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Chitin Micro Particles Regulate Splenocytes Immune Response in Experimental Autoimmune Encephalomyelitis

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ABSTRACT

Contrasting studies are reported on the induction of IL-10 and IFN-γ via chitin microparticles (CMPs) during immune stimulation. Our previous studies have shown marked protection among CMP treated *Leishmania*-infected mice via regulated IL-10/IFN-γ response, at the present study, once more, examined the inconsistent responses regarding the immunologic response of CMPs.

To verify whether CMPs could indeed up-regulate IL-10/IFN-γ axis, isolated spleen cells from the myelin oligodendrocyte glycoprotein (MOG) induced experimental autoimmune encephalomyelitis (EAE) mice were cultured in the presence of MOG peptide and/or CMPs. The effects of CMPs on IL-10, IFN-γ and IL-17 production were evaluated by Enzyme-linked Immunosorbent Assay (ELISA). Moreover, GATA binding protein 3 (Gata3), T-box transcription factor TBX21 (Tbx21), and RAR-related orphan receptor gamma (RORγT) expressions (real-time PCR) were investigated.

MOG alone stimulated the production of IFN-γ ($p \leq 0.004$) but not, IL-10 ($p \leq 0.140$). MOG/chitin stimulation resulted in a significant increase in IFN-γ and IL-10 levels, respectively; ($p \leq 0.004$ and $p \leq 0.003$) rather than MOG. Additionally, the expression of Tbx21 ($p \leq 0.001$), but not Gata3 ($p \leq 0.08$), was increased in the MOG/chitin-treated spleen cells. All in all, CMP supports Gata3 independent IL-10 production and promotes Tbx21 dependent IFN-γ induction.

These results, alongside our previous data, indicate that CMPs has particular adjuvant effects.

Keywords: Chitin; Cytokine; Experimental autoimmune encephalomyelitis; Immunomodulation; Transcription factor

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INTRODUCTION

The second most recognized natural polysaccharide after cellulose is chitin. This poly [β-(1-4)-N-acetyl-D-

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glucosamine] holds the potential for widespread applications in medicine because of its non-toxicity and biodegradability effects.[1] According to the data, chitin microparticles (CMPs) are known by TLR2, dectin-1, mannose receptor, FIBCD1, TLR-9, ficolin, and NOD2.[2] Therefore, several immune cell subsets could identify the chitin stimulant for redirecting immune response.

In fact, to investigate chitin immune-stimulating properties, chitin fractions are applied to different murine models, and various immunomodulatory capacities are identified ranging from anti-inflammatory[3,4] to pro-inflammatory.[3] Nevertheless, its immunomodulatory properties are not fully understood.

Small size CMPs (<40 μm diameter) were found to drive immunomodulatory effects through IL-10 reduction but IFN-γ induction among murine asthma model;[4] nonetheless, many researchers attained that CMPs induce anti-inflammatory IL-10 expression.[5,6] Mizoguchi et al demonstrated that CMPs up-regulates IFN-γ and IL-10 production in a murine colitis model.[7] Interestingly, Shibata et al showed that mouse splenic macrophages produce IL-10 when stimulated by CMPs,[8] and in sharp contrast, they demonstrated that CMPs could not induce an elevated IL-10 production in their in-vitro macrophage studies.[9] In contrary, there are different data on the induction of IL-10 by CMPs immune stimulation.[10]

Shibata et al considers that CMPs are IFN-γ inducer (T_H1 adjuvants) and could redirect T_H2 allergic responses.[4] However, Dasilva et al introduced chitin as a TLR-2 agonist to function as an effective multifaceted adjuvant for T_H2/T_H17 responses.[5] They demonstrated that chitin acts size-dependent and CMPs inducts IL-10 production. These researchers, together with some others, hypothesized that innate recognition of chitin by TLR2 conducts to the induction of T_H2/T_H17 cytokines. They believe that, the chitin with the release of pro-inflammatory cytokines triggers the secretion of chitinases. Therefore, large chitin particle digests and leads to the generation of CMPs that induce IL-10 secretion. The IL-10, as an anti-inflammatory cytokine, dampens the T_H2/T_H17 response by down-regulating pro-inflammatory cytokine production. They do not believe in the redirection of T_H2 responses via IFN-γ production by CMPs.[11] Therefore, exact regulatory mechanisms of IFN-γ and IL-10, which are controlled by the chitin treatment, remain to be elucidated.

