologic Analysis

Animals were euthanized with sodium pentobarbital. The chest wall was opened and the animals were exsanguinated by cardiac puncture. The trachea was cannulated after blood collection. Bronchoalveolar lavage (BAL) was performed four times with 1 ml of sterile PBS, instilled and gently harvested. Lavage fluid was collected, centrifuged at 300 g for 10 min, and the pellet was resuspended in 0.5 ml PBS. Total cell yield was quantified and BAL differential cell counts were performed on cytocentrifuge slides prepared by centrifugation of samples at 80g for 5 min (Cytospin 4; Shandon, Pittsburg, PA). These slides were fixed and stained with a modified Wright-Giemsa stain (Tinción 15, Biopur SRL, Rosario, Argentina), and a total of 200 cells were counted for each sample by microscopy. Macrophages, lymphocytes, neutrophils, and eosinophils were quantified. After lavage the lungs were instilled with 10% buffered formalin, removed and fixed in the same solution. Following paraffin embedding, sections for microscopy were stained with H&E and PAS. Indices of pathologic changes were obtained as previously described (Fenoy et al., 2008). Briefly, index of inflammatory infiltrates around the airways and vessels was calculated by scoring severity (0, normal; 1, < 4 cells diameter thick; 2, 4–10 cells diameter thick; 3, >10 cells diameter thick) and overall the extent (0, normal; 1, <25% of sample; 2, 26–50%; 3, 51–75%; 4, >75%) The index was calculated by multiplying

severity by extent. An histological goblet cell score was obtained in Periodic acid-Schiff (PAS)-stained lung sections by examining 10 to 20 consecutive airways from all groups of mice at 40x magnification and categorized according to the abundance of PAS-positive goblet (0, < 5% goblet cells; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4,>75%). The index was calculated by dividing the sum of the airway scores from each lung by the number of airways examined for the histological goblet cell score.

Proliferation Assays and Cytokine Production

Thoracic lymph nodes (TLN) were removed. Single cell suspensions were made using a cell strainer and $3x10^5$ cells were cultured in 200 ml of medium RPMI 1640 supplemented with 20% FBS (GIBCO), 1% antibiotics (GIBCO) and $5x10^{-5}$ M 2-mercaptoethanol alone or in the presence of OVA (200 mg/ml) (grade V, Sigma-Aldrich) or concanavalin A (ConA) (5 mg/ml) (Sigma-Aldrich). Cytokine production was measured in supernatants at 72 h by capture ELISA commercial kits (IL-10: BioLegend ELISA MAX™ kit and TGF-β: Pharmingen, BD Bioscience OptEIA™ kit). ConA stimulated supernatants were harvested at 48 h. Proliferative responses of TLN cells cultured with medium or OVA (concentrations as above) were determined after addition of methyl-3Hthymidine (1 mCi/well, PerkinElmer, Argentina) for the last 18 h of a 5 days culture period. To evaluate *in vitro* suppression activity, TLN cells from allergic ($2.5x10^5$/well) mice were co-cultured with TLN cells from TO, T, or naive ($2.5x10^5$/well) mice and stimulated with OVA or medium. To obtain cell supernatant (CS), TO, T and naïve TLN cells ($2.5x10^5$) were stimulated for 4 days with OVA and medium alone or 2 days with ConA and medium alone. This CS was

next added to TLN cells from allergic mice ($2.5x10^5$), stimulated with OVA (200 mg/ml) and cultured for 5 days. Incorporated radioactivity was measured in a liquid scintillation beta-counter (Beckman). From the row data obtained, an index was calculated as cpm incorporated by stimulated cells over those cultured with medium.

Adoptive Transfer Experiments

TLN were removed from TO, T, and naïve mice. Single-cell suspensions in RPMI–3% FBS were made using a cell strainer. $5x10^6$ cells were injected i.v. in PBS. Receptor mice received TLN cells 7 days after a second OVA ip sensitization. Twenty four h later, mice were exposed to aerosols of allergen, 3% (w/v) OVA in PBS, for 10 min on 3 consecutive days.

Flow Cytometry Analysis

Intracellular staining of Foxp3 was performed using phycoerythrin- conjugated anti-Foxp3 mAb and the Foxp3 staining buffer set (Pharmingen, BD Biosciences) according to the manufacturer's protocol. Fluorescein isothiocyanate-conjugated anti-CD4 mAb (clone RM4-5) from Pharmingen, BD was used. Cells were acquired on a FACScan cytometer (Becton Dickinson, Mountain View, CA). Data were analyzed by using WinMDI 2.9 software.

Statistical Analysis

Each experimental group had at least four mice and each experiment was repeated at least 4 times. Data are presented as mean ± SEM. Statistical analysis was performed using ANOVA analysis of differences among groups with Bonferroni test *a posteriori* as indicated in the figure legends. Statistical analysis for semi-quantitative scoring was done using Kruskal Wallis with Dunn's test a posteriori. Statistical significance was accepted when $p<0.05$.

