

High IFN- α expression is associated with the induction of experimental autoimmune uveitis (EAU) in Fischer 344 rat

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ABSTRACT

Th1-response plays a crucial role in determining pathogenesis of organ-specific autoimmune diseases. It is believed that both IL-12 and INF- α are initiators to regulate Th1- response. In our experimental autoimmune uveitis (EAU) model, both Lewis and Fischer 344 rats share the same MHC class II molecules, while Lewis rat is EAU susceptible and Fischer 344 rat is EAU resistant. However, under the same condition of immunization, if pertussis toxin (PTX) was injected intraperitoneally as an additional adjuvant, Fischer 344 rat can develop EAU. In this study we investigate which mechanisms are involved in the induction of EAU in CFA+R16+PTX-treated (CRP-treated) Fischer 344 rats. In vivo and in vitro data demonstrated that Th1-cytokine, IFN- γ mRNA expression was significantly increased in disease target tissue-eyes and in draining lymph node cells of CRP-treated Fischer 344 rat. When IL-12 and IFN- α mRNA expression were compared in the experimental groups, only IFN- α mRNA expression was associated with EAU development. To distinguish the sources of IFN- α producing cells, it was observed that IFN- α expression was mainly produced by macrophages. It was further confirmed that normal macrophage from Fischer 344 rat was able to produce significant IFN- α in the presence of PTX. The data strongly suggested that IFN- α might be involved in initiating Th1-cell differentiation and in turn contribute to the induction of EAU. High IFN- α expression induced by PTX may represent a novel pathway to initiate Th1 response in Fischer 344 rat.

Key words: *Experimental autoimmune uveitis(EAU), fischer 344 rat, IFN- α , pertussis toxin(PTX).*

INTRODUCTION

The CD4⁺ T cells can be subdivided into Th1 and Th2 subsets based on their secreted cytokine profile. Th1 cells characteristically secrete Interferon- γ (IFN- γ), whereas Th2 cells mainly produce IL-4[1]. IL-12 polarizes the differentiation of naïve, CD4⁺T cells towards Th1 pathway, in contrast IL-4 directs T cell

differentiation towards Th2 pathway. The broken balance between Th1 and Th2 immune responses and predominant Th1 response are crucial factors in initiation of organ-specific autoimmune diseases.

Human uveitis is an intraocular inflammatory disease that mostly affects children and young adults. It is one of the major causes of blindness in young individuals in the world. It causes about 10 per cent of total visual impairment. Experimental autoimmune uveitis (EAU) is a prototypic Th1 cell-mediated and organ-specific autoimmune disease, in which the target tissue is the neural retina[2]. EAU can be induced in animals by immunization with retinal antigens such as the interphotoreceptor retinoid-binding protein (IRBP) or its fragments such as

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Abbreviations: IFN- α , Interferon-alpha; CFA, complete Freund's adjuvant; TCS, trichosanthin; PTX, pertussis toxin; LPS, Lipopolysaccharide; R16, a fragment of protein IRBP; CRP-treated, CFA+R16+PTX treated; CR-treated, CFA+R16 treated; CP-treated, CFA+PTX treated

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peptide R16. Previous study indicated that genetic susceptibility to EAU in the rat is strictly strain dependent. Lewis and Fischer 344 rats share the same MHC class II (RT1B) that allow them to recognize pathogenic peptide-R16 of IRBP[3]. In vitro assays of cytokine profile clearly showed that the Lewis rats developed EAU with a predominant Th1 cell response, whereas the Fischer 344 rats failed to develop EAU with low Th1 response. However, under the same condition of immunization, when Fischer 344 rat was treated with pertussis toxin (PTX) as an additional adjuvant, Fischer 344 rats developed EAU with an increased Th1 response. These results suggested that PTX was involved in the initiation of Th1-response in Fischer 344 rat.

