Trakia Journal of Sciences, Vol. 3, No. 1, pp 43-48, 2005

Available online at:
http://www.uni-sz.bg

ISSN 1312-1723

Original Contribution

THE IMMUNOMODULATORY C3 BINDING GLYCOPROTEIN INDUCES IL-12 AND CHANGES IL-12/IL-10 RATIO IN STIMULATED HUMAN PBMC

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ABSTRACT

The induction of IL-12 and IL-10 production by the immunomodulatory C3 binding glycoprotein (C3bgp) was studied on human peripheral blood mononuclear cells (PBMC). Isolated PBMC from healthy human donors were cultured 24 hours with C3bgp isolated from *Cuscuta europea*. We studied also the influence of C3bgp on the cytokine production in LPS-, PHA- and PWM-stimulated PBMC. The levels of these cytokines in the various culture supernatants were subsequently determined using ELISA.

Results showed that amount of IL-12 was significantly higher in C3bgp-stimulated cultures in comparison with LPS-, PHA- and PWM-stimulated PBMC. C3bgp also significantly increased IL-12 in PHA- or PWM-stimulated cultures, but not in LPS-stimulated culture. C3bgp alone was a weak inducer of IL-10 production, compared to the other stimuli. Furthermore it inhibited IL-10 production after LPS, PHA and PWM stimulation. Taken together these results indicate that immunomodulatory C3 binding glycoprotein has the ability to change IL-12/IL-10 ratio in stimulated human PBMC.

Key Words: C3bgp, PBMC, IL-12, IL-10, PHA, LPS, PWM

INTRODUCTION

The immune response to foreign agents involves complex cell-cell interactions among different cell types, and is critically regulated by a network of soluble molecules, cytokines, released by activated immune cells. Early in the immune response professional antigen-presenting cells produce IL-12, a cytokine that provides an important functional bridge between innate and adaptive immune responses (1) The biologically active IL-12 is a 70 kD heterodimer (p70) composed of two covalently linked polypeptide chains, p35 and p40 (2). The production of IL-12 heterodimer requires coordinated expression of both p40 and p35 chains. The expression of *p40* gene is restricted to the cells which are able to produce biologically active heterodimer; in contrast *p35* gene is constitutively expressed in many cell types, including lymphocytes which are not known to produce IL-12 (2). Early IL-12 production can be induced mainly by intracellular pathogens or by their products, including LPS, lipoteicholic acid, protein extracts, and heat-shock proteins (3, 4). IL-12 mediates several biological activities on T and NK cells, including the induction of IFN-γ production and the enhancement of the cell-mediated cytotoxicity. The development of Th1 -cells is driven mainly by IL-12 (5). IL-12 production is tightly regulated by a positive feedback mechanism mediated through IFN-γ and by negative feedback through IL-10 (6).

IL-10 is the most important anti-inflammatory cytokine found within the human immune response. It is a potent suppressor of Th1 development, but is a prominent factor promoting Th2 immune response. IL-10 is also a potent inhibitor of monocyte/macrophage activation and of proinflammatory cytokine synthesis, including IL-12 and IFN-γ (6).

C3 binding glycoprotein (C3bgp)

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isolated from the parasitic plant *Cuscuta europea*, has a lectin – like characteristics (7). The experimental evidence presented in our previous reports show that C3bgp has strong immunostimulatory properties and enhanced specific antibody response (8). In mice C3bgp induced a specific group of cytokines including the hallmark cytokine of Th1 type response, IFN-γ (9).

To clarify immunomodulatory properties of C3bgp with a view to clinical and laboratory application, it was of interest to investigate its influence on IL-12 and IL-10 production by human immune cells.

MATERIALS AND METHODS

Subject selection

Twenty-two (22) healthy human volunteers with age ranging from 20 to 45 years (male and female) were recruited for this study. Informed consent was obtained from each participant. Subsequently 10 ml of peripheral venous blood was collected in sterilised ethylenediamine tetraacetic acid (EDTA) tubes.

C3bgp isolation

C3bgp was isolated from a total water extract of the seeds of the parasitic plant *Cuscuta europea* by affinity chromatography as described previously (7).

PBMC isolation

Each blood sample was diluted 1:1 with sterile phosphate buffered saline (PBS), layered carefully over Histopaque-1077 (Sigma) and followed by centrifugation at 1800 revolutions per min at 4°C for 20 min. The interface containing peripheral blood mononuclear cells (PBMC) was harvested and washed twice with cold serum free RPMI-1640 medium (Sigma). The washed cell pellet was re-suspended and PBMC viability was done using the trypan blue exclusion method. Finally, cells were counted according to the cell configuration.

