IL-10-producing B cells regulated 1,3-β-glucan induced Th responses in coordinated with Treg

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Research

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Abstract

Background: Repeated exposure to fungi contaminated dust can lead to multiple adverse effect on lung, such as hypersensitivity pneumonitis, granuloma even irreversible fibrosis. 1,3-β-glucan, as the major cell wall component of fungi, was considered as its exposure biomarker. Existing studies showed that series of Th response were involved in 1,3-β-glucan induced hypersensitivity pneumonitis, in which macrophages, regulatory T cells (Treg) and IL-10 producing B cells were reported to participate. The reciprocal interaction among those critical immune cells in 1,3-β-glucan induced inflammation were not investigated yet.

Results: To clarify the regulatory mechanism of IL-10 producing B cells on Th and Treg, current study set up a primary cell co-culture system. Anti-CD22 antibody was injected intraperitoneally to generate IL-10 producing B cells deficiency mouse model. Cells were isolated and purified from different groups' mice. Flow cytometry was used to check the phenotype of different cell subtypes. CBA assay and realtime PCR were used to examine the levels of multiple cytokines. Our results indicated that IL-10 producing B cells were involved in modulating 1,3-β-glucan induced inflammatory response. The modulation of IL-10 producing B cells on Th response after 1,3-β-glucan treatment was independent on cell-cell contact. What's more, the modulation pattern of IL-10 producing B cells might be impaired without Treg response.

Conclusions: IL-10-producing B cells regulated 1,3-β-glucan induced Th responses in coordinated with Treg.

Introduction

The Inhalation of the fungal or bacteria polluted organic dust could induce various inflammatory disease, such as hypersensitivity pneumonitis (HP). And the harmful effects of polluted organic dust have drawn an increasing attention recently [1,2]. Repeated polluted organic dust exposure could even lead to granulomas or pulmonary fibrosis. An epidemiology study using HP data from 2004 to 2013 in U.S. showed that approximately one-fourth HP cases were fibrotic HP, which were related to a higher mortality rate [3]. Reports showed that increasing HP incidence was associated with urban air pollution [4]. The gradually increased morbidity of those organic dust-induced disease was not only related to its wildly existence in living environment, but also because more and more occupational environments were at risk of organic dust exposure. Traditionally, livestock farmers, crop farmers, bagasse workers and birds breeding workers were considered as the majority of whom suffer from HP [5,6]. However, some previously ignored occupations also took risk to organic dust exposure. Study showed that both the fungal load and the bacteria load in air in veterinary environment exceeded the WHO limit [7].

1,3-β-glucan, the major structure components of fungal cell wall, was accepted as a biomarker of organic dust exposure, and was widely used in animal experiments [8-10]. Furthermore, the concentration of 1,3-β-glucan in patients serum was reported to be closely related to the prognosis [11]. Our previous studies have set up a lung inflammation animal model induced by 1,3-β-glucan instillation. The existing studies

demonstrated that 1,3-β-glucan instillation could activate the innate immune system of mice. 1,3-β-glucan could be recognized by alveolar macrophages through Dectin-1 and TLRs, which could sent signals to recruit more macrophages and neutrophils to exacerbate the inflammation. Tremendous cytokines and chemokines were secreted to evoke the adaptive immune response, in which $CD4^+$ T cells played a crucial role. Th1, Th17 and Th2 responses were reported to be predominant in different stage of 1,3-β-glucan induced inflammation. The orchestration among three Th responses modulated the progression of 1,3-β-glucan induced lung inflammation. In addition, $CD4^+CD25^+$ regulatory T cells (Treg) was known as a special subset of T cell, which could regulate other effect T cells (Teff). Neutralization of Treg's critical membrane cluster differentiation antigen CD25 could obviously exacerbate the Th immune responses mediated inflammation after 1,3-β-glucan treatment [12].

Recent studies indicated a novel regulatory B cell subset, characterized by IL-10 secretion (also known as B10), was capable of regulating allergic disease, tumorigenesis and autoimmune disease [13-16]. Restoration regulatory B cell could help relieve the symptom of relapsing multiple sclerosis [17]. Activation of regulatory B cells could alleviate allergic inflammation, and was related with their complication such as pneumonia [18-20]. The modulation of ovalbumin-induced airway inflammation could be abrogated by adoptive transfer of IL-10-/- B cells [21]. Regulatory B cells were showed to modulate T cell-mediated inflammation through influencing multiple Th types' cytokines, including IFN-γ, IL-12 and IL-17 [22]. Our previous experiments demonstrated that restriction of IL-10-producing B cells could aggravate Th immune responses and limit Treg responses during 1, 3-β-glucan-induced lung inflammation in mice. However, the actual role of IL-10-producing B cells in 1, 3-β-glucan-induced $CD4^+$ T cells responses still need further study.

In order to clarify the regulative mechanism of 1, 3-β-glucan induced IL-10-producing B cells on Teff and Treg respectively, the comprehensive experiments in vitro were carried out. Anti-CD22 mAb was used to restrict the induction of IL-10-producing B cells as previous [23-25]. Teff($CD4^+CD25^-$), Treg($CD4^+CD25^+$), macrophages and B cells($CD19^+$) were isolated from different group mice. 1, 3-β-glucan was used to induce inflammatory response *in vitro*. Our results showed that IL-10-producing B cells were involved in modulating the inflammatory response after 1,3-β-glucan treatment in vitro. The modulation of IL-10-producing B cells on multiple Th responses was independent on cell-cell contact. Furthermore, the modulation pattern of IL-10-producing B cells might be impaired without Treg response.