Results

Thoracic lymph node cells from acute *T. gondii* infected mice diminished allergic lung inflammation

Previously we showed that acute *T. gondii* infection can induce a deviation to a Th1 immune response detected as increased IFN-γ levels in thoracic lymph node (TLN) cells and brochoalveolar lavage and IgG2a antibodies (Fenoy et al., 2008). These data let us hypothesize that immune deviation may be responsible for allergy protection when sensitizing in acute phase. In order to further study the mechanism underlying the protective effect of acute *T. gondii* infection on allergic airway inflammation and to investigate whether regulatory mechanism would also be involved adoptive transfer studies were performed to evaluate whether TLN cells from mice sensitized during acute *T. gondii* infection (TO) could suppress an allergic lung inflammation. TLN cells from this group were *iv* inoculated in mice that had received two OVA/Al *ip* injections. The control group included OVA sensitized mice transferred with TLN cells from naïve (N) and from non-sensitized *T. gondii* infected mice (T). One day later, receptor mice were aerosol challenged with the allergen for three consecutive days (Figure 1 A). Animals transferred with TLN cells from TO mice showed a decrease in total BAL cells compared with those transferred with naïve cells, interestingly animals transferred with TLN cells from T mice also showed a trend toward less BAL cells (Figure 1 B). The differential count after allergen challenge showed a substantial decline in BAL eosinophilia in mice receiving TLN cells from TO mice. Interestingly, transferring cells from non-sensitized infected animals (T) also induced a strong reduction in BAL eosinophils compared to those receiving naïve TLN cells (Figure

1 C). Haematoxylin and PAS lung-stained sections were analyzed to evaluate whether diminished BAL eosinophilia correlated with reduced lung pathology. Mice transferred with naïve TLN cells showed characteristic pathological changes of pulmonary allergic inflammation: inflammatory cell infiltration around airway and vessels, and goblet cell hyperplasia (Figure 2 A). While no significant differences were observed in peribronchial and perivascular infiltrates, a reduction in mucus production was detected in both groups of animals transferred with cells from TO and T mice compared to those transferred with naïve cells (Figure 2 A). The results of semiquantitative scoring of histology support the qualitative changes described (Figure 2 B and C).

Suppressive activity by thoracic lymph node cells from acute *T. gondii* infected and sensitized mice

Given the above findings, we then moved to *ex vivo* studies to evaluate whether acute *T. gondii* infection could modulate allergen specific T cell proliferation. Two days after allergen challenge, proliferation of TLN cells was assayed by [^{3}H]thymidine incorporation during *in vitro* culture in the presence of OVA. Sensitization during acute phase of *T. gondii* infection resulted in a decrease in antigen-specific T cell proliferation compared to allergic mice (Figure 3 A).

These results along with the adoptive transference experiments strongly suggested the presence of regulatory cells. Hence, we next studied the ability of TLN cells from *T. gondii* infected (T) or *T. gondii* infected and OVA sensitized (TO) mice to *in vitro* suppress

T cell proliferation. TLN cells from allergic mice were co-cultured with TLN cells from T, TO or naive mice and stimulated with OVA. T cell proliferation from allergic mice was significantly diminished when co-cultured with TLN cells from *T. gondii* infected plus OVA sensitized mice (TO). TLN cells from normal mice did not affect proliferation levels (Figure 3 B). Noteworthy, similarly to that observed in the *ex vivo* proliferation assay, a suppressor activity was also observed when cells were co-cultured with TLN cells from *T. gondii* infected mice (T). These results suggest that TLN cells from both *T. gondii* infected plus OVA sensitized (TO) and from acute *T. gondii* infected non sensitized (T) animals have a suppressor activity on allergen-specific T cell proliferation. To assess whether the suppressor activity of TLN cells from TO and T mice was cell-cell contact dependent, conditioned medium from cultures from TO or T TLN cells stimulated for 4 days with OVA was added to TLN cells from allergic mice. Supernatant from TO TLN cells was able to diminish allergic TLN cells proliferation against OVA (Figure 3 C). However, no decrease was observed when TLN cells from allergic mice were incubated with supernatant from non-sensitized *T. gondii* infected (T) or naïve animals. To rule out the possibility that the *in vitro* OVA stimulation of TLN cells from infected animals (T) do not result in a successful activation of regulatory cells and, for this reason do not release the soluble mediator involved in the suppression, we next prepared the conditioned medium by stimulating with ConA. This supernatant was added to TLN cells from allergic animals and the culture was stimulated with ConA. As can be observed in Figure 3 D significantly reduced proliferative levels were detected when regulating with conditioned medium not

only from TO TLN cells but also when cells from allergic mice were cultivated with supernatant from non-sensitized *T. gondii* infected mice (T) TLN cells.

Analysis of Treg $CD4^+FoxP3^+$ cells

Many studies show $CD4^+FoxP3^+$ Tregs as central players in the immunoregulatory network and have highlighted a functional role of in controlling allergic Th2 cell responses (Robinson et al., 2009; Lloyd et al., 2009). To assess whether the allergen specific suppressor activity observed in thoracic lymph nodes from acute *T. gondii* infected OVA sensitised group (TO) correlates with the presence of $CD4^+FoxP3^+$ cells, we evaluated the percentage of this population in all experimental groups. Figure 4 shows that Treg cell frequency within the $CD4^+$ population in TLN cells are higher in both acute *T. gondii* infected mice, OVA sensitized (TO) and non-sensitized (T), compared with the allergic and naïve animals.

Production of local regulatory cytokines

We showed above that TLN cells from acute *T. gondii* infected and sensitized mice (TO) are able to suppress an allergic lung inflammation and also *in vitro* allergen-specific proliferation. In order to study whether the soluble mediator involved in suppression correlated with higher levels of regulatory cytokines, we analyzed IL-10 and TGF-β cytokines in supernatants from *ex vitro* cultures. TLN cells from the different groups were *in vitro* stimulated with OVA or ConA. OVA stimulated TLN cells from allergic mice

secreted high levels of IL-10 compared to naïve animals, while mice sensitized during acute phase of infection showed a marked decreased in the production of this cytokine. The same tendency was detected when cells were stimulated with ConA (Figure 5 A). Thus, IL-10 production behaved in the same way as the Th2 cytokines previously measured (Fenoy et al., 2008). No significant differences were observed in allergen specific TGF-β production between the TO mice and the other groups when *in vitro* stimulated either with OVA or ConA (Figure 5 B).