Recently, we reported that injection of CMPs in a murine model of leishmaniasis induced IL-10 and IFN-γ production, which supports marked protection oppose to leishmaniasis. We hypothesized that CMPs induced regulated response by the IL10/IFN-γ axis.[12,13] The present investigation assessed the potential of chitin to up-regulate IL10 and IFN-γ production, we utilized a mouse model of autoimmune encephalomyelitis elicited by MOG35-55 peptide. MOG35-55 induced EAE is an immune-mediated disease that is considered as an IFN-γ mediated and IL-10 suppressed disease.[14]

MATERIALS AND METHODS

CMPs Preparation

The production of fragments (<40 μm) of small size chitin was done according to the previously explained procedure.[13,15-17] In summary, pure chitin powder (C-7170, Sigma Chemical Co. St. Louis, MO) was milled; consequently, they were suspended in the 1X sterile phosphate buffered saline (PBS). The suspension was sonicated and filtered with 40 μm sterile cell strainers (Cell Strainer, BD Falcon, Mexico). Laser particle size analyzer (Malvern MasterSizer, Malvern Instruments, Ltd, Worcestershire, UK) was used to measure the size of particles. The CMPs suspension was monitored for endotoxin levels using a Limulus Amebocyte Lysate (LAL) assay (Cambrex, USA).

Ninety percent of the CMPs were smaller than 40 μm in size and the endotoxin level was less than 0.25EU/ml in the CMPs suspension.

Induction of EAE

By using the common method, EAE was induced in female C57BL/6 mice.[18,19] Briefly, 200 μg of myelin oligodendrocyte glycoprotein peptide (MOG35–55; KJ Ross-Petersen ApS, Copenhagen, Denmark) was emulsified with the same volume of complete Freund's adjuvant (CFA; Sigma, F5881, USA) comprising 500 μg of heat-killed *Mycobacterium tuberculosis* (100 μl total volume); consequently, the subcutaneously emulsion was injected in both hind flanks of the mice. The animals as well received intraperitoneal injections of pertussis toxin (300 ng in 100 μL PBS; List Biological Lab, Campbell, CA, USA) when immunization occurred. Then, 48 h later the same persuader was done. Demyelination of the CNS in EAE drives clinical disease symptoms, which is characterized by a grading scale of ascending paralysis.

Monitoring the clinical EAE disease scores were performed by using the grading scale as follows: 1) loss of tail tonicity; 2) mild hind limb weakness; 3) partial hind limb paralysis; 4) complete hind limb paralysis; 5) complete limb paralysis with the moribund.[20,21]

The present research is approved by the ethical committee of the Shahid Beheshti University of Medical Sciences, (Ethical Code: IR.SBMU.MSP.REC.1395.336).

Histological Assessment of the Spinal Cord

To verify EAE induction, 25 days post-induction, mice were humanely sacrificed; the entire spinal cord was removed[21] and then axonal loss and inflammatory cell infiltration were identified using routine luxol fast blue (LFB) and hematoxylin and eosin (H&E) techniques with some modifications according to newcomer-supply protocol.

Cytokine Assessment

Spleen cells were taken from MOG-induced EAE animals; accordingly, the cells were prepared from the spleen by grinding on 70 μm sterile cell strainers (Cell Strainer, BD Falcon, Mexico) in complete RPMI 1640 medium. They were treated with RBC lysis buffer (1.55 M NH4Cl, 0.1 M NaHCO3, 1 mM EDTA, pH 7.4.) for tow minute at room temperature. Then, the cells were washed twice using the complete RPMI 1640 medium. Cells were counted using the trypan-blue exclusion method and then using sterile 24-well flat-bottomed tissue culture plates. Splenocytes suspensions ($2*10^6$ cells/mL) from five individual mice were seeded in duplicate. The spleen cells were divided into four groups. The primary group was selected as the untreated control. The second group was treated by MOG (10μg/mL), the third group by CMPs (100μg/ml), and the fourth group by MOG-CMPs for 72 h at 37°C, 5% CO2. Then; we determined IL-10 (U-CYTech, Netherlands), IFN-γ, and IL-17 (Mabtech, Sweden) using ELISA in supernatants of spleen cell suspensions, according to the manufacturer's protocol.