Results

IL-10-producing B cells were involved in modulating the inflammatory response after 1,3-β-glucan treatment *in vitro*.

To establish the pro-inflammatory role of 1,3-β-glucan in vitro, the mice primary macrophage was treated with three doses of 1,3-β-glucan at two time points. The expressions of representative inflammatory cytokines were checked by realtime-PCR. All the three doses of 1,3-β-glucan (10, 50 and 250 μg/ml) could obviously increase the expression of IL-6 at both 24h and 72h (fig 1a). Besides, 10 μg/ml 1,3-β-glucan

could induce the enhanced expressions of IFN-γ and IL-4 in vitro at 24h and 72h respectively (fig 1b,c), which is consist with the previous animal study, without obvious toxicity on macrophage in vitro (fig 1d).

Next, to further identify the role of IL-10-producing B cells in vitro, a cell co-culture model was set up (fig 2a). Macrophages and isolated B cells with different proportions of IL-10-producing B cells were co-cultured and treated with 10 μg/ml 1,3-β-glucan or saline for 12h. Then, the stimulated B cells were extracted and cultured with T cells from vehicle group mice for 24h. The results of flow cytometry showed that 1,3-β-glucan treatment significantly increased the percentage of IL-10-producing B cells. And antiCD22 injection in mice restricted the induction of IL-10-producing B cells in vitro (fig 2b,c). And, the group with lower proportion of IL-10-producing B cells showed higher secretions of pro-inflammatory cytokines IL-6 (fig 2d) and TNF-α (fig 2e), which indicated that IL-10-producing B cells were involved in modulating the inflammatory response after 1,3-β-glucan treatment in vitro.

The modulation of IL-10-producing B cells on multiple Th responses was independent on cell-cell contact.

Our previous studies demonstrated that multiple Th responses were involved in 1,3-β-glucan-induced lung inflammation, and IL-10-producing B cells could take part in regulating the Th1/Th2/Th17 responses in mice. To further check the actual regulatory effect of IL-10-producing B cells on multiple Th responses, stimulated B cells from different groups were cultured with T cells from vehicle mice. As showed in figure3, antiCD22 B+glucan group showed higher expressions of pro-inflammatory cytokines IL-6 and TNF-α compared with both vehicle B+saline group and vehicle B+glucan group (fig 3b,c). Specifically, antiCD22 B+glucan group showed significantly increased expressions of typical Th1 cytokines IL-2 and IFN-γ in the co-cultured system (fig 3d,e). The same trend was observed on the expression of IL-4, a typical Th2 cytokine (fig 3f). The levels of IL-17 and Ror-γt were all elevated in antiCD22 B+glucan group (fig 3g-i), which indicated Th17 response was also amplified by insufficient IL-10-producing B cells.

What's more, this regulatory effect of IL-10-producing B cells on multiple cytokines was not vanished when the transwell plates were used in the co-cultured system (fig 3a). In general, the expressions of IL-6 and TNF-α in antiCD22 B+glucan group remained higher than vehicle B+glucan group and vehicle B+saline group in transwell plates after 1,3-β-glucan treatment (fig 3b,c). Although the expression of IFN-γ seemed to be similar between antiCD22 B+glucan group and vehicle B+glucan group with transwell plates, the amplified levels of other representative Th1/Th2/Th17 cytokines, such as IL-2, IL-4, IL-17 and Ror-γt, were not abrogated when transwell plates were used (fig 3d-i). These data suggested that blocking the cell-cell contact by using transwell plates did not alter the amplification of Th1/Th2/Th17 responses by the insufficient IL-10-producing B cells.

The modulation pattern of IL-10-producing B cells on multiple Th responses might be impaired without Treg response.

Existing studies demonstrated that Treg played a crucial role in regulating 1,3-β-glucan induced lung inflammation in mice. As showed in figure4a, the expression of Foxp3, the typical nuclear transcriptor of Treg, was significantly decreased in antiCD22B+glucan group compared with vehicle B+glucan group.

Meanwhile, the expression of Treg functional factor CTLA4 was restricted by insufficient IL-10-producing B cells (fig 4b). The expression of Treg typical cytokine IL-10 was also decreased in antiCD22B+glucan group (fig 4c). These data indicated that insufficient IL-10-producing B cells could inhibit the 1,3-β-glucan induced Treg response. What's more, the attenuated Treg response was not disturbed when the transwell plates were used (fig 4a-c). The difference of IL-10 secretion between antiCD22B+glucan group and vehicle B+glucan group became more significant when cultured with transwell, which further indicated that the impact of IL-10-producing B cells on Treg response was not depended on cell-cell contact (fig 4d).

Since T cells in co-cultured system above was a mixture including both $CD4^+CD25^-$ Teff and $CD4^+CD25^+$ Treg, whether the regulation of B10 on Treg was related with Teff was still under investigated. In following experiments, the purified Treg cultured with B cells either from vehicle mice or from antiCD22 injected mice was treated with 1,3-β-glucan or saline respectively (fig 4e). As showed in figure 4f and 4g, the expressions of Foxp3 and IL-10 in Treg+antiCD22 B+glucan group decreased significantly compared with that in Treg+vehicle B+glucan group. Furthermore, insufficient IL-10-producing B cells could even restrict the increase of CTLA4 and TGF-β when co-cultured with purified Teff(fig 4h,i). These might imply that the Teff was not indispensable in the modulation of IL-10-producing B cells on Treg response.