Cell culture and stimulation

$1x10^6$ PBMC were cultured in sterile polystyrene tubes containing 2 ml RPMI 1640 media supplemented with: 100 U/ml penicillin, 100 μg/ml gentamycin and 0.3 mg/ml L-glutamine. The cultures were stimulated with: 30 μg/ml C3 binding glycoprotein (C3bgp), 1 μg/ml lipopolysaccharide from *E. coli* (serotype 026-B6) - LPS (Sigma), 10 μg/ml phytohemagglutinin – PHA (Sigma), 20 μg/ml pokeweed mitogen – PWM (Sigma); and co stimulated with: C3bgp+LPS, C3bgp+PHA, C3bgp+PWM with the indicated concentrations. Non-stimulated cultures were used as a control. All cultures were incubated at 37° for 24 h.

Cytokine determination

The quantitative determination of IL-12p40 and IL-10 after 24 h in culture supernatants was performed by ELISA using commercially available kits purchased from *BioSource, Austria*, following the manufacturer's protocol. Colour reaction developed was measured as OD units at 450 nm on an ELISA reader (*Rosys Anthos 2010, Austria*). The concentration of cytokines in pg/ml was obtained using the standards supplied in the kit.

Statistical analysis

The data were expressed as means and standard error of the mean. Student's t-test was used to determine the statistical differences between mean values. Differences were considered significant when the p value was less than 0.05.

RESULTS

C3bgp induced higher level of IL-12 in human PBMC compared to both LPS and plant lectins: PHA and PWM

The result presented in **Figure 1** shows that all stimulating substances induced IL-12 in human PBMC and significantly enhanced the quantity of IL-12 in the culture supernatants. There are no significant differences in IL-12 level in cultures containing LPS, PHA and PWM. In contrast, we observed that C3bgp induced significantly higher level of IL-12 compared to both LPS and plant lectins - PHA and PWM.

C3bgp induced lower quantity of IL-10 compared to both LPS and the plant lectins: PHA and PWM

IL-10 level also depends on the stimuli used. PWM stimulation produces the highest, followed by LPS, PHA and C3bgp (**Figure 2**). C3bgp induces significantly lower level of IL-10 compared to the other stimuli. PWM is a stronger inducer of IL-10 in human PBMC and significantly increases the quantity of this

cytokine in culture supernatants compared to C3bgp and PHA. The experimental data demonstrates that C3bgp is a weak inducer of IL-10 production compared to both LPS and plant lectins - PHA and PWM.