Since evidences indicate that cytokines (IL-12 and IFN- α) can positively regulate Th1 cell differentiation and IFN- γ production[4],[5], in this work we intend to examine which cytokine, IL-12 or IFN- α plays more important role in initiating Th1 response in EAU of CRP-treated Fischer 344 rat. IL-12 and IFN- α mRNA expressions were determined by semi-quantitative RT-PCR from draining lymph node cells and inflamed eye tissue in different groups. The data demonstrated that IFN- α mRNA expression was highly associated with Th1 response in CRP-treated Fischer 344 rat, but IL-12 did not. It was further confirmed that the adherent cells (macrophages) isolated from R16-primed draining lymph node cells and spleen cells produced high amounts of IFN- α after re-stimulation. The normal macrophages from Fischer 344 rat stimulated in vitro with PTX were capable of producing significant IFN- α . Our primary data is the first time to demonstrate that the effect of PTX as an additional adjuvant to induce EAU in Fischer 344 rats may be related to up-regulation of IFN- α which promotes IFN- γ production and Th1-response resulting in EAU expression.

MATERIALS AND METHODS

Rats

Female Fischer 344 rats at age of 6 to 8 w were purchased from Frederick Cancer Research Center (Frederick, MD, USA) and were kept in pathogen-free conditions in the animal center of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. They were bred according to the Institution guidelines.

Antigen and reagents

Peptide R16 of bovine IRBP (sequence ADGSSWEGVGVDPDV, residues 1177-1191) was kindly gifted by Dr. Rachel R. Caspi (National Eye Institute, NIH, USA). Other reagents such as pertussis toxin (PTX), and Lipopolysaccharide (LPS) and complete Freund's adjuvant (CFA) containing 2.5 mg/ml Mycobacterium tuberculosis H37RA (Difco, Detroit, MI, USA) were purchased from Sigma. Crystallized trichosanthin(TCS), a plant protein, was purchased from Jin-San Pharmaceutical Factory (Shanghai, China). TRIzol Reagents was purchased from Life Technologies (Gibco BRL). ^3H -thymidine was purchased from Beijing YaHui company.

Immunization and EAU scoring

Fischer 344 rats were divided into three groups. Group I rats were immunized with 100 μl of emulsified CFA 1/1 v/v with PBS into one hind footpad and were intraperitoneally injected with 1 μg of PTX in 0.1ml of RPMI 1640 medium (abbreviation: CP-treated). Group II rats were immunized with 100 μl of emulsified 1/1 v/v CFA with 30 μg of peptide R16 in PBS into one hind footpad (abbreviation: CR-treated). Group III rats were immunized with 100 μl of emulsified 1/1 v/v CFA with 30 μg of peptide R16 in PBS and injected i.p. with 1 mg of PTX (abbreviation: CRP-treated).

Eyes were collected on day 14 (disease peak time) after immunization and were fixed in 4% phosphated-buffered glutaraldehyde for 1 hour and transferred into 10% phosphated-buffered formaldehyde until processing. Degree of diseases was classified by histopathology. Eyes were graded on a scale of 0 to 4 according to the extent of inflammation and tissue damage[6].

Primary culture of R16-primed lymphocytes

Draining lymph node cells from popliteal and inguinal lymph nodes were collected on day 14 after immunization. 4×10^6 cells were cultured with or without peptide R-16 (2 $\mu\text{g}/\text{ml}$) for 48 h in 24 well tissue culture plate in RPMI 1640 medium containing 1% syngeneic rat serum. The cells were harvested for mRNA isolation and RT-PCR assay was performed as described[7],[8].

Lymphocyte proliferation Assay

Draining lymph node cells were collected on day 14 after immunization. 2×10^5 cells were cultured with 2 $\mu\text{g}/\text{ml}$, 0.2 $\mu\text{g}/\text{ml}$, 0.02 $\mu\text{g}/\text{ml}$ or without R-16 peptide, respectively, in 96 well tissue culture plate in RPMI 1640 medium containing 1% syngeneic rat serum. After 72 h, the cultures were pulsed with 0.5 μCi ^3H -thymidine/well 18 h before harvest and the ^3H -TdR incorporation was counted by standard liquid scintillation[17].