Nevertheless, Treg response seems to be essential in the modulation of IL-10-producing B cells on Teff. Besides of the increased expressions of IL-6 and TNF-α, the expressions of Th2 typical cytokines IL-4 and IL-13 were elevated when different B cells were co-cultured with isolated Teff(fig 5a-d). Th1 and Th17 responses was not amplified in Teff+antiCD22 B+glucan group compared with that in Treg+vehicle B+glucan group(fig5e,g). The expressions of Th1 and Th17 transcript factor T-bet and Ror-γt were even decreased in Teff+antiCD22 B+glucan group (fig 5f,h). It seemed that the modulation pattern of IL-10-producing B cells on Teff might be impaired without Treg response.

Discussion

The Account for only 1-3% in peripheral lymphoid organ, regulatory B cells became a bright star in maintaining immune homeostasis of inflammatory disease since firstly reported decades ago. Although regulatory B cells exhibited various phenotypes and were involved in immune modulation in different ways, IL-10 secretion was a remarkable character in autoimmune disease, tumor, inflammation and infection [22,27-29] . Adoptive transfer of IL-10 deficient B cells failed in controlling inflammatory responses [30]. Thus, CD19 and IL-10 were used to label regulatory B cells in this experiment, which was consist with our previous studies [31].

And CD22 antibody was injected to restrict IL-10-producing B cells, which was testified by many researches [32,33] . As a classical inhibitory receptor, CD22 was clarified to inhibit parts of BCR signal. Some CD22 antibody was showed to be able to skew B cells into secreting inhibitory inflammatory cytokines [34,35]. However, others demonstrated CD22 antibody could preferentially limit the induction of regulatory B cells. This paradoxical functions might due to the different IgG isotype of CD22 antibody

Regulatory B cells were known to be capable to modulate multiple Th responses in many diseases' development, as well as Treg. However, the mutual relation between them has not been deeply investigated. Some showed the regulatory role of IL-10-producing B cells was not dependent on Treg, others believed that Treg might assist the regulatory function of IL-10-producing B cells [36,37] .Our previous animal study showed that IL-10-producing B cells acted as the upstream of Treg, since Treg depletion failed to affect the number of IL-10-producing B cells [23]. In this experiment, insufficient IL-10-producing B cells indeed restricted the number of 1,3-β-glucan induced Treg, also limited the levels of Treg functional factors Foxp3 and IL-10 in $CD4^+$T cells and B cells co-cultured system in vitro. And the suppression trend of IL-10-producing B cells on Treg was not changed much when only $CD4^+CD25^+$Treg and different group B cells were co-cultured together. This indicated that the suppressive function of IL-10-producing B cells on Treg did not depended on Th cells response.

Another interesting phenomenon was that Th1, Th2 and Th17 responses were all affected by insufficient IL-10-producing B cells when T cells and B cells were cultured. And the inhibitory function of IL-10-producing B cells on Th responses was barely dependent on cell-cell contact. Whereas, only Th2 response was amplified by insufficient IL-10-producing B cells when B cells were cultured with $CD4^+CD25^-$ Teff cells. It seemed that the modulation of IL-10-producing B cells on Th1 and Th17 response partly depended on Treg, which might be the direct suppressor on these two Th types' responses. The intimate relationship among Th1, Th17 and Treg were checked in many studies [38,39]. Treg could amplify the Th1 or Th17 response through promoting the expressions of their transcript factors T-bet or ROR-γt [40]. The heterogeneity of regulatory function of IL-10-producing B cells on multiple Th responses indicated that the detailed regulatory mechanism of IL-10-producing B cells was diverse based on multiple pathogenesis. Taken together, as showed in figure5i, our study suggested a regulatory pattern of IL-10-producing B cells on 1,3-β-glucan induced inflammatory responses, through either modulate Teff responses directly, or regulating Treg cells, which further affect Teff responses after 1,3-β-glucan treatment.

Conclusions

In general, our study demonstrated that IL-10-producing B cells were involved in modulating the inflammatory Th responses after 1,3-β-glucan treatment in vitro. And its modulation on multiple Th responses was independent on cell-cell contact. Furthermore, the modulation pattern of IL-10-producing B cells might be impaired without Treg response in 1,3-β-glucan induced inflammatory response.

Materials And Methods

Animals

C57BL/6 female mice at 6-8 weeks age were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Mice were housed in a specific-pathogen-free environment and maintained on standard mouse chow at an environmental temperature of 24±1ºC, with 12 h light /12 h dark cycles, and

water ad libitum. All animal procedures were approved by the Animal Care and Use Committee at the China Medical University, which complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The study was carried out in accordance with the approved guidelines.

Cell treatment

C57BL/6 mice were randomly allocated into 2 groups according to weight: antiCD22 group and vehicle group as needed. Macrophages were isolated from vehicle group mice intraperitoneally, and purified by a 2 hrs adherence culture as previous described [26]. $CD4^+$ T cells were first purified from vehicle group mice spleen by MACS, and then Treg($CD4^+CD25^+$) and Teff ($CD4^+CD25^-$)were separately isolated by positive selection and negative selection according to a magnetic column-based system Treg/Teff cell isolation kit (Miltenyi Biotech, Auburn, CA).

