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Immunomodulatory activity accompanying chicken egg yolk immunoglobulin Y

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ABSTRACT Immunity transfer from a mother to the newborn does not depend exclusively on immunoglobulins. Peptides, which are characterized by immunoregulatory properties that accompany IgG_2, known as proline-rich polypeptide complex (PRP), have been discovered for the first time in ovine colostrum. In this report we present new data showing that some immunoregulatory peptides associated with the main immunoglobulin class, IgY, are also present in the avian immune system. Cytokine-inducing activity of particular fractions obtained from ovine colostrum, IgG+ (IgG_2 containing PRP), IgG− (IgG_2 free of PRP), and purified PRP, was compared with that of crude egg yolk IgY (IgY+), additionally purified egg yolk IgY (IgY−), and polypeptides accompanying IgY named Yolkin (Y), using an ex vivo model of whole human blood cells. It was shown that both IgG+ fraction and PRP, but not IgG−, stimulated the whole blood cells to release tumor necrosis factor-α and interleukin-1β cytokines. Similar experiments performed with hen's egg IgY preparations showed that IgY+ and Y samples showed higher cytokine-inducing activity than samples additionally purified with the use of size exclusion chromatography (IgY−). The IgY+ at a dose of 100 μg was even more active than the positive lipopolysaccharide control. It was also found that Y is able to stimulate macrophage cell line J774.2 to release nitric oxide. The results obtained suggest that IgY, the main chicken immunoglobulin fraction, is accompanied by additional polypeptides and plays a role of a transporter of biologically active substances, which was observed in the case of colostral IgG.

Key words: chicken egg yolk, immunoglobulin Y, cytokine induction

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INTRODUCTION

The immune system of newborns is not fully developed. In humans and rodents IgG is transferred through the placenta, whereas in ruminants the immunity (mainly IgG) is transferred with colostrum and milk. The bird's immune system consists mainly of primary and secondary lymphoid organs. Functionally, it may be divided into innate, nonspecific and adaptive, specific. The noncellular branch includes 3 immunoglobulin classes: IgA, IgM, and IgY. The IgA and IgM are similar in molecular weight, structure, and electrophoretic mobility to their mammalian counterparts. The major avian immunoglobulin class IgY present in the serum and egg yolk shows fundamental structural differences in comparison with IgG molecule (e.g., higher molecular weight than that of mammalian IgG, the presence of an additional C_H domain and 2 carbohydrate side-chains, a lower content of β-sheet structure, and more disordered structure compared with mammalian IgG). It also differs in proteolysis, temperature, pressure, and freezing stability (Chalghoumi et al., 2009). The IgY binds neither to proteins A and G, nor to the mammalian complement, and does not cross-react with mammalian immunoglobulins (Jensenius et al., 1981; Larsson et al., 1991).

In mammals, the transfer of maternal antibodies can take place after birth. However, in the chicken, the maternal antibodies must be transferred to the developing embryo to give acquired immunity to the chick. The IgA and IgM are incorporated into the egg white during egg formation (Rose and Orlans, 1981; Warr et al., 1995; Aveskogh and Hellman, 1998). Serum IgY is selectively transferred to the yolk via a receptor on the surface of the yolk membrane that is specific for IgY translocation. Hen's eggs are very convenient tool for antibody production. The serum concentration of IgY have been reported to be 5.0 mg/mL. Antigenic stimulation can yield more than 100 mg of IgY from one egg. A laying hen produces approximately 20 eggs per month; therefore, more than 2 g of IgY per month can

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be obtained from a hen. Hyperimmune or immune eggs are laid by hens that have typically been stimulated with inactivated microorganisms or purified antigens. Stimulation of the hens results in the formation of eggs containing high level of antibodies, predominantly the IgY class (Warr et al., 1995; Aveskogh and Hellman, 1998; Schade et al., 2005; Nilsson et al., 2007; Dias da Silva