Allergy protection induced by acute *T. gondii* infection is not mediated by IL-10

The lower levels of IL-10 observed in supernatants from acute OVA sensitized TLN cells stimulated with OVA compared to TLN cells from allergic animals suggest that this cytokine would not be participating in the allergic protection induced by *T. gondii* infection. However, and considering previous results showing increased levels of this cytokine in BAL from *T. gondii* infected mice respect to normal or allergic animals [8], IL-10-deficient mice were employed to corroborate whether IL-10 was mediating the protection from allergic inflammation in mice sensitized during acute infection. Examination of pulmonary inflammation yielded results similar to those seen in wild type mice (Fenoy et al., 2008). Allergic sensitization during the acute stage of *T. gondii* infection resulted in a strong reduction of BAL airway eosinophilia (Figure 6 A). Moreover, TO mice lung histopathology exhibited significantly less peribronchiolar and perivascular inflammation and reduced goblet cell hyperplasia than allergic animals (Figure 6 B). As previously observed in mice sensitized during chronic infection, these results suggest that

T. gondii-induced protection from allergy is not mediated by IL-10 also when sensitization occurs during acute phase of infection.

Discussion

Our thinking of how infectious diseases can be linked to atopic disease can be tracked to the eighties when a link between decreased childhood infections and increased allergy in Western societies (Strachan, 1989) lead to the postulation of the original version of the hygiene hypothesis. This hypothesis allows an appealing synthesis of biologic data with epidemiologic observations that asthma has increased as 'hygiene' has improved (Strachan, 1989). However, the influence of infections on the onset and progression of allergic disease is still under exhaustive investigation in both epidemiological and experimental studies (Gilstrap et al., 2013).

Within the infections associated with the incidence of atopic disorders, epidemiological studies showed that respiratory allergies are less common in individuals exposed to orofecal microorganisms such as *Toxoplasma gondii* (Matricardi et al., 2000). This parasite is an obligate intracellular protozoan that, after an acute phase, sets in immunocompetent individuals an asymptomatic chronic infection (Gaddi et al., 2007). Infection with *T. gondii* induces a strong cellular immune response with a marked polarization towards a Th1 response (Gaddi et al., 2007). By using a well-known model of allergic lung inflammation we have previously demonstrated that both acute and chronic *T. gondii* infection can diminish the development of allergic airway inflammation (Fenoy et al., 2008). In agreement with our results, Wagner et al. showed that *T. gondii* infection prior to sensitization with Bet v 1 showed lower eosinophils and Th2 cytokines in BAL fluids and reduced Bet v 1-specific IgE antibodies along with elevated levels of rBet v 1-specific IgG2a compared to allergic mice (Wagner et al., 2009). Based on the high levels of IFN-γ

characteristic of the infection along with the reduction of the Th2 phenotype, we first hypothesized that the protective effect may be given by a deviation of the immune response towards a Th1 profile. In effect, a shift in allergen specific Ig isotypes from Th2 to Th1 and lower IL-4 and IL-5 with increased IFN-γ levels were detected in mice allergen sensitized during acute *T. gondii* infection (Fenoy et al., 2008; Wagner et al., 2009). This deviation in cytokine OVA specific secretion was not detected when sensitizing during the chronic stage (Fenoy et al., 2008). In line with this result we then demonstrated that when sensitizing in chronic infection, regulatory cells in thoracic lymph nodes could decreased allergen specific *ex vivo* T cell proliferation and could also ameliorate allergic lung inflammation after adoptive transference to allergen sensitized mice (Fenoy et al., 2012). To further study the mechanisms involved in *T. gondii* allergy protection we extended our studies to mice sensitized during acute *T. gondii* infection.

By using an adoptive cell transfer experiment, we observed that allergen sensitized mice that received TLN cells from infected plus sensitized (TO) mice showed decreased lung inflammation. This effect was also observed when the adoptive transfer was performed with cells from non-sensitized acute *T. gondii* infected animals (T). Suppression of *in vivo* allergy correlated with suppression of *ex vivo* allergen specific proliferation. These data indicated that the TLN cells not only from the TO group but also from non-sensitized infected animals (T) can suppress both lung inflammation and the proliferation of effector T cells from allergic mice. Moreover this effect seemed stronger than the one obtained in mice sensitized in chronic infection and particularly when comparing non-sensitized acute vs. chronic infected animals (Fenoy et al., 2012). These results show that,

similar to what we previously observed in chronic *T. gondii* infection (Fenoy et al., 2012), acute *T. gondii* infection induce regulatory cells in lung draining lymph nodes able to diminished an OVA induced allergic airway inflammation. To further study the mechanism of suppression we performed *ex vivo* proliferation experiments with conditioned medium from cultures from TO or T TLN cells stimulated with OVA. Only supernatant from TO TLN cells could suppress allergen specific proliferation. This suggests that regulatory cells in infected and sensitized mice (TO) are cell contact independent but the suppression exerted by non-sensitized infected TLN cells (T) is contact dependent. However, another interpretation could be that these latter regulatory cells were not *in vitro* appropriately stimulated. Hence, we performed the same experiment but instead of culturing with OVA, the conditioned medium was obtained after stimulation with ConA. With this protocol, not only TO TLN cells but also supernatant from non-sensitized infected TLN cells (T) could suppress T cell proliferation. Therefore, we can hypothesize that two regulatory cells populations would be involved in acute infected OVA sensitized mice (TO): one induced against the parasite and acting in a non antigen-specific way and the other would be allergen-specific and induced when sensitizing during acute *T. gondii* infection.