Transcription Factor (Gata3, Tbx21, and RORγT) Expression

By using RNeasy Mini Kit (Qiagen, Germany), the total RNA was extracted from splenocytes. Single-stranded cDNA was made using the Primscript First Strand cDNA Synthesis Kit (TAKARA Japan), according to the manufacturer instructions.

The relative gene expression of each transcription factor was examined through real time-PCR amplification using respective specific primers set (Table 1).[22-25]

Thermal parameters applied for DNA amplification in real-time PCR machine were: 95°C for 30 s to activate Taq DNA polymerase followed by 40 cycles including denaturation at 94°C for 30 s, annealing 30 seconds at 53°C for Gata3, at 55°C for Tbx21, at 63.5°C for RORγT, and at 55°C for beta-actin. The extension was done at 72°C for 45 s.

Assessment of Culture Supernatant Nitrite Concentrations

Nitrite concentrations were determined according to Griss technique that was previously described elsewhere .[17] Briefly, samples and NaN02 standards were incubated with the Griess reagent (zistruyesh, Iran) in 96-well plates, gently shaken for 20 minutes at room temperature. Afterward, they were read in a microplate reader at 550 nm. Nitrite concentrations of experimental samples were read off the standard curve generated by NaNO2 absorbance. The assay is sensitive to about 3μM.

Statistics

Data were presented as the mean±standard deviation (SD). Data analysis with IBM SPSS Statistics for Windows, version 23 (IBM corp., Armonk, N.Y., USA) and drawing diagrams were done with Prism (GraphPad, version 5.0) software $p \leq 0.05$ were considered significant, n=5 (mice per each group).

The latest version of REST (Relative Expression Software Tool) was used to compare mRNA alteration.[26]

RESULTS

EAE Induction and Histological Data

The induction of EAE was done in C57BL/6 mice through immunization with an emulsion of MOG in CFA. EAE onset was 13 days after immunization, with the peak of disease 6 days after onset (Figure 1). Then, 25 days post-induction, the mice were humanely sacrificed and histological analysis was performed. Spinal cord white matter in EAE mice showed demyelinated areas into the LFB staining (Figure 2).

The inflammatory cell wasn't seen in the damaged area of H&E section. It seems that after the acute phase of the disease (25th day's post of induction) the inflammatory cells have left CNS.[27]

CMPs Regulate IL-10 and IFN-γ Production

In order to evaluate the hypothesis that chitin could act as an IL-10 inducer among spleen cell culture of mice sensitized with MOG, we assessed the results of MOG stimulation in comparison with CMPs plus MOG. Level of IL-10 in the culture of the cell from groups without CMPs were similar to each other (media (51.45±2.11 pg/mL) and MOG (56.46±4.42 pg/mL) p=0.14). Incubation of cells by CMPs, caused a rise in the amount of IL-10 secretion (chitin (2232.96±796.13 pg/mL) and MOG-CMPs (1398.56±399.52 pg/mL)). We found basal IL-10 production in the absence of CMPs stimulation. In other cases, IL-10 production

