

What should be taken into account during study on immunoregulatory effects of helminths: a critical analyzing on "Downregulation of immune responses in asthmatic patients by ES products of *Marshallagia marshalli*"

Dear Editor,

We read with great interest the article by Jabbari Azad *et al.* entitled, 'Downregulation of immune responses in asthmatic humans by ES products of *Marshallagia marshalli*,' which was recently published in *The Clinical Respiratory Journal* (CRJ) (1). Their findings are intriguing in that they showed excretory-secretory (ES) antigens of *M. marshalli* are able to alter cytokines expression in peripheral blood mononuclear cells (PBMCs), but we have some concerns about the interpretation of the results.

First, in most investigations aimed to study helminth-derived antigens, it is an important issue that ES products used in such an experimental model, should be lipopolysaccharide (LPS) free (2, 3). Thus prior to study on immunoregulatory effects of helminth-derived antigens, LPS content in ES should be determined by endotoxin detection assays such as Limulus Amebocyte Lysate test. The endotoxin quantity needs to be under the sensitivity level of cell stimulation (<0.4 U/mg protein). In high levels of LPS contamination, it requires to be removed by commercial kits. In this study, ES products have been extracted from worms living in abomasum of ruminants that is full of gram-negative bacteria, thus, undoubtedly ES products are contaminated with LPS. Moreover, the level of LPS contamination has not been determined. In fact, LPS contamination can significantly influence the cytokines gene expression and Th1/Th2 balance. Thus, we believe the results concerning the expression of cytokines mRNA in PBMCs can significantly be affected by LPS contaminations of ES products. Therefore, without addressing this issue in the discussion part of the article, the article lacks enough evidence to make such a conclusion.

Second, there are no data and graphs concerning the *in vitro* proliferation assay and 50% lethal concentration (LC₅₀) value of (ES) antigen of *M. marshalli*. Another discrepancy is mentioning only GAPDH as

housekeeping gene in Material and Methods, but showing also B2M as housekeeping gene in Table 1.

Third, to provide more reliable results the level of IL-4, IL-10, TGF- β , and IFN- γ expression in PBMCs of asthmatic patients should have been evaluated before and after exposure of PBMCs to ES. There are no data concerning the levels of cytokine expression in PBMCs of asthmatic patients before exposure to ES. Thus, it is still unknown whether alteration in gene expression is because of the exposure to ES or not. Comparing the levels of gene expression in PBMCs of asthmatic patients before and after exposure to ES can provide meaningful results. In other words, we think absence a negative control in asthmatic patients group is a flawed. It would have been better that PBMCs of asthmatic patients were divided into two groups. One group could have been exposed to ES and another group could have been exposed to PBS or culture medium in which *M. marshalli* were cultured for ES extraction.

Fourth, there is no information concerning the methods by which asthmatic patients and healthy persons have been distinguished. Importantly, it needs to be mentioned how patients with moderate chronic asthma differentiate from patients with severe chronic asthma. More evidence concerning the diagnostic methods used could allow readers to assess whether they were appropriately and correctly selected.

In the following, we express comments that believe would be useful for discussion of this article.

First, given the increased expression of IL-10 and TGF- β in PBMCs of asthmatic patients, the authors have proposed that ES products are able to enhance Treg cells. Thus, in discussion, the authors have focused on the mechanisms by which Tregs suppress asthma. But, there are publications that report the percentage of CD4⁺FoxP3 Tregs in the BAL of patients with moderate to severe asthma is more than both mild asthmatic patients and healthy subjects (4). Indeed, the number of cells displaying regulatory capacity, either through FoxP3 expression or CTLA4 expression, is increased in moderate to severe asthma (4). Also, increase of Tregs in asthmatic patients

Conflict of interest

The authors declare no conflicts of interest.

because ES treatment does not absolutely provide protection against asthma development. In fact, it should be taken into account that helminths exploit various mechanisms to regulate host immune responses and some mechanisms might be Treg-independent.

Second, it has been shown that corticosteroids can increase Treg population and immunoregulatory cytokines in asthmatic patients (5, 6). In addition, corticosteroids are able to increase Tregs suppressive capacity in subsequent allergen-stimulated cultures, in part through an increase in IL-10 production (7). Given the fact that corticosteroids are widely used by asthmatic patients, in this study, it should have been determined whether elevated expression of IL-10 and TGF- β mRNA in PBMCs arises from the use of corticosteroids or ES treatment. This flaw is because of the lack of negative control in PBMCs of asthmatic patients which was discussed earlier.

Third, in this study, only the expression at mRNA level of four genes has been evaluated, and we think this does not provide solid evidence to propose that ES products of this worm can downregulate immune responses in asthmatic patients. Cytokine mRNA level does not usually predict its protein level (8). So, other assays such as enzyme linked immunosorbent assay (ELISA) and flow cytometry, which detect proteins instead of mRNA, would provide more reliable data to opine on regulatory effects of *M. marshalli*.

Amin Zakeri and Alireza Haghparast
Immunology Section, Department of Pathobiology,
Faculty of Veterinary Medicine, Ferdowsi University of
Mashhad, Mashhad, Iran

References

1. Jabbari Azad F, Kiaee F, Rezaei A, Farid Hosseini R, Soleimani N, Borji H. Downregulation of immune responses in asthmatic humans by ES products of *Marshallagia marshalli*. Clin Respir J. 2015; doi: 10.1111/crj.12309.
2. McSorley HJ, Blair NE, Smith KA, McKenzie AN, Maizels RM. Blockade of IL-33 release and suppression of type 2 innate lymphoid cell responses by helminth secreted products in airway allergy. Mucosal Immunol. 2014;7(5): 1068–78.
3. Ebner F, Hepworth M, Rausch S, et al. Therapeutic potential of larval excretory/secretory proteins of the pig whipworm *Trichuris suis* in allergic disease. Allergy 2014; 69(11): 1489–97.
4. Smyth LJ, Eustace A, Kolsum U, Blaikely J, Singh D. Increased airway T regulatory cells in asthmatic subjects. CHEST J 2010;138(4): 905–12.
5. Singh AM, Dahlberg P, Burmeister K, et al. Inhaled corticosteroid use is associated with increased circulating T regulatory cells in children with asthma. Clin Mol Allergy 2013;11(1): 1.
6. Barrat, FJ, Cua DJ, Boonstra A, et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J Exp Med. 2002;195: 603–616.
7. Robinson D, Nguyen D. Fluticasone propionate increases suppression of allergen-driven T cell proliferation by CD4+ CD25+ T cells. J Allergy Clin Immunol. 2004;113(2): S322.
8. Perrini S, Ficarella R, Picardi E, et al. Differences in gene expression and cytokine release profiles highlight the heterogeneity of distinct subsets of adipose tissue-derived stem cells in the subcutaneous and visceral adipose tissue in humans. PLoS One 2013;8(3): e57892.