New evidence, product from research in murine models and humans, supports the possibility that regulatory T cells normally prevent and control the development of atopic disorders (Robinson et al., 2009; Lloyd et al., 2009). Adoptive transfer of $CD4^+CD25^+$ cells specific for the allergen prevented the development of allergic lung inflammation and airway hyperresponsiveness. This suppression was dependent on the induction of a $CD4^+$ population in the lung able to secrete IL-10 and different from the regulatory cells

transferred (Kearley et al., 2005). Interestingly, when the adoptive transference was performed after the establishment of the asthma phenotype, regulatory T cells were able to reverse the allergic inflammation and prevent airway remodeling (Kearley et al., 2008). Also, the elimination of the regulatory T cell $CD4^+CD25^+$ population previous to the sensitization resulted in an increase in the severity of inflammation and airway hyperresponsiveness (Lewkowich et al., 2005). Several lines of evidence also indicate that the function of $Foxp3^+$ Treg cells is altered in patients with allergies, particularly with asthma (Ryanna et al., 2009)

Characterization of the regulatory cells triggered during acute and chronic toxoplasmosis is still under study. Conventional T-bet^+ $FoxP3^-$ Th1 lymphocytes induced in spleen and peritoneum after i.p. *T. gondii* infection are capable of secreting biologically active IL-10 (Gaddi et al., 2007). However, though high levels of IL-10 were detected in BAL from *T. gondii* infected (T and TO) mice (Fenoy et al., 2008), no increases in IL-10 were detected in TLN cells from acute *T. gondii* infected and sensitized animals (TO). Moreover, IL-10 KO *T. gondii* infected BALB/c mice were protected at least as well as wild-type infected mice from OVA-induced asthma demonstrating that IL-10 would not be involved in allergy suppression. It was recently demonstrated that $CD4^+FoxP3^+$ T cells play an important role in the modulation of the protective immune response against *T. gondii* infection during acute phase (Tenorio et al., 2010; Morampudi et al., 2011). Depletion of Treg $FoxP3^+$ cells in orally infected BALB/c mice led to an increase in parasite burden and higher production of pro-inflammatory cytokines (Morampudi et al.,

2011). Recently, Tenorio et al showed in B6 mice that *T. gondii* infection induced an immunosuppression affecting $CD4^+$ and $CD8^+$ T-cell proliferation which correlates with a reduction in $CD4^+FoxP3^+$T cell number. Nevertheless, the residual Treg cells are activated and display an increased suppressive capacity. The numbers of IL-10-producing Treg cells also increased during infection, although the *in vitro* neutralization of this cytokine did not modify T-cell proliferation suggesting that, in agreement with our results, IL-10 does not mediate the Treg-mediated suppression (Tenorio et al., 2011).

Herein, the fact that increased percentage of $CD4^+FoxP3^+$ in the $CD4^+$ T cell subset was detected in both OVA sensitized and non-sensitized *T. gondii* infected groups suggests that this population could be mediating allergy protection. On the contrary, it can be argue that as it is widely accepted that $FoxP3^+$ T cells need to contact responder cells to exert its activity (Shevach et al., 2009), these regulatory cells would not be involved. On the other hand, our data argue against a suppression dependent of the TGF-β pathway since no differences in this cytokine levels were detected in the supernatant of *ex vivo* OVA or ConA stimulated TLN cells. Nevertheless, although TGF-β plays a critical role in the induction of $Foxp3^+$ Treg cells *in vivo* and *in vitro* and in Treg cell homeostasis, its role as a suppressor effector molecule remains controversial (Shevach et al., 2009). Therefore, FoxP3 Tregs could still be involved in *T. gondii* induced allergy suppression since *in vitro* models of cell function suggest that Treg cells may use multiple mechanisms to suppress immune responses (Shevach et al., 2009).

As we have already mentioned, TLN cells from non sensitized *T. gondii* infected mice could suppress *in vitro* cell proliferation and *in vivo* allergic lung inflammation. The fact that cells from infected mice can suppress an allergen specific response is not surprising. Previous studies in mouse models of allergic lung inflammation also showed this non-specific effect (Wilson et al., 2005; Layland et al., 2013; van der Vlugt et al., 2012; Wilson et al., 2010). In line with this, different studies agree that although the different types of Treg cells are antigen-specific, they all exert suppressive activity in a non antigen-specific way (Romagnani et al., 2006). In fact, it has been shown that the regulatory cells do not need to be antigen-specific *in vivo* to suppress an allergic lung inflammation. The adoptive transfer of $CD4^+$ $CD25^+$ $Foxp3^+$ cells from *naive* mice (natural Tregs) reduced pulmonary allergic inflammation, decreased TLN cells IL-5 and IL-13secretion and the levels of IgE and IgG1 (Leech et al., 2007).

Studies with different types of infections show that in addition to $CD4^+$ regulatory T cells, other regulatory populations are induced by infections, and have a role in preventing the development of an asthma phenotype (van der Vlugt et al., 2012; Wilson et al., 2010; Marsland et al., 2004). Studies with influenza virus show that the infection induces a population of memory $CD8^+$ T cells residing in the lung able to prevent the development of an allergic lung inflammation (Marsland et al., 2004). More recently, work with the helminth *Schistosoma mansoni* and *Heligmosomoides polygyus* show that in response to infection regulatory B lymphocytes secreting IL-10 are induced in spleen and mediastinal lymph node respectively. The adoptive transfer of these cells prevented the development of allergic lung inflammation (Wilson et al., 2010; Romagnani et al., 2006).

Our results extend earlier work (Fenoy et al., 2008; Wagner et al., 2009) and show that acute *T. gondii* infection can suppress asthmatic airway inflammation through both immune deviation (Fenoy et al., 2008) and, as demonstrated in this study, through immune suppression. These results provide further experimental support for the hygiene hypothesis. Indeed, the current view of cellular and molecular mechanisms of this phenomenon includes changes in the balance of the Th1, Th2 and regulatory immune responses, which are triggered by a lack of activation or an altered activation of the innate immune system. Finally, investigations with defined experimental models are essential to dissect the mechanisms that are likely to occur in humans, and to exploit any molecular principle from microbes that may be beneficial in ameliorating and preventing different immunopathologies.