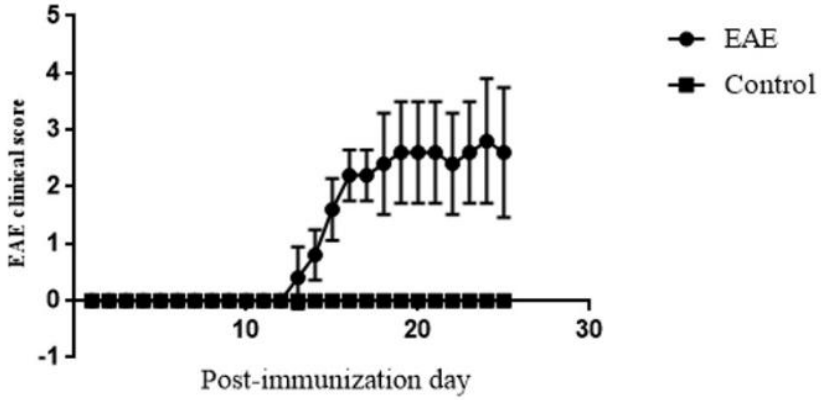

Figure 1. Representative experimental autoimmune encephalomyelitis (EAE) results in C57BL/6 female mice. Clinical signs were recorded daily. EAE onset was 13 days after immunization, with the peak of disease 6 days following the onset. The control group did not show any symptoms (n=5 for each group).

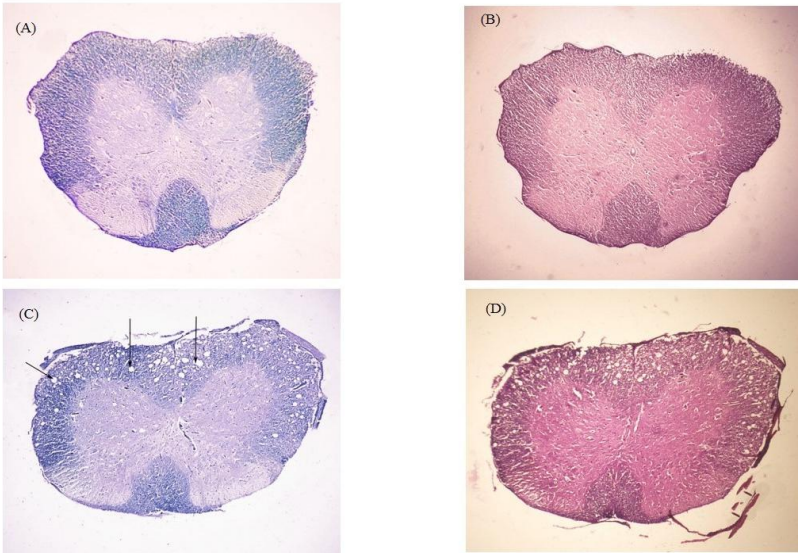

Figure 2.Histopathological examination of the EAE-developed spinal cordsLuxol Fast Blue (LFB) and Hematoxylin Eosin (H&E) Staining of the spinal cord from experimental autoimmune encephalomyelitis (EAE) mice. The figures show the normal and damaged spinal cord using two different staining methods (40 x magnifications). One representative of normal tissue staining by Luxol Fast Blue (LFB) (up-left) and Haemotoxylin and Eosin (H&E) (up-right) (A & B) besides representative tissue sections of spinal cord from mice at the 25th day of classic EAE stained with LFB (down-left) and H&E (down-right) were shown. Arrows indicated the lesions site(C). The demyelinated area was seen in LFB section.

was drastically enhanced by CMPs stimulation. The combination of chitin and MOG shifted the cytokine response to a more pronounced IL-10 response in comparison with MOG (p=0.003) (Figure 3A). This finding shows that chitin can act as an enhancer for IL-10 mediated responses.

In order to distinctly assess the immunomodulatory effect of CMPs, the IFN-γ levels were compared among different groups, as well. The IFN-γ levels in different groups significantly were increased in comparison with the media group. MOG (91.43±17.05 pg/mL) and CMPs (34.33±4.1 pg/mL) both served as IFN-γ inducers. The most increase in IFN-γ induction (p=0.004) was seen in MOG-CMPs group (678.66±118.11 pg/mL)(Figure 3B). These findings indicate that CMPs augment IFN-γ production and as an IL-10 regulator agent, did not down-regulate MOG-induced IFN- γ response.