Preparation of macrophage fraction and lymphocyte fraction

Preparation of macrophage fraction and lymphocyte fraction from R16-primed draining lymph node and preparation of normal macrophage fraction from peritoneal cavity of Fischer 344 rat for the in vitro induction of IFN- α are as follows.

The protocols for isolation of peritoneal macrophages were according to reference[9]. Briefly, 50 ml of ice-cold PBS was intraperitoneally injected into the Fischer 344 rats killed by cervical

dislocation and the cells in peritoneal fluid were collected. After centrifugation the macrophages were purified by adherence to 90 mm culture dishes (Nunc, USA) in RPMI-1640 with 0.5% normal syngeneic rat serum for 1 h in 5% CO₂ at 37°C. Non-adherent cells (mainly lymphocytes) were removed by washing with PBS for 3 times. The adherent cells (mainly macrophages) were collected and stimulated in vitro with 10 ng/ml of TCS, 10ng/ml LPS, or 10 ng/ml PTX for 4 h, respectively.

The isolation of macrophages from spleen and lymph nodes of CRP-treated and CR-treated Fischer 344 rats according to following protocol. The 1×10^6 cells/ml of spleen cell or lymph node cell suspension were prepared from immunized rat on day 14. Red blood cells were destroyed with 5ml of red blood cell lysing buffer. The cells were washed twice with PBS and then the macrophages were adherent to 50 mm culture dish as above and were harvested. Non-adherent cells were removed by washing with PBS for 3 times and collected as the lymphocyte fraction.

RT-PCR

Total RNA of freshly eye tissue and of cultured cells was extracted according to the instruction of TRIzol Reagent manufacturer. RNAs were dissolved with 30 μ l of DEPC-treated H₂O and the concentration was measured. Total RNA was reverse-transcribed into cDNA using random hexamer primers as described elsewhere[10].

The amount of cDNA in each sample was standardized by a preliminary amplification for the glyceraldehydes 3-phosphate-dehydrogenase (GAPDH). mRNA expressions for IFN- γ , IL-4, IL-12p40, IFN- α and interferon regulator factor-3(IRF-3) were measured in the standard cDNA samples by PCR as previously described[8],[10], [11]. Briefly, 30 μ l of total reaction volume was used. 20.5 μ l H₂O, 3 μ l of $10 \times$ buffer, 1.8 μ l of 25 mM MgCl₂, 1.5 μ l of 2.5 mM each of dNTPs, 0.2 μ l of 1 unit Taq DNA polymerase (Promega, USA), and 1 μ l of 10 mM each primer (Synthesized by BioAsia Company, Shanghai, China) were mixed and then 1 μ l of cDNA sample was added. The PCR cycling condition was 2 minutes at 94°C for pre-denaturation, 1 minute at 94°C for denaturation, 45 seconds at 60°C for annealing, 1 minute at 72°C for extension, and then 7 min at 72°C for the last cycle. cDNA samples were amplified for 25 cycles for GAPDH, 30 cycles for IFN- γ , IL-4, IFN- α , IL-12p40, and IRF-3. The sequences of PCR primers for each gene are GAPDH (sense, 5' CCA TGG AGA AGG CTGG GG 3'; antisense, 5' CTG CCC CAC GGC CAT CAC 3'; 297 bp), IFN- γ (sense, 5'ATG AGT GCT ACA CGC CGC GTC TTGG 3'; antisense, 5' GAG TTC ATT GAC AGC TTT GTG CTG G 3'; 405 bp). IL-4 (sense, 5' TGA TGG GTC TCA GCC CCC ACC TTG C 3'; antisense, 5' CTT TCA GTG TTG TGA GCG TGG ACT C 3'; 378 bp). IL-12p40 (sense, 5' TGC TGG TGT CTC CAC TCA TCA TG 3'; antisense, 5' CTC GGT GGA CCA AAT TCC 3'; 302 bp). IFN- α sense, 5' CAT GGC TCG GCT CTG TGC TTT C 3'; antisense, 5' TTT GAC CAC CTC CCA GGC ACA G 3'; 505 bp). IRF-3 (sense, 5' TAA GGG AGA TCG GCT GGC TG 3'; antisense, 5' TGA TGG AGA GGT CCC CAA GG 3'; 287 bp). The PCR products were electrophoresed, stained with ethidium bromide and photographed. The band density was calculated by FURI Smart View 2000 (Shanghai). The ratio of cytokines/GAPDH was used to reflect target mRNA expression