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Conflict of Interest

The authors have declared that no competing interests exist.

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Figure Legends

Figure 1: Thoracic lymph node cells from *T. gondii* infected mice diminished bronchoalveolar lavage infiltrate. A) Experimental design: TLN were removed from infected and sensitized (TO), only infected (T), and naïve (N) mice and injected iv in mice previously ip sensitized twice with OVA. Twenty four hours later, mice were exposed to aerosols of allergen on 3 consecutive days. B) Bronchoalveolar lavage was performed 48 h. after the last exposure to OVA. C) BAL differential cell counts were performed on cytocentrifuge slides, fixed and stained with a modified Wright-Giemsa stain. * $p<0.05$ and ***$p< 0.001$ vs N, ANOVA with Bonferroni's test *a posteriori*.

Figure 2: Thoracic lymph node cells from *T. gondii* infected mice ameliorate lung histopathology. A) After lavage, lungs were instilled and fixed with 10% buffered formalin. Following paraffin embedding, sections for microscopy were stained with Hematoxylin and PAS. Original magnification 200X B) An index of pathologic changes in H&E slides was obtained by scoring the inflammatory infiltrate around the airways and vessels for greatest severity (0, normal; 1, <4 cells diameter thick; 2, 4–10 cells diameter thick; 3, >10 cells diameter thick) and overall extent (0, normal; 1, <25% of sample; 2, 26–50%; 3, 51–75%; 4, >75%). The Index was calculated by multiplying severity by extent. C) An histological goblet cell score was obtained in Periodic acid- Schiff (PAS)-stained lung sections by examining 10 to 20 consecutive airways from all groups of mice at 40x magnification and categorized according to the abundance of PAS-positive goblet (0, <5%

goblet cells; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4, >75%). The Index was calculated by dividing the sum of the airway scores from each lung by the number of airways examined for the histological goblet cell score. *p<0.05 vs N, Kruskal-Wallis test with Dunn's test *a posteriori*.

Figure 3: Thoracic lymph node cells from *T. gondii* infected mice suppress T cell proliferation. Proliferative responses of thoracic lymph nodes cells from naïve (N), non-sensitized *T. gondii* infected (T), allergic (O) and infected/sensitized (TO) mice were determined by 3H-thymidine incorporation after a 5-day culture period upon stimulation by OVA (A). TLN cells from naïve (N), non-sensitized *T. gondii* infected (T) and infected/sensitized (TO) mice were *in vitro* co-cultured with thoracic lymph nodes cells from allergic (O) mice. Proliferative responses were determined by 3H-thymidine incorporation after a 5-day culture period upon OVA stimulation (B). TLNC cells from naïve N, T and TO mice were *ex vivo* cultured during 4-days upon OVA (C) or 2 days ConA (D) stimulation. Cell supernatants were collected and used to culture thoracic lymph nodes cells during 5 days upon OVA stimulation or 2 days upon ConA stimulation. Proliferative responses were determined by 3H-thymidine incorporation. Results are expressed as an Index (incorporation by cells stimulated over those cultured with medium alone). a p<0.01 vs N, T and TO; b p<0.05 vs N; c p<0.01 vs N; ANOVA with Bonferroni's test *a posteriori.*

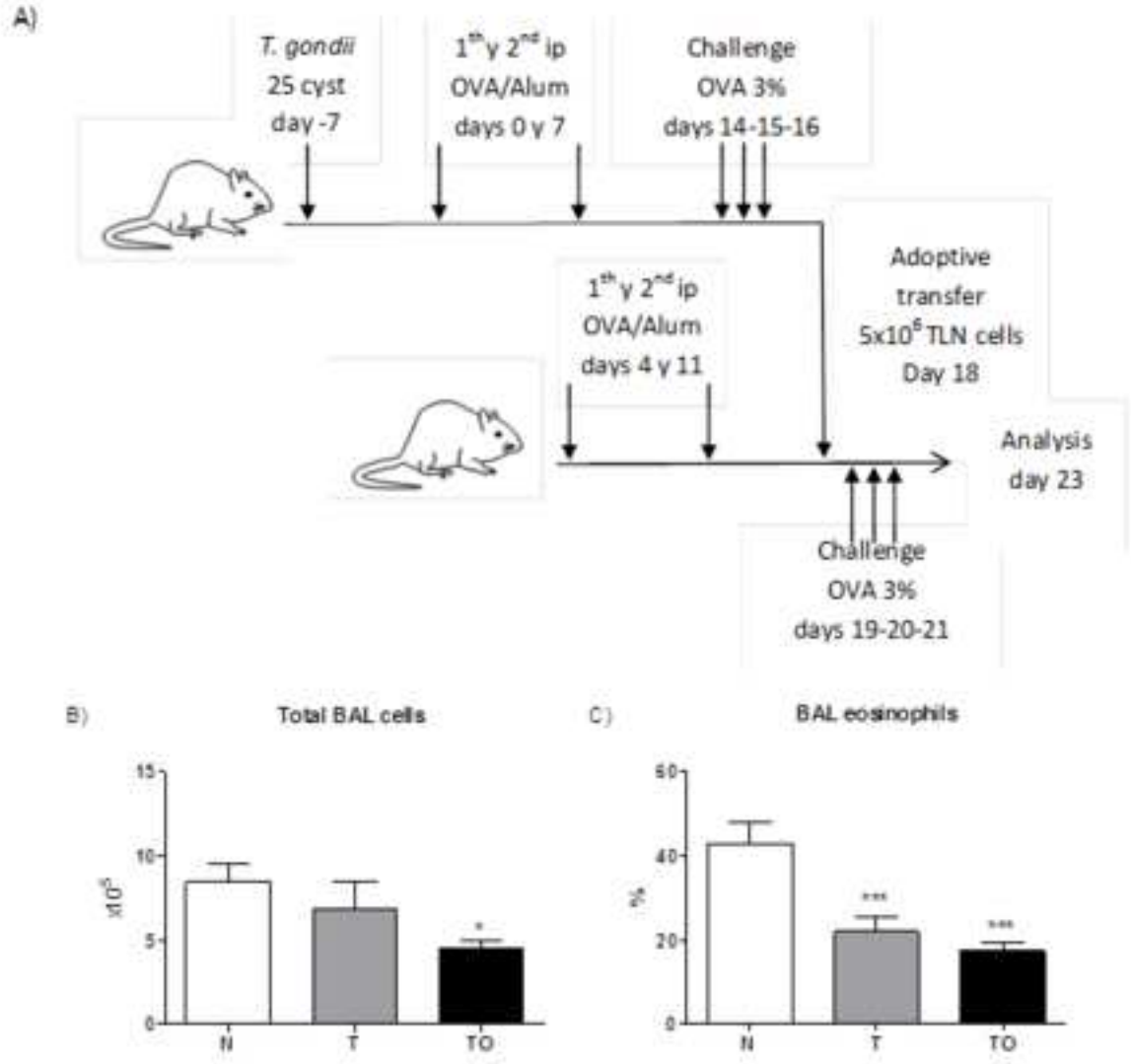
Figure 4: Expansion of T CD4$^+$Foxp3$^+$ regulatory cells. Flow cytometry analysis of TLN cells from naïve (N), *T. gondii* infected (T), allergic (O) and *T. gondii* infected/OVA sensitized mice (TO) stained with anti-CD4 and Foxp3. Representative dot blots from each group (A) and bar charts showing percentage of TLN cells expressing Foxp3 on CD4$^+$ subset (B). *p<0.05, **p<0.01 vs O; ANOVA with Bonferroni's test *a posteriori*.