The MOG immunization has been known to be involved in establishing a pathologic T_H17 response. As expected there was a significant difference (p=0.003) between media (30.7±2.82 pg/mL) and MOG (613.44±102.56 pg/mL) groups. We also analyzed the capability of chitin to increase or block IL-17 secretion. Co-incubation of MOG together with CMPs did not have any effect on the IL-17 level (MOG (613.44±102.56 pg/mL) and MOG-CMP_S (673.66±124.5 pg/mL) p=0.63) (Figure 3C).

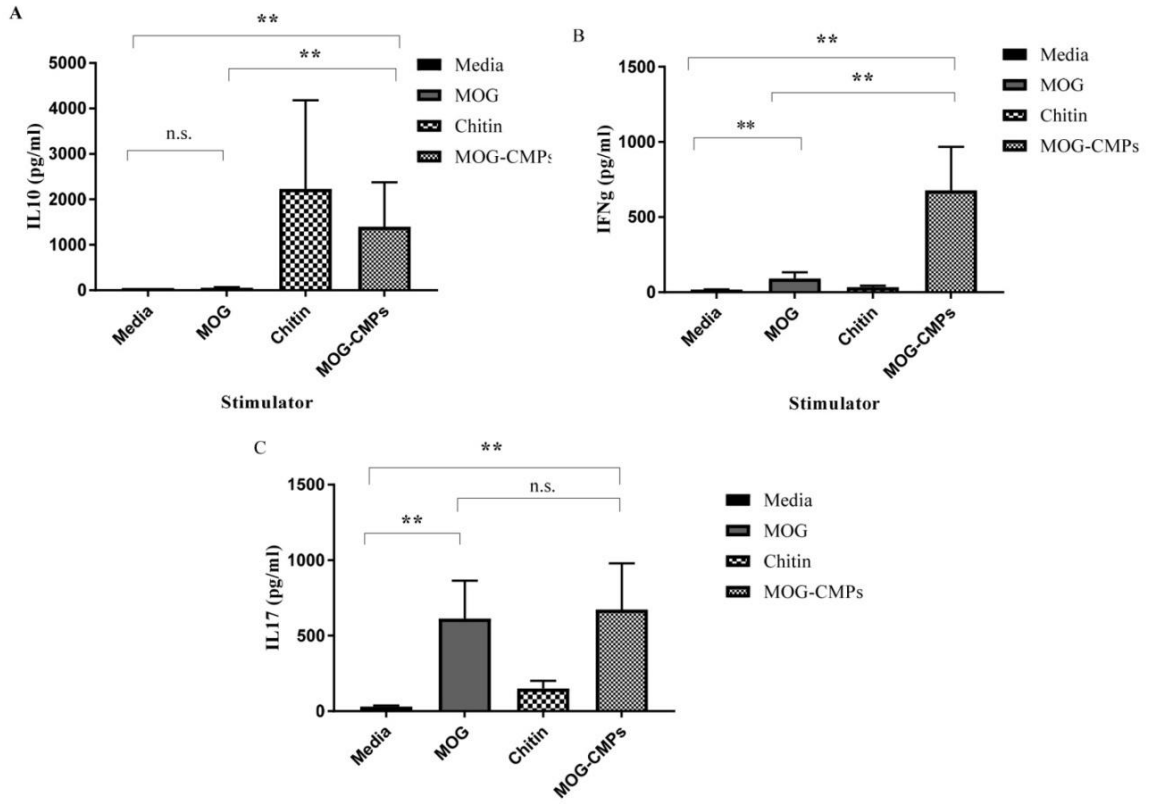

Figure 3. Cytokine production by spleen cell from C57 BL/6 mice treated with MOG, MOG-CMPs, and CMPs. The supernatants were isolated from spleen cell culture stimulated by MOG, MOG-CMPs, and CMPs. IL-10(A) , IFN-γ (B), and IL-17(C) levels were measured using ELISA. Mean± SD; *n*= 5. *$p \leq 0.05$.Incubation of murine spleen cells with chitin induces too much to be expected IL-10 secretion. MOG-CMPs resulted in much more IL-10 induction compared with MOG, $p \leq 0.003$(A). The highest level of IFN-γ was related to the MOG-CMPs stimulated cells. Ranks of the means were 21.5(MOG-Chitin)>15.5(MOG)>9.5(Chitin)>3.5(media) in Kruskal Wallis analysis ($p \leq 0.001$) (B). As expected a significant difference was present between media and MOG groups in IL-17 levels .There was no significant difference observed between MOG and MOG-CMPs in IL-17 levels. (C).