level.

Experiments were repeated for three times and were reproducible. Graphs show representative experiments.

RESULTS

Fischer 344 rat can be induced to develop EAU by peptide R16 plus PTX immunization

In all three repeated experiments, only group III (CRP-treated rats) developed EAU as compared to other two groups (CP-treated and CR-treated rats). The EAU scores were examined by histopathology on day 14 following the immunization (Fig 1). During the induction of EAU, a hazy or opaque anterior chamber, the hallmark of infiltration by inflammatory cells, occurred in the CRP-treated rats. The histopathology of this group showed a mass of infiltrated cells in retinal lesions (data not shown). In contrast, the histopathology examination from other two groups (CP-treated and CR-treated rats) showed that their retinas were normal. The data suggested that PTX was a trigger to initiate the induction of EAU in Fischer 344 rats.

PTX significantly enhanced proliferation of immunized lymphocytes in response to peptide R16

In order to test the Influence of PTX on the proliferation of immunized lymphocytes. The draining lymph node cells were harvested on day 14 (disease

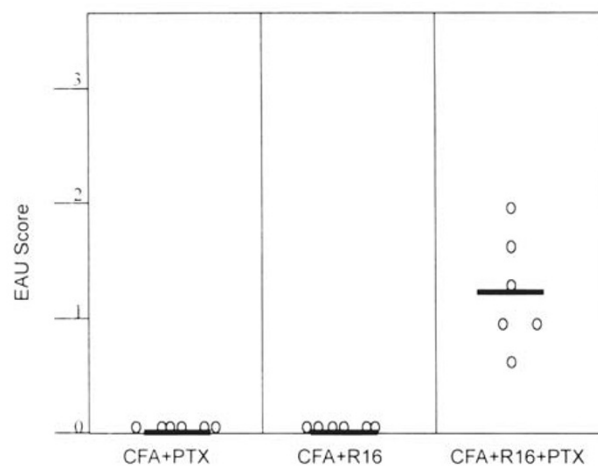


Fig 1.

The EAU score of Fischer 344 rats. EAU scores were evaluated by histopathology on day 14 after immunization with CFA+PTX, CFA+R16 and CFA+R16+PTX. Each dot represents average of two eyes in each rat. The horizontal bar is the average score of the group.

peak time) and cultured with peptide R16 at different concentrations for 72 h. The proliferation results showed that lymph node cells from the CRP-treated rats generated high cpm value of $^3\text{H-TdR}$ incorporation (Fig 2). Whereas the values in two groups (CP-treated and CR-treated) were significantly lower. The data suggested that PTX may increase R16-specific T-lymphocytes from the CRP-treated rats.