Figure 5: Local production of regulatory cytokines. Cytokine production by TLNC cells cultured *ex vivo* with OVA or ConA were measured in OVA-sensitized (O), *T. gondii* infected/OVA sensitized (TO) (both groups aerosolized with OVA), *T. gondii* infected (T) or naïve (N) mice. **p<0.05 vs N, T and TO; ANOVA with Bonferroni's test *a posteriori*.

Figure 6: Reduced allergic lung inflammation in IL-10 deficient mice. Mice were OVA sensitized during acute *T. gondii* infection. A) BAL was performed 48 h after the last exposure to OVA. Differential cell counts were performed on cytocentrifuge slides, fixed and stained with a modified Wright–Giemsa stain. Semiquantitative analysis of histopathological changes. After lavage, lungs were instilled and fixed with 10% buffered formalin. Following paraffin embedding, sections for microscopy were stained with H&E and PAS.B) An index of pathologic changes in H&E slides was obtained by scoring the inflammatory infiltrate around the airways and vessels for greatest severity (0, normal; 1, <4 cells diameter thick; 2, 4–10 cells diameter thick; 3, >10 cells diameter thick) and overall extent (0, normal; 1, <25% of sample; 2, 26–50%; 3, 51–75%; 4, >75%). The Index

was calculated by multiplying severity by extent. An histological goblet cell score was obtained in Periodic acid-Schiff (PAS)-stained lung sections by examining 10 to 20 consecutive airways from all groups of mice at 40x magnification and categorized according to the abundance of PAS positive goblet (0, <5% goblet cells; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4, >75%). The Index was calculated by dividing the sum of the airway scores from each lung by the number of airways examined for the histological goblet cell score. *p<0.05 vs N, Kruskal-Wallis test with Dunn's test *a posteriori*.