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Table 1. Gata3, RORγT, Tbx21 and Beta-actin primers used in representative experimental autoimmune encephalomyelitis (EAE) model

Gene	Reverse Seq.	Forward Seq.	Ref.
Gata3	5'-GGATACCTCTGCACCGTAGC-3 '	5'-CTCGGCCATTCGTACATGGAA-3'	25
ROR-γT	5'-AGTAGGCCACATTACACTGCT-3'	5'-GACCCACACCTCACAAAT TGA-3'	22
Tbx21	5'-GGAGTCTGGGTGGACATATAAGC-3'	5'-CCACAAGCCATTACAGGATGTT-3'	23
Beta-actin	5'-CCTAGCACCATGAAGATCAAGATCA-3'	5'-AAGCCATGCCAATGTTGTCTCT-3'	24

CMPs Didn't Increase Gata3 Expression

Previously, the involvement of various transcription factors in activation of the Il-10gene was indicated, including Gata3 (the T_H2 master regulator).[28] The production of IL-10 gets control by Gata3 in T_H2; however, the expression of Gata3 among T_H1 and T_H17 cells is not significant. To verify that T_H2 is not a cellular source of CMPs induced Il-10, the effects of CMPs treatment on the expression of Gata3 was evaluated, besides Tbx21 and RORγT. The analysis revealed that Gata3 and RORγT expression were not increased while Tbx21 overexpressed significantly ($p \leq 0.001$) by MOG-CMPs treatment in comparison with media. Furthermore, ROR- treatment in comparison wild ($p \leq 0.022$) among MOG stimulated group (Figure 4).

CMPs Induce Nitric oxide (NO) Production

Splenic cells are iNOS-expressing cells.[29,30] Our data showed that CMPs stimulation generated nitrite but no nitrite was generated if cells were stimulated with MOG instead (Figure 5). This finding raises the possibility that in the expansion of pathogenic MOG-reactive cells, NO production is blunted; however, in the presence of CMPs, iNOS-expressing cells were activated and generated NO (Figure 5).