EAU in Fischer 344 rats was mediated by Th1-

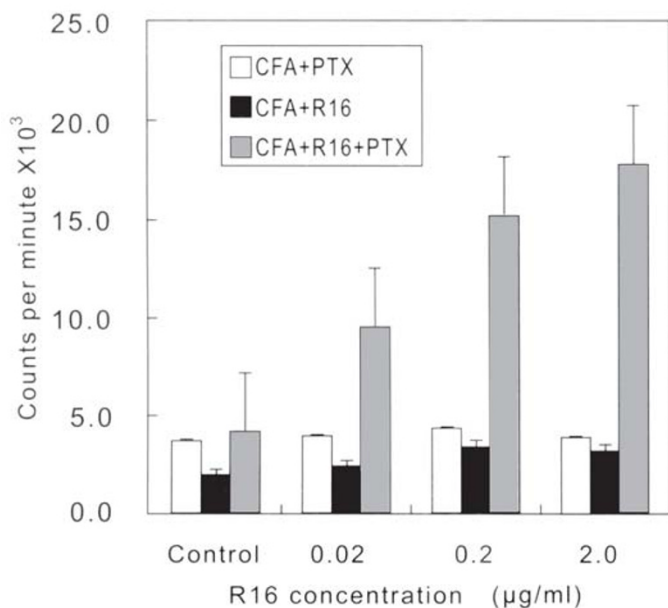


Fig 2.

Proliferation of draining lymph node cells to peptide R16. Draining lymph node cells (LNC) were harvested on day 14 following immunization and re-stimulated with different concentrations of peptide R16 in the culture for 72 h. Three groups (CFA+PTX, CFA+PTX and CFA+R16+PTX) were done in all the experiments. The results are expressed as cpm × 10⁻³.

response

In our previous work, we have demonstrated that all EAU susceptible strains of rat are Th1 responder [7],[8]. To test the cytokine profiles, IFN- γ (Th1 type cytokine) and IL-4 (Th2 type cytokine) were determined by RT-PCR from draining lymph node cells and inflamed eyes. As compared to CP-treated and CR-treated rats, CRP-treated rats produced predominant IFN- γ either in the eyes or in draining lymph node (Fig 3). The ratios of IFN- γ /GAPDH are 0.73 (in the eyes) and 0.54 (in the lymph node) which were significantly higher than those of the CP-

treated or CR-treated rats. In contrast, the difference of IL-4 expression was insignificant among the three groups. These results suggested that EAU in Fischer 344 rats was a Th1-responder and the data confirmed our previous work that Th1-type response was required to induce EAU.

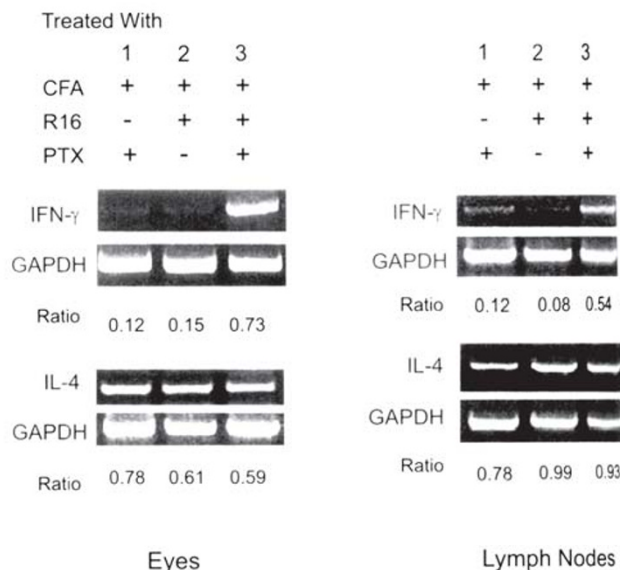


Fig 3. The mRNA expression of IFN- γ and IL-4 in the eyes and in draining LNC. mRNAs were isolated from eyes and LNC of three groups on day 14 following immunization. High ratio indicates high expression of target mRNA. These data is one representative result of three experiments.