Figure 1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OVA/Alum
days 0 y 7
Challenge
OVA 3%
days 14-15-16
Adoptive
transfer
5x10^6 TLN cells
Day 18
1th y 2nd ip
OVA/Alum
days 4 y 11
Analysis
day 23
Challenge
OVA 3%
days 19-20-21
B)
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C)
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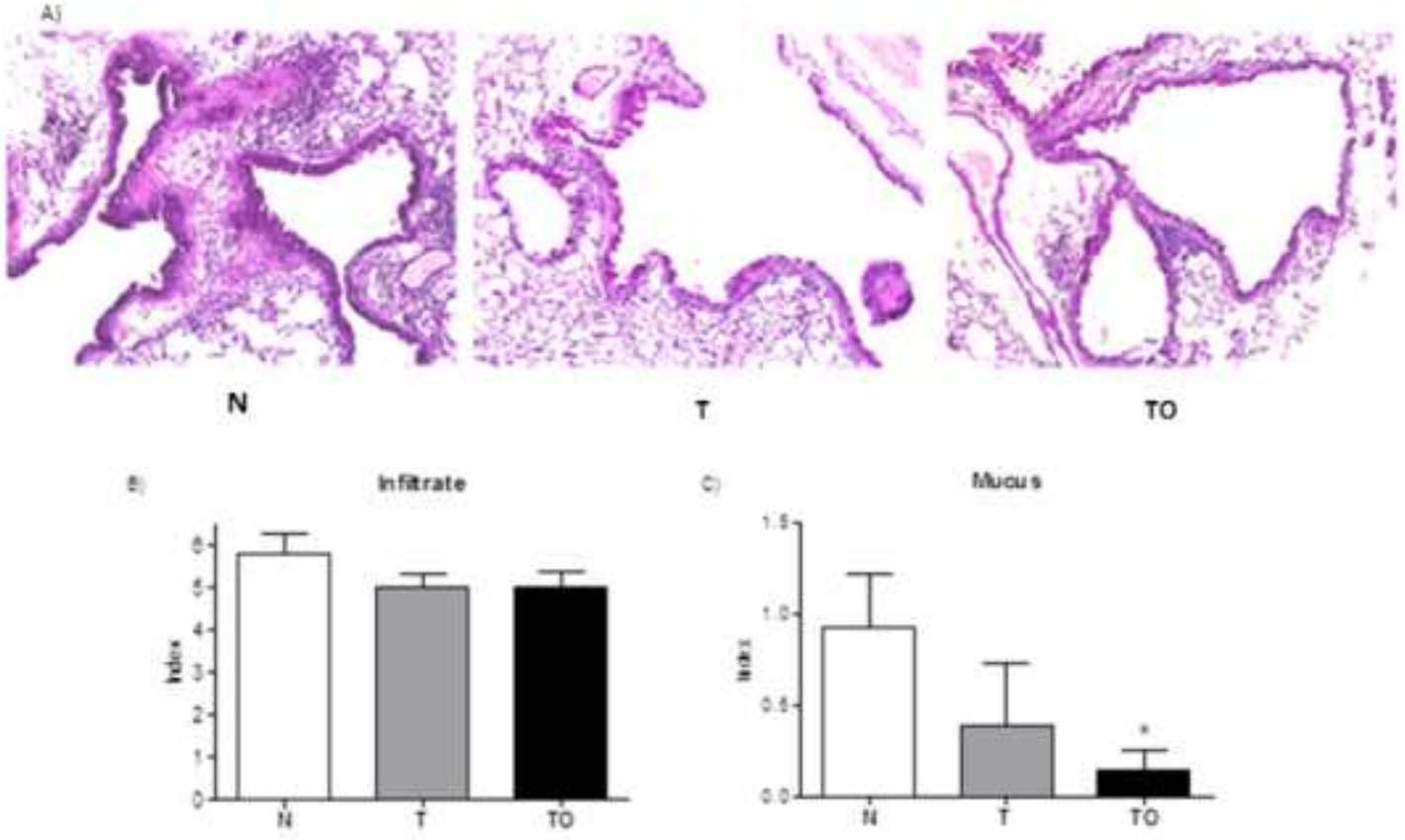
Figure 2
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Mucus
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Figure 3