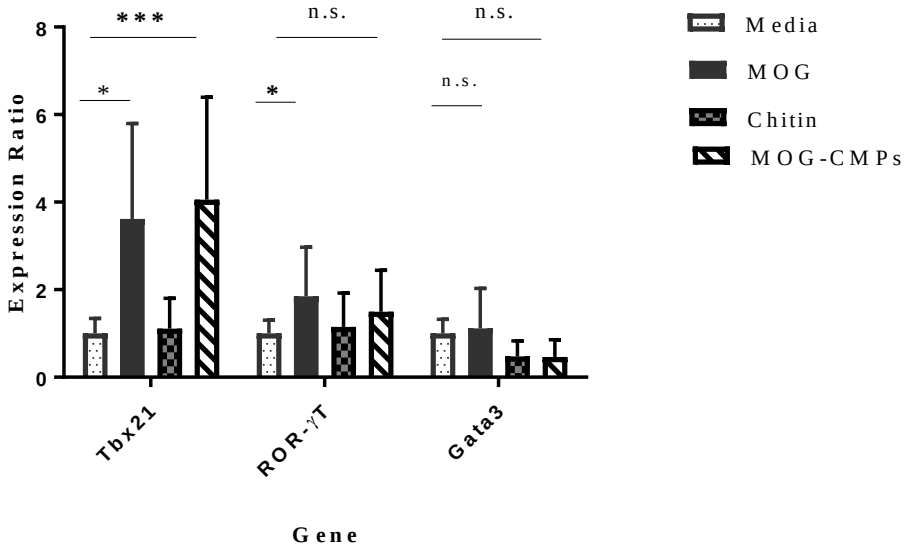

Figure 4. Comparison of the expression of Tbx21, RORγT and GATA3 Mrna between myelin oligodendrocyte glycoprotein (MOG), Chitin and myelin oligodendrocyte glycoprotein and chitin microparticles (MOG-CMP) treated. Spleen cells were exposed to the different stimulator and mRNA expressions of transcription factors in comparison to media group were evaluated by using real-time PCR. The fold raise in mRNAs were evaluated using REST method. MOG exposures do not upregulate Gata3 but RORγT & Tbx21 mRNA expression ($p<0.022$ and $p<0.05$ respectively) increased as expected. Tbx21 was up-regulated 4.060 (S.E. range is 2.340 - 6.510) times in MOG-CMPs group as well ($p<0.001$) but Gata3 & RORll ((S.E. range is 2.340 - 6.e in mRN Furthermore, when MOG treated group was considered as the control to calculate any differences in the expression of transcription factors in MOG-CMPs, the analysis revealed that Gata-3 expression was not increased, (data not shown).

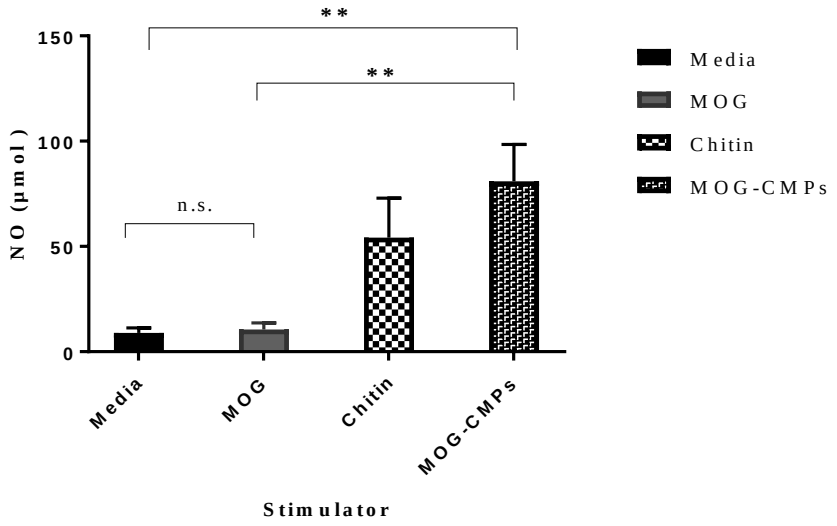

Figure 5. Nitric Oxide (NO) production by spleen cell from C57 BL/6 mice treated with MOG, MOG-CMPs, and CMPs. Chitin induces NO production by splenocytes from MOG-immunized mice. Spleen cells were cultured in the presence of MOG (10 µg/ml), chitin (100 µg/ml), or MOG mixed with chitin (MOG-chitin) for 3 days. The amount of NO in the supernatants was measured via the Griess reagent. NO production was significantly increased following the chitin treatment. There were significant differences between chitin (54.22±7.66 µM) and MOG+Chitin (80.90±7.15 µM) in comparison with MOG (10.52±1.30 µM) and Media (8.93±0.96 µM) in NO levels.

DISCUSSION

Unbridled activation of T_H1 and T_H17 happen in EAE C57BL/6 mice model.[31] In the current study, *in vitro* approaches were used to identify the capability of CMPs in the induction of IL-10 and regulation of the unbridle T_H1/T_H17 responses. In contrast with the former studies, which explained chitin as an immunologically inert particle[32,33] our results show that CMPs induce Gata3 independent IL-10 production and promote IFN-γ production striking finding in our study is that CMPs stimulated IL-10 in a representative of T_H1/T_H17 immune response model. The attained data suggested that the addition of CMPs under TH1/TH17 skewing conditions caused the initiation of IL-10 production. The obtained data is quite similar to the other studies.[7]