High IFN- α mRNA expression was associated with Th1-type response in CRP-treated Fischer 344 rats, but IL-12 did not

In many autoimmune diseases, especially in the Th1-mediated autoimmune diseases, IL-12 signal pathway plays a crucial role in initiating Th1-type response. Recently, there are many evidences suggested that Type I IFN (IFN- α) also plays an important role in the regulation of Th1-cell differentiation[12],[13]. To address which pathway was involved in the induction of Th1-type response in EAU of Fischer 344, IL-12 and IFN- α were measured in draining lymph node and eye tissues. Initially, we supposed that IL-12 played a determining role in initiating Th1-response, but the data showed that IL-12p40 mRNA levels were not obviously different among different groups (Fig 4). It was interesting to observe that IFN- α was highly expressed in the eyes of CRP-treated rat. To further confirm this

finding, IL-12p40 and IFN- α mRNA expression were also measured in draining lymph node from immunized rats. IL-12p40 mRNA levels was not significantly different while the IFN- α mRNA levels was greatly different among them (Fig 4). The results demonstrated that PTX had a capacity to induce IFN- α mRNA expression. The data suggested that it was IFN- α rather than IL-12 was associated with EAU of Fischer 344 rats. IFN- α is associated

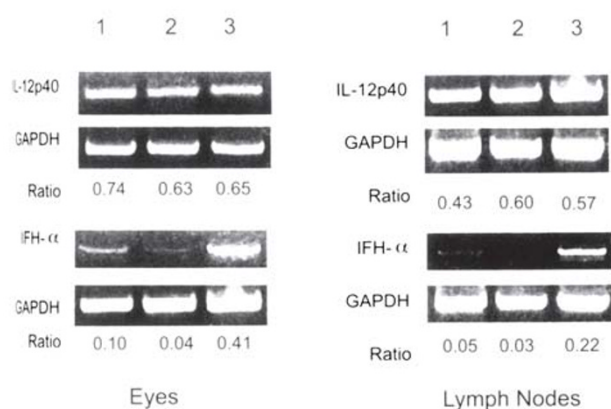


Fig 4. The mRNA expression of IL-12p40 and IFN- α . The mRNA expression was determined in the samples described in the legend to Fig 3. The ratio of IL-12p40/GAPDH and IFN- α /GAPDH were measured for the eyes and LNC.

with IFN- γ expression in response to R16 re-stimulation in vitro

Since IFN- α is believed to be involved in induction of Th1-type response, and promotes the typical Th1-cytokine, IFN- γ production[5]. Peptide R16-induced IFN- γ expression was analyzed by RT-PCR. Draining lymph node cells were collected on day 14 following immunization and stimulated in vitro with or without R16 (2 mg/ml) for 48 h. Both IFN- α and IFN- γ expressions were measured in parallel. The results showed that when the cells were cultured without antigen (peptide R16), IFN- α and IFN- γ were significantly co-expressed in CRP-treated rats. Furthermore when cultured cells were re-stimulated with R16, IFN- γ expression was strongly induced in both CR-treated and CRP-treated rats. It was obviously found that IFN- α is associated with IFN- γ expression. The higher IFN- α expression, the higher IFN- γ expression (Fig 5). The data suggested that IFN- α also had a potential role in promoting IFN- γ production in vitro.

IFN- α is mainly produced by macrophages

Since IFN- α has a potential role in initiating Th1-type response, it is important to know which cells produce IFN- α . We are assuming that IFN- α secreted cells may be related to antigen-presenting cells. To test our hypothesis, cell fractions were prepared from spleen and draining lymph node cells of EAU rats. Non-adherent cells mainly containing lymphocytes and the adherent cells mostly containing macrophages were separated as described in the methods. The purity of macrophage is up to 92% via phagocytosis assay (data not shown). The data showed that IFN- α was expressed higher in macrophages than that of lymphocytes either in spleen or in lymph node cells (Fig 6). Furthermore, the higher expressed IFN- α in macrophages from lymph node suggested that lymph node was a major location for Th1-cell activation and IFN- α was involved