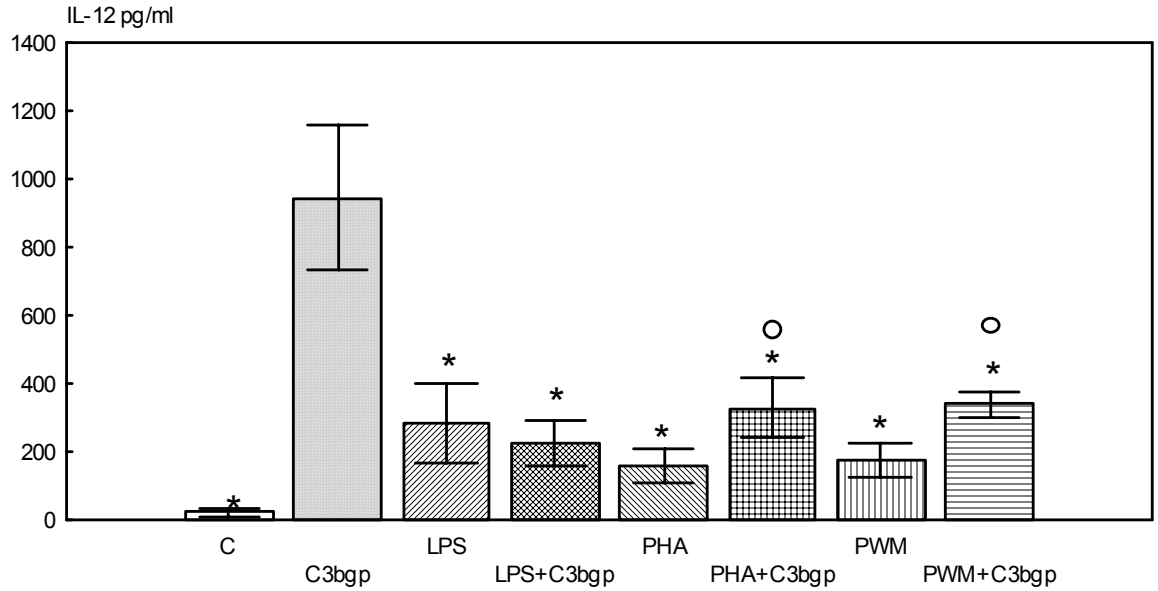

Figure 1: IL-12 production in human PBMC after stimulation with C3bgp, LPS, LPS+C3bgp, PHA, PHA+C3bgp, PWM, PWM+C3bgp and non-stimulated PBMC (C). The cytokine production is determined in culture supernatants after 24 h by ELISA. ∗ Significantly less than in C3bgp ($p<0.05$); ° Significantly greater than in cultures stimulated with corresponding stimulus (PHA and PWM) alone ($p<0.05$).

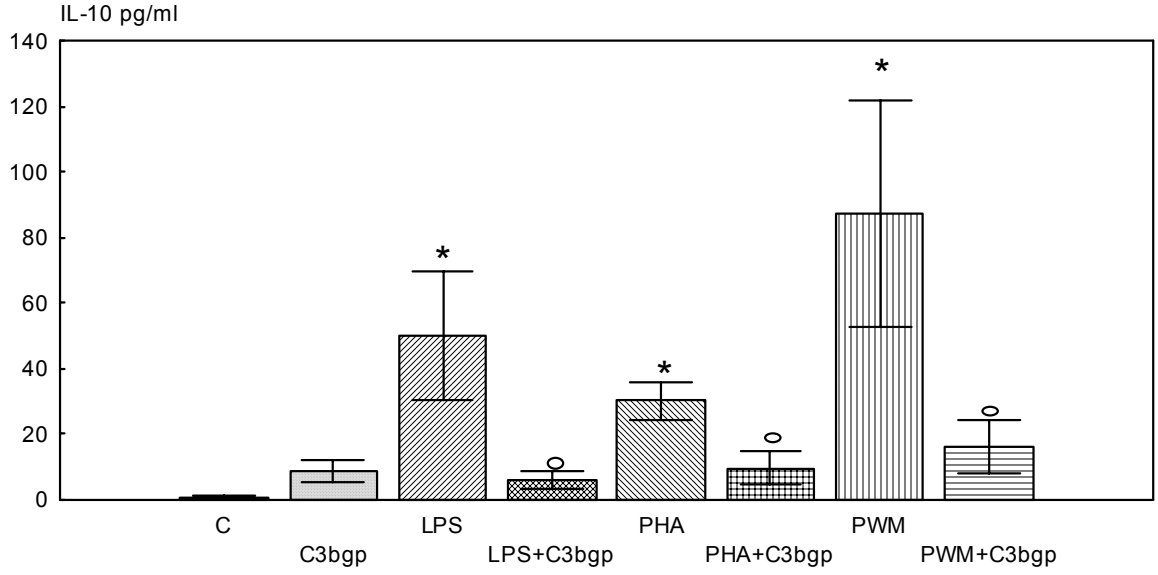

Figure 2: IL-10 production in human PBMC after stimulation with C3bgp, LPS, LPS+C3bgp, PHA, PHA+C3bgp, PWM, PWM+C3bgp and non-stimulated cultures (C). The cytokine production is determined in culture supernatants after 24 h by ELISA. ∗ Significantly greater than in C3bgp ($p<0.05$); ° Significantly less than in cultures stimulated with corresponding stimulus (LPS, PHA and PWM) alone.

C3bgp increased IL-12 production from PHA and PWM and decreased IL-10 production in both LPS and PHA or PWM stimulated human PBMC

We observed that C3bgp has different effects towards IL-12 production in LPS and PHA or PWM stimulated PBMC (**Figure1**). The results demonstrate that the addition of C3bgp slightly decreases IL-12 production in LPS stimulated cultures. In contrast, the effect of C3bgp on PHA and PWM stimulated PBMC is in opposite direction. The addition of C3bgp in PHA and PWM stimulated cultures significantly increases IL-12 production compared to cultures stimulated with

corresponding stimulus (PHA or PWM) alone.

The results obtained for IL-10 production from co-stimulated PBMC are presented in **Fig 2**. The data demonstrate that the effect of C3bgp on LPS, PHA and PWM stimulated PBMC is the similar. In all co-stimulated cultures the presence of C3bgp significantly decreases the level of the IL-10 production compared to cultures stimulated with LPS, PHA and PWM alone.