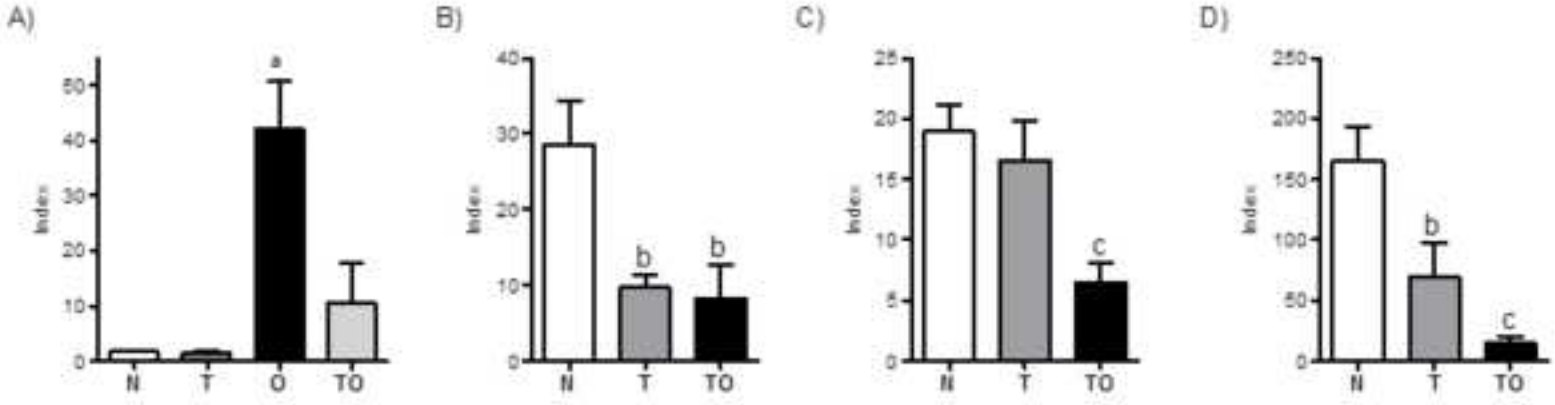

Figure 4

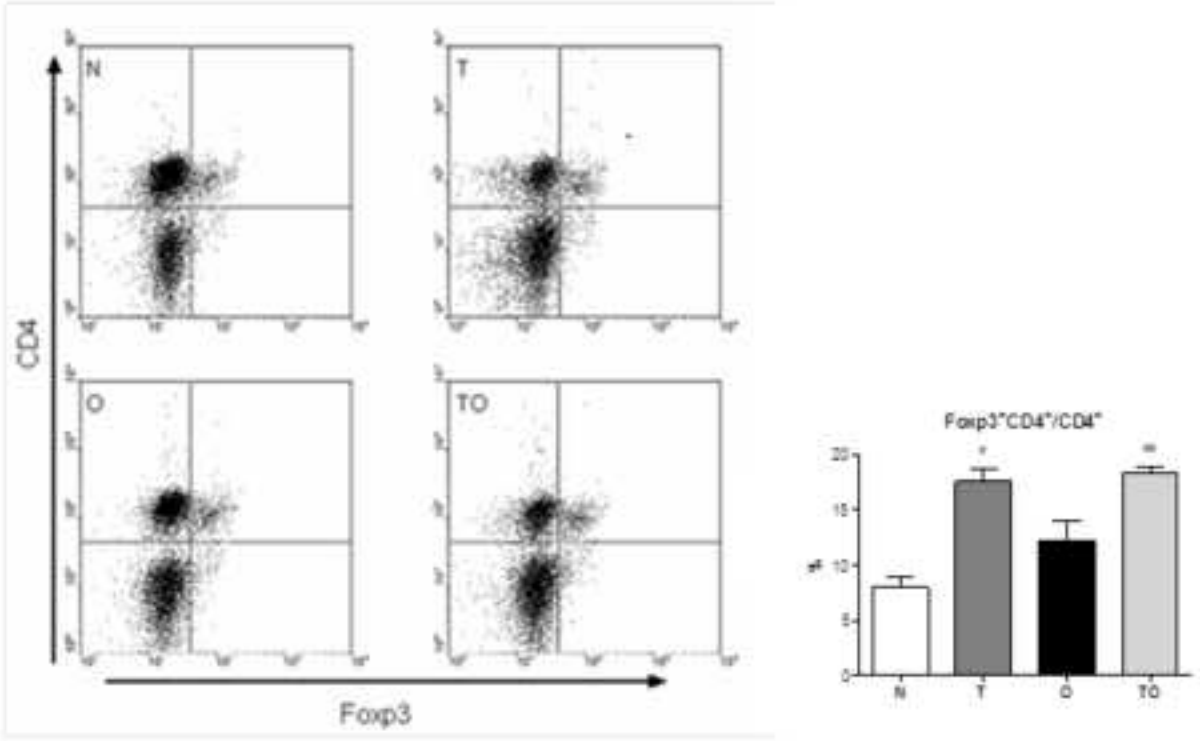

Figure 5

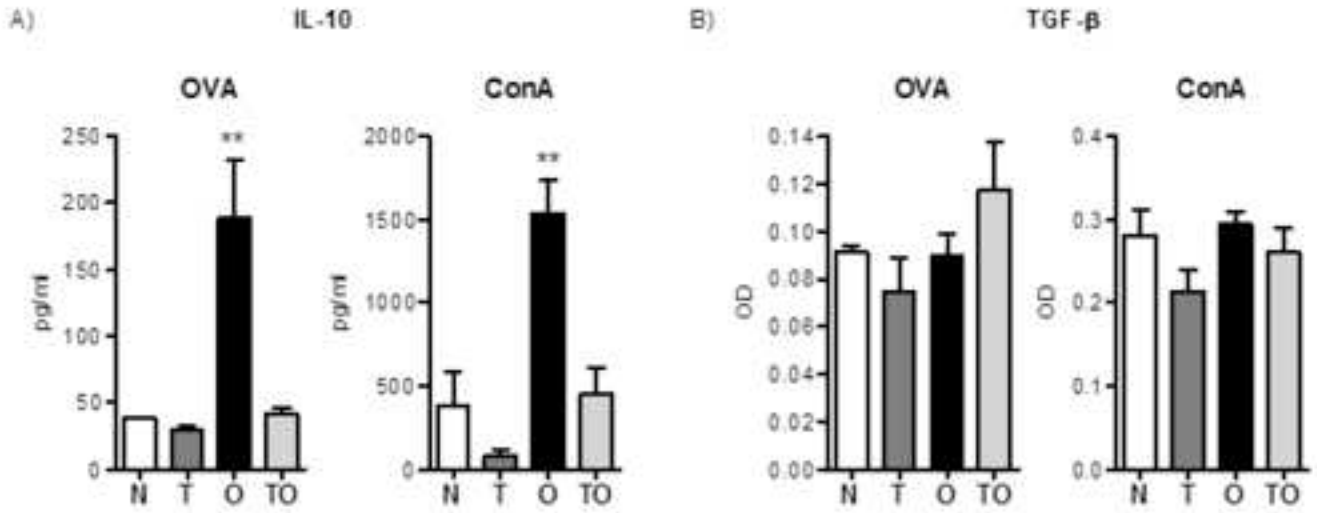
A)
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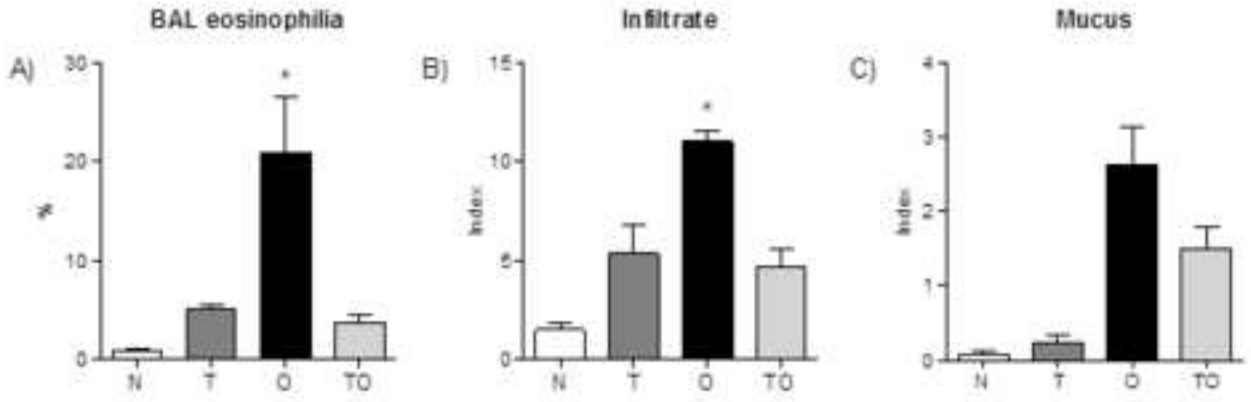
Figure 6


BAL eosinophilia
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