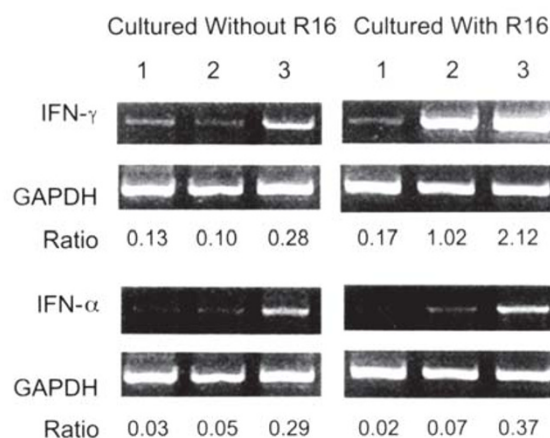


Fig 5. The mRNA expression of IFN- γ and IFN- α in LNC. LNC were harvested on day 14 after immunization. The cells were re-stimulated with R16 (2 μ g/ml) for 48 h and IFN- α and IFN- γ expression were determined and described in the legend to Fig 3. Lane 1, from rats immunized with CFA+PTX; Lane 2, from rats immunized with CFA+R16; Lane 3, from rats immunized with CFA+R16+PTX.

in the disease process.

IRF-3 gene expression was associated with increased IFN- α expression

If IFN- α expression is really high, other factors that specifically regulate IFN- α gene expression should be highly expressed. Since Interferon regulator factor 3 (IRF-3) transcription factor has been

shown to regulate the activation of IFN- α gene transcription and was specifically involved in IFN- α gene expression[14], we examined the expression of IRF-3 in the macrophages of spleen and draining lymph nodes from CR-treated and CRP-treated rats on day 14 following immunization. The results showed that IRF-3 was highly expressed in macrophages of spleen and lymph node from CRP-treated rats (Fig 7). The data confirmed that high IRF-3 ex-

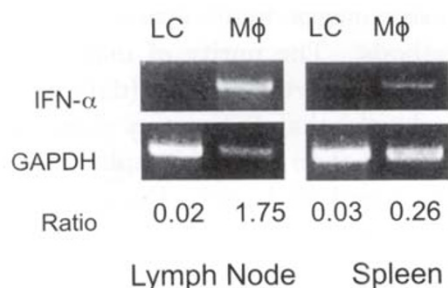


Fig 6. The mRNA expression of IFN-a in the fractions of draining LNC and spleen cells. Non-adherent cells (mainly lymphocytes) and adherent cells (mainly macrophages) were prepared from the draining LNC and spleen cells of CRP-treated rats and IFN-a expression was determined as described in the legend to Fig 3. M ϕ : adherent cells, LC: Non-adherent cells.

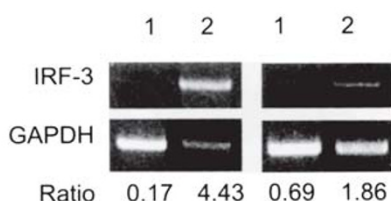


Fig 7. IRF-3 expression in macrophages from lymph nodes and spleen of CR-treated or CRP-treated rats. IRF-3 expression was determined as described in the legend to Fig 3. Lane 1: macrophages from CR-treated rats . Lane 2: macrophages from CRP-treated rats .

pression may result in IFN- α expression and in turn initiate Th1-type response.

PTX induced significant IFN- α production by macrophages in Fischer 344 rat

We have observed that only after PTX treatment, CFA and R16 immunized Fischer 344 rats were able to initiate Th1-type response and developed EAU. However CFA is not sufficient to induce EAU in Fischer344 rat, and PTX is required to be an addi-

tional adjuvant to initiate Th1-type response. Since IFN- α has a potential to induce Th1-type response and it is mainly produced by macrophages, the isolated macrophages from peritoneal cavity of naive Fischer 344 rat were stimulated in vitro with different stimuli as described in methods. The purity of macrophages separated from rat peritoneal cavity is up to 96% tested by phagocytosis assay (Data not shown). Trichosanthin (TCS) is a toxic plant protein served as a negative control. Lipopolysaccharide (LPS) served as a bacterial derivative control and it had been used as adjuvant for the induction of autoimmune disease[21]. IFN- α expression was detectable in LPS and PTX treated macrophages and their ratios of IFN- α /GAPDH were 0.21 and 0.74, respectively (Fig 8). The data clearly demonstrated that PTX did have a potential role to induce significant IFN-a expression and this effect may be related