To restrict the induction of $CD19^+IL\text{-}10^+$ regulatory B cell, mice were injected intraperitoneally with 300 μg antiCD22 antibody (KH2014176, F239, Sangon Biotech, Shanghai, China) one day before scarified as previous described [23]. IgG1 was used as control. $CD19^+$ B cells were immediately isolated from two groups after the mice were sacrificed by using magnetic-activated cell sorting (MACS) technology (Miltenyi Biotech, Auburn, CA). Zymosan A (1,3-β-glucan) from Saccharomyces cerevisiae (Z4250), purchased from Sigma–Aldrich.Inc (St. Louis, MO63103, USA) and were used to induce the inflammatory responses.

MTS assay

To check the toxicity of certain dose of zymosan A in vitro, CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, USA) was performed. In brief, the cell culture medium in 96-well plates was removed and replaced with 100 μL of fresh culture medium. 20μl of CellTiter 96® AQueous One Solution Reagent were added into each well. Incubate the plate at 37°C for 2 hours. The absorbance was record at 490nm under a 96-well plate reader.

Flow cytometry

Cells from different culture groups were stimulated with a leukocyte activation cocktail (BD Pharmingen, San Jose, CA, USA) for 5 h, followed by treatment with anti-mouse CD16/CD32 (BD Pharmingen) for 10 min at 4°C to block nonspecific binding. Anti-CD19-PerCP-Cy5.5 (BD Pharmingen) and anti-IL-10-PE (BD Pharmingen) were used to label regulatory B cells. Anti-CD4-PerCP-Cy5.5 (BD Pharmingen) and anti-CD25-PE (Miltenyi Biotech, Auburn, CA) were used to mark different T cell subsets. In general, cells were first stained with CD4-PerCP-Cy5.5, CD-25-PE, or CD19-PerCP-Cy5.5 respectively as described previously [23]. Next, cells were fixed with fixation/permeabilization kit (eBioscience, USA) and were stained intracellularly with anti-IL-10-PE. Stained cells were washed, resuspended and analyzed by using a FACSCanto II system (BD, Franklin Lakes, NJ, USA). Dead cells were excluded depending on forward scattering (FSC) and side scattering (SSC) through FlowJo software.

Cytokine analysis

The protein levels of multiple cytokines in cell culture medium were examined by CBA assay (mouse Th1/Th2/Th17 cytokine kit, BD Pharmingen) as follow: series of capture beads for IL-17, IL-2, IFN-γ, IL-6, TNF-α and IL-10 were firstly mixed together. Then co-culture the capture beads mixture, detection reagent and 50 μl sample for 2 h. Wash the beads carefully and resuspend it for analysing using a FACSCanto II system (BD Biosciences). Data was analyzed with FCAP Array software.

Realtime-PCR analysis

Total RNA of cultured cells was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), based on the manufacturer's protocol. The RNA concentration and quality were determined under a UV spectrophotometer. Primers were designed with Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/), and were rechecked with a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). PrimeScript RT kit (DRR047A, Takara, Japan) and Premix Ex Taq II RT-PCR kit (DRR081A; Takara) were used for real time RT-PCR analysis. Amplification efficiency differences were checked and adjusted by comparing the slopes of the standard curves. Data analyses were performed by the 7500 system software (Applied Biosystems).

Statistics

Data were analyzed for statistical significance using Prism v.7.0 (GraphPad Inc., USA). The differences between groups were analyzed through a one-way analysis of variance (ANOVA) followed by pair-wise comparison with the Student-Newman-Keuls test. All experiments were performed in triplicate. $P<0.05$ was considered statistically significant. All values are means ± SEM.

Declarations

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None

Authors' contributions

FL designed experiments and prepared the manuscript. YL and XZ carried out experiments. FL and JC interpreted the findings. All authors participated in its revision. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