DISCUSSION

In the present study, we demonstrated that C3bgp alone is a powerful inducer of IL-12 production. The high level of IL-12 obtained from stimulation by C3bgp suggested a preferential stimulation of the monocytes. As is well known, the main source of IL-12 in PBMC fraction is the activated mononuclear phagocytes (1). C3bgp, in contradistinction to both PHA and PWM, has the ability to induce significant higher quantities of IL-12. As had been reported by other authors, PHA and PWM are weak inducers of the proinflammatory cytokines in PBMC cultures, because PHA primarily functions as a nonspecific activator of T lymphocytes while PWM stimulates helper T cells and, in association – the B cells (10, 11). Furthermore, the addition of C3bgp to PHA- or PWM-stimulated cultures significantly enhanced IL-12 production. These results and our previously published data for cytokine production from mouse peritoneal macrophages after *in vitro* stimulation with C3bgp confirmed our suggestion that C3bgp activates mainly the monocyte cells (9).

Further, we showed that C3bgp had a different effect on the IL-12 production compared to LPS. In contrast to LPS, C3bp was a very strong inducer of IL-12 production. The results also demonstrated that PBMC, after LPS stimulation under serum-free conditions, did not produce significantly higher quantity of IL-12, compared to both plant lectins PHA and PWM. The reason for using serum-free conditions was to avoid binding of C3bgp to the serum C3 component of complement that results in the formation of a stable C3:C3bgp complex (7). This complex might be recognised by monocyte complement receptors and can mediate the immunostimulatory activity of C3bgp, as we proposed in our previous paper (8). Therefore, to avoid the effect of C3bgp through complement receptors on monocytes we used PBMC stimulation in serum free conditions.

IL-12 is released during the early stages of infections caused by a large variety of bacteria, intracellular pathogens, fungi, and certain viruses. This early production of IL-12 is T cell-independent and is caused by direct interaction of pathogens or their products with phagocytic cells (2). The macrophage receptors involved with this interaction are not clearly understood. Gantner et al., 2003, indicated that Dectin-1 macrophage receptor which coupled with fungal β-glucans was involved with early IL-12 production (12). Other authors suggested that the mannose receptor may be involved with the secretion of this cytokine (13, 14).

IL-12 plays a key role in the modulation of the immune response by providing the stimuli for CD4+ T cells to differentiate toward Th1, IFN-γ secreting cells. Previously, we have reported that C3bgp had ability to induce a specific group of cytokines in mice, including higher level of IFN-γ (9). Therefore, C3bgp has capacity to induce in vivo and/or *in vitro* the key cytokines directing the immune response to Th1 cell-mediated response.

C3bgp increased negligible anti-inflammatory IL-10 production in human PBMC. Furthermore, the addition of C3bgp significantly decreased IL-10 production induced by LPS, PHA or PWM. IL-10 provided the main negative regulatory feedback on proinflammatory cytokine production (15). C3bgp not only had the ability to generate IL-12 from human mononuclear cells by direct stimulation, but simultaneously inhibited the cytokine feedback control mechanism.

Because a Th1 type immune response is crucial to the successful elimination of intracellular pathogens, such as certain bacteria (*Mycobacterium tuberculosis*), viruses (HIV), protozoa (*Plasmodium falciparum*), the changes in IL-12/IL-10 ratio influenced the outcome and immunopathology of infections caused by these pathogens (16, 17, 18). Both *in vitro* and *in vivo* experiments have demonstrated also that IL-12 plays a crucial role in the development of specific immunity against intracellular pathogens, including *Leishmania major, Mycobacterium tuberculosis, Listeria monocytogenes, and Toxoplasma gondii* (19, 20, 21, 22). Inverse modulation of IL-12/IL-10 ratio is reported also in some type of cancer with various malignancies (23, 24). In cancer patients treatment with exogenous IL-12 or pharmacological modulation of endogenous IL-12 production augment cytolytic activity of NK cells and direct immune response to Th1

type immune response (25, 26).

In summary, these studies demonstrate that C3bgp has the ability to change IL-12/IL-10 ratio by stimulation of IL-12 and inhibition of IL-10 production from stimulated human PBMC. Finally, the results may be of use in the development of immunomodulatory agents, of therapeutic importance, capable of modulating IL-12/IL-10 ratio as in the experimental models.

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