IL-10 is produced by a large array of cell types, containing B10 cells, NK cells, DCs, monocytes, macrophages and T cells.[28] Interestingly, T_H1 cells themselves were found to secrete IL-10. The cellular source of CMPs induced IL-10 and physiological relevance of these different IL-10 sources is uncertain and needed to be defined. Chitin could act as a polyclonal activator of B cells.[34] It might be that IL-10 source could be B10 cells.[35] CMPs induce IL-10 in a TLR2 and Dectin-1 dependent manner[7] so it might be that macrophages are a source of IL-10. However, more refined experimental systems are required to determine the cell type(s) responsible for the increased IL-10 productions. Although, it might be that some cell types in combination are required. It would be interesting to determine the cell type responsible for increased IFN-γ production under CMPs stimulation as well. All in all our data indicate that CMPs stimulation may lead to deviation of T_H cells esponse[36].

Chitin is an indispensable determinant of T_H2 cell-mediated responses. In the T_H2 cells, IL-10 is induced by the Gata3 as part of the T_H2 differentiation program.[37]However, appropriately sized chitin fragments (CMPs) have been shown to decrease type 2 responses[37] and CMPs stimulate IL-10 production to down-regulating T_H2 responses[6,38] or act as IFN-γ inducer (a T_H1 adjuvant) to inhibit T_H2 inflammation.[4,9] Our findings: IFN-γ/IL-10 production and Tbx21 but not Gata3 overexpression indicate CMP is the polyhedral adjuvant. Future investigations are required to analyze the complex transcriptional regulation of IL-10, but our work identified that Gata3 isn't an essential factor for IL-10 production by CMPs stimulation.

Murray et al found that overexpression of IL-10 does not inhibit but rather enhances IFN-γ secretion by stimulated spleen cells.[39]It has been shown that IL-10 can be produced by T_H1 cells without diminishing IFN-γ production and is indeed essential for self-regulation of T_H1 immunity.[40] Such modified or equipoised T_H1 cells lose their harmful inflammatory capacity.

Currently, our findings suggest that in the context of a pre-formed TH1/TH17 response, the CMP can cause IL-10 production. Isolated cells should be stimulated using CMPs and cytokine production should be assessed using intracellular flow cytometry to determine if there are any T_H cells that produce both cytokines. In order to conclude about the IL-10/IFN-γ axis, more experiments using inhibitory antibody should be performed in ex-vivo studies. Future studies should investigate if MOG-specific IL-10 responses might be established in cultures of T cells of CMPs treated EAE mice.

Equipoised T_H1-like immune responses are a well-known inducer of NO[30] and NO, contributes to suppressive effects on lymphocyte over activation.[41,42] In the EAE model, inhibition of NO intensifies the severity of disease.[42,43] C57BL/6 mice treated by complete Freund's adjuvant (adjuvant immunotherapy) are resistant to subsequent attempts to induce EAE because of the suppressive effects of NO on lymphocyte activation.[41,44]Further studies are needed to elucidate that NO and IL-10 productions by CMPs play roles in blocking the pathogenic capacity of MOG-reactive T cells.CMPs could cause distinct possibilities for therapeutic intervention in T_H1-induced pathology.

Our research was an *in-vitro* study employing mice spleen cells, thus it pertains obscure whether the same effects would be seen with *in-vivo* studies. To better understand IL-10 and NO regulation during EAE disease and in order to evaluate the therapeutic potency of CMPs treatment, oral and subcutaneous administrations of CMPs among EAE mice model are in progress in our laboratory.

This study is the first to examine CMPs treatment in the regulation of the unbridle T_H1/T_H17 responses and our idea is that immune response to CMPs, setback the development of such pathogenic immune responses. In summary, our findings suggest that in the context of a pre-formed T_H1/T_H17 response, the CMP can modulate the immune response. All in all, CMP supports Gata3 independent IL-10 production and promotes Tbx21 dependent IFN-γ induction. These results, together with our previous data, contribute to clarify the immunomodulatory effect, which has been attributed to CMPs.

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