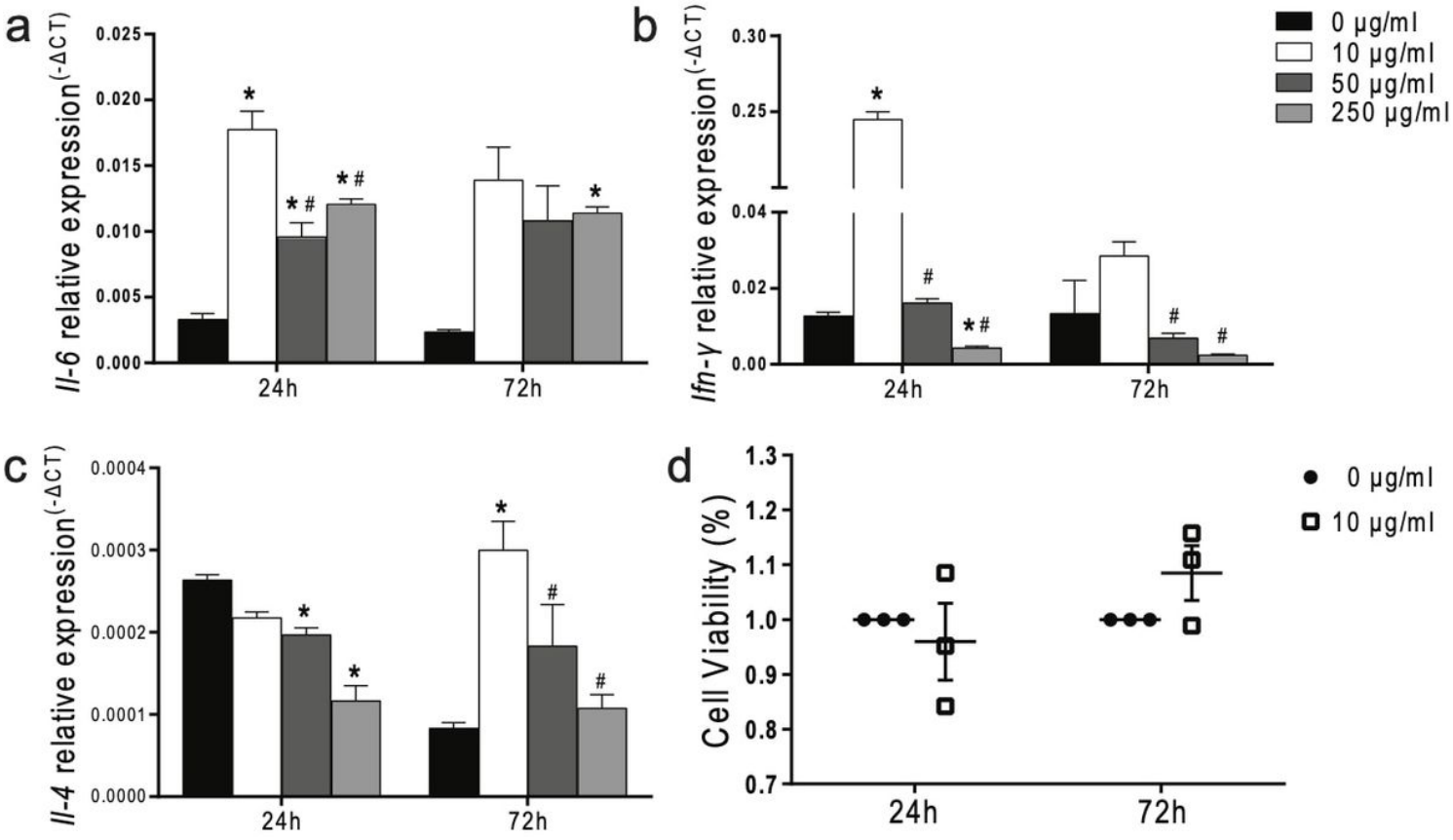

Figure 1

1,3-β-glucan could induce tremendous expressions of pro-inflammatory cytokines in macrophages. Different dose of zymosan were used to treat macrophages isolated from wildtype(WT) C57BL/6 mice for 24h or 72h. Pro-inflammatory cytokines in the supernatant were checked. The expressions of IL-6 (a) and IFN-γ (b) in culture primary macrophages were increased after 24h treatment with 10μg/ml zymosan. The expressions of IL-4 (c) were also elevated by 10μg/ml zymosan after 72h treatment. No significant changes in cell viability by zymosan treatment at both time points (d). (Data was presented as mean ± SEM (n=3~4); *P<0.05 compared with the 0μg/ml group; #P<0.05 compared with the 10μg/ml group).