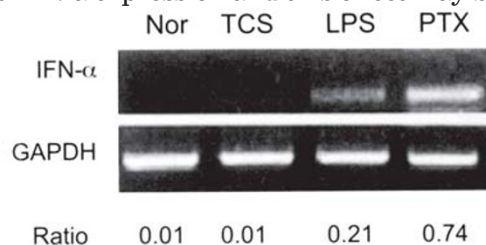


Fig 8. IFN- α was measured in macrophages in vitro. The cells were stimulated with different stimuli for 4 h and IFN-a expression were examined and described in the legend to Figure 3. Nor: no stimulation; TCS: TCS stimulation; LPS: LPS stimulation; PTX: PTX stimulation.

to the initiation of Th1-type response in Fischer344 rat.

DISCUSSION

Previous data indicated that Fischer 344 rat is EAU resistant[8]. However, if pertussis toxin (PTX) was given as an additional adjuvant, Fischer 344 rat is able to generate Th1-response and develop disease under the same conditions of immunization[3]. In this study we focused on examining what mechanism was involved in initiating Th1 response of EAU in Fischer 344 rat. We showed that, in the target tissue and draining lymph node, high IFN- α expression was associated with Th1-response, but IL-12 did not.

Since Ag-specific T-cells has a determining role in the induction of immune response, the high lymphocyte proliferation from the CRP-treated rats indicate that PTX may provide some signaling that is

required to induce T-cell proliferation in the induction of EAU disease.

According to our previous work, Th1-response is essential to induce EAU[3],[7], therefore next we asked whether EAU-developed Fischer 344 rats treated with additional PTX had a predominant Th1-type cytokine profiles. As we expected, Th1-cytokine (IFN- γ) was highly expressed in the disease target eye and draining lymph node. The results suggested that additional PTX had a synergic effect to initiate Th1-response and induce disease.

Then we asked which factor plays a crucial role in initiating Th1-reponse. IL-12 and IFN- α are two major regulators to control Th1-cell differentiation. IL-12 is a critical cytokine for induction of Th1-mediated immune response[15], and is found to be essential for Th1-cell-mediated autoimmune disease [16]. We originally believed that PTX was able to promote IL-12 secretion and then initiate Th1-response. To our surprise, the IL-12p40 mRNA expression was similar among three groups. These results suggested that there is an additional signaling pathway that contributes to initiate Th1-response in CRP-treated Fischer 344 rats.

Currently, it has been reported that type I interferon (IFN- α /IFN-b) also has the ability to induce Th1 differentiation in human. The evidences suggested that IFN- α could activate human T cells to favor the Th1 cell development[18]. However this regulatory role is not confirmed in rat system. In human, IFN- α binds to IFNAR (IFN-areceptor), IFNAR activation involves JAK-STAT pathway to promote IFN- γ production[19]. In our work, we demonstrated that IFN- α expression was associated with Th1- response in vivo and its expression paralleled to IFN- γ expression in response to R16-restimulation in vitro. Furthermore PTX was able to stimulate macrophages of Fischer 344 rat to produce IFN- α in vitro. All these data strongly suggested that IFN- α may involved in initiating Th1-response. IRF-3 expression was used to confirm IFN- α gene expression, since IRF-3 are specifically involved in IFN- α gene expression[5]. We confirmed that IRF-3 expression was also associated with IFN- α expression in draining lymph node cells and spleen cells of EAU rats. IRF-3 has been shown to regulate the IFN- α transcription by binding to the IRF-E elements of IFN- α promoters[20]. In our study,

high IRF-3 expression in EAU rat may reflect that it had a potential to promote IFN- α secretion.

In summary, in this study we hypothesized that additional PTX treatment may initiate IFN- α production and then promote IFN- γ production. This observation may represent one of mechanisms to explain that PTX+CFA+R16 immunized Fischer 344 rats were able to generate Th1-reponse and develop EAU.

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