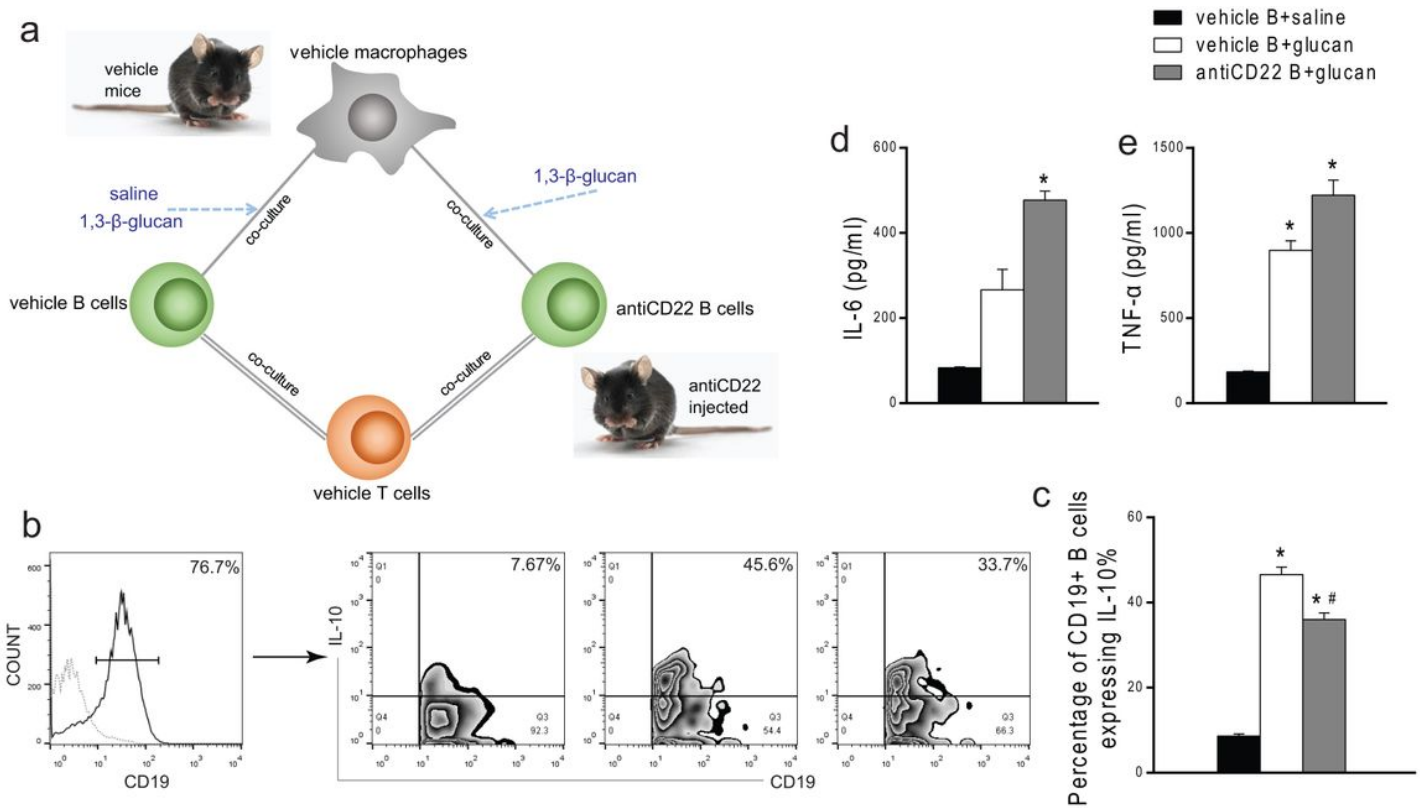

Figure 2

Insufficient IL-10 producing B cells could exacerbate 1,3-β-glucan induced inflammatory response in vitro. Macrophages were isolated from WT C57BL/6 mice and co-cultured with B cells purified from either wildtype mice or anti-CD22 injected mice for 12h treated with zymosan. Then the activated B cells from different groups mice were cultured with T cells from WT mice for 24h(a,b). Anti-CD22 injection restricted the increase of 1,3-β-glucan induced IL-10 producing B cells in vitro(c). The secretions of pro-inflammatory cytokines IL-6 (d) and TNF-α (e) were elevated by insufficient IL-10 producing B cells. (Data was presented as mean ± SEM (n=3); *P<0.05 compared with the vehicle B+saline group; #P<0.05 compared with the vehicle B+glucan group).

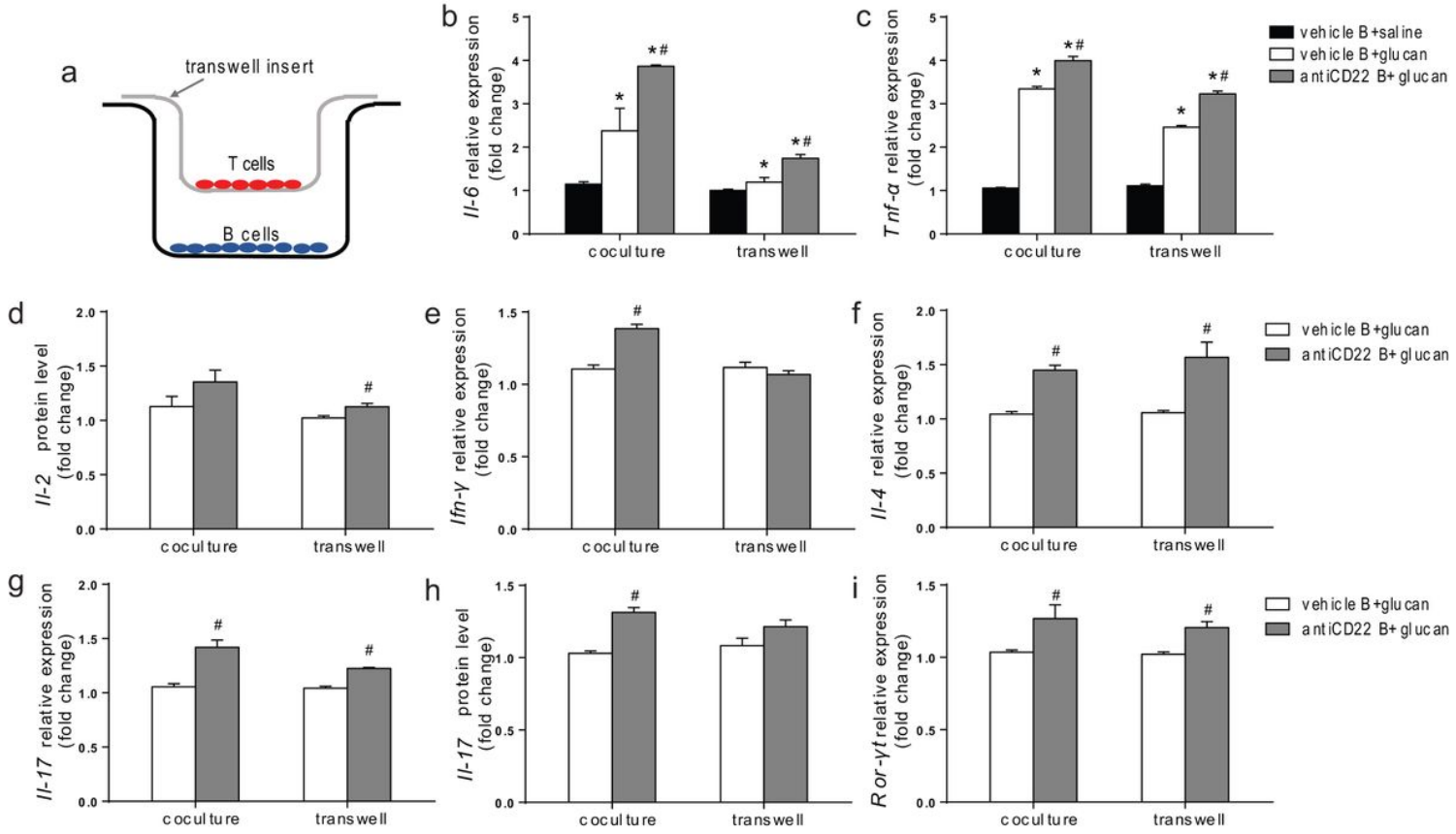

Figure 3

The modulation of IL-10 producing B cells on Th1/Th2/Th17 responses was independent on cell-cell contact. A schematic diagram of different groups B cells and T cells co-cultured pattern with transwell insert plates(a). The expression of IL-6(b) and TNF-α(c) were significantly increased by insufficient IL-10 producing B cells in B cell and T cell co-culture system with or without transwell plates. The secretions of IL-2(d) and IL-17(h) in supernatant of the B cell and T cell co-culture system with or without transwell plates were assayed by CBA. The expression of multiple Th cytokines IFN-γ(e), IL-4(f), IL-17(g) and ROR-γt (i) in different groups of the B cell and T cell co-culture system with or without transwell plates were assayed by real-time PCR. (Data was presented as mean ± SEM (n=3~5). *P<0.05 compared with the vehicle B+saline group; #P<0.05, compared with the vehicle B+glucan group).

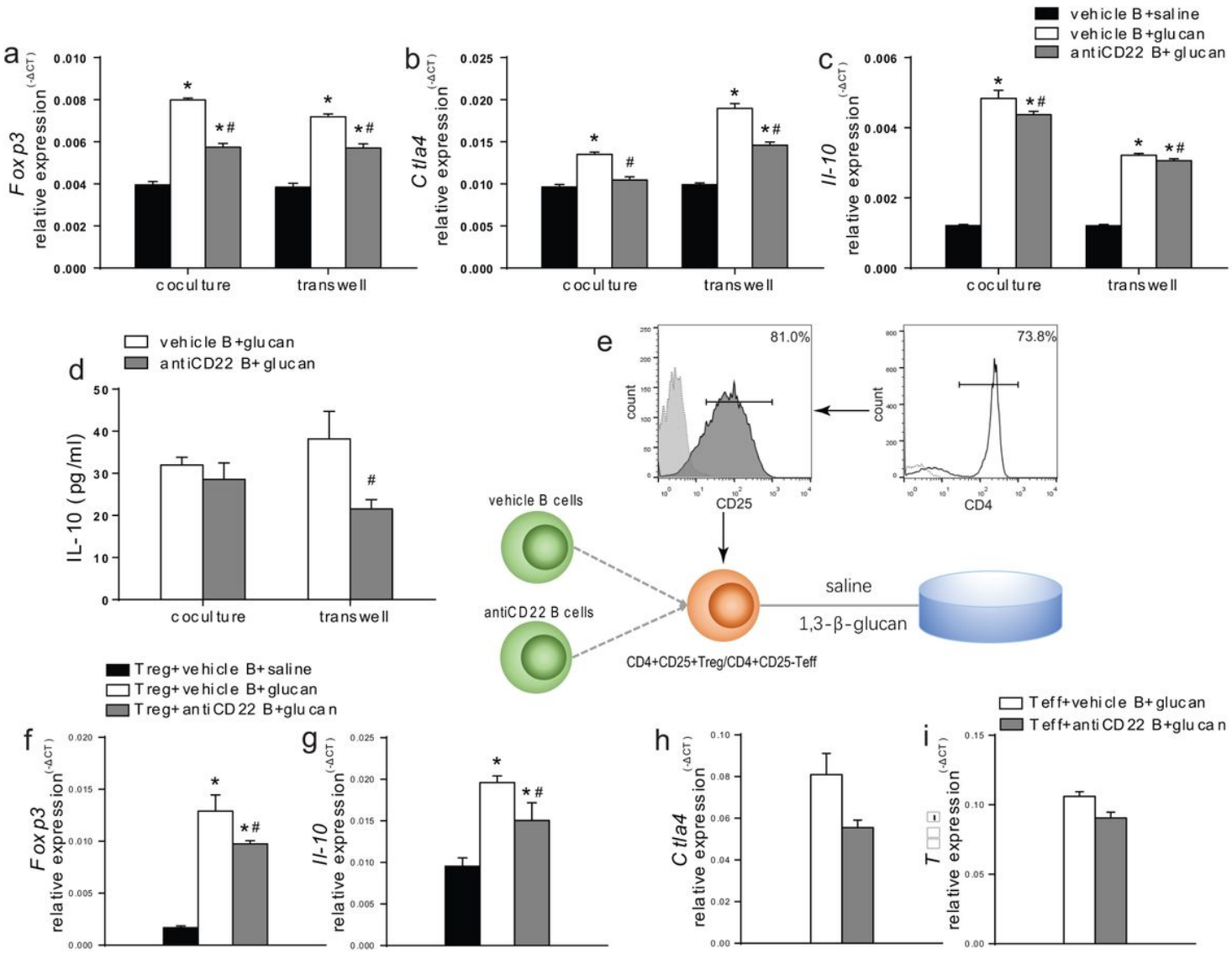

Figure 4

Insufficient IL-10 producing B cells could restrict 1,3-β-glucan induced Treg responses in vitro. Insufficient IL-10 producing B cells limited the expressions of Foxp3(a), CTLA4(b) and IL-10(c) in B cell and T cell co-culture system with or without transwell plates. The secretion of IL-10 were also inhibited by insufficient IL-10 producing B cells in B cell and T cell co-culture system with transwell plates(d). A schematic diagram of CD4+CD25+Treg cells or CD4+CD25-Teff cells co-cultured with different groups B cells treated with 1,3-β-glucan(e). Insufficient IL-10 producing B cells restrict 1,3-β-glucan induced Foxp3(f) and IL-10(g) expressions in Treg and B cells co-cultured system. The expressions of 1,3-β-glucan induced CTLA4(h) and TGF-β(i) also were suppressed by insufficient IL-10 producing B cells in Teff and B cells co-cultured system. (Data was presented as mean ± SEM (n=3~5). *P<0.05 compared with the vehicle B+saline group; #P<0.05, compared with the vehicle B+glucan group).

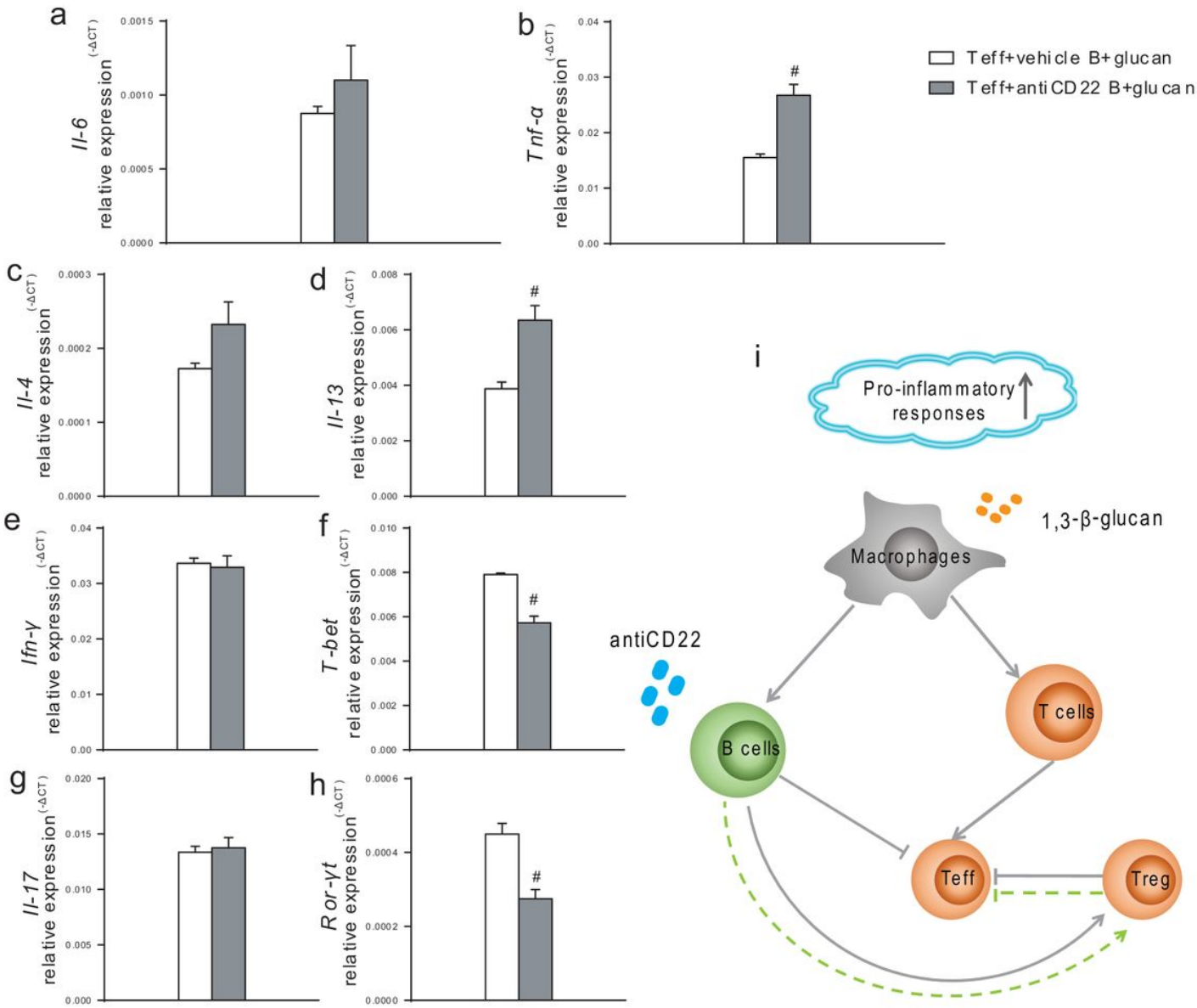

Figure 5

The modulation pattern of IL-10 producing B cells could be impaired without Treg. The expression of IL-6(a) and TNF-α(b) were amplified by insufficient IL-10 producing B cells in B cell and Teff co-culture system in vitro. The expression of Th2 cytokines IL-4(c) and IL-13(d) were promoted by insufficient IL-10 producing B cells in B cell and Teff co-culture system. Although the expressions of IFN-γ(e) and IL-17(g) were not affected by insufficient IL-10 producing B cells in B cell and Teff co-culture system. The expressions of Th1 transcript factor T-bet(f) and Th17 transcript factor ROR-γt(h) were decreased by insufficient IL-10 producing B cells in B cell and Teff co-culture system. A schematic representation of the role of IL-10 producing B cells in modulating 1,3-β-glucan induced Treg/Teff immune responses(i). (Data was presented as mean ± SEM (n=3~5). #P<0.05, compared with the Teff+vehicle B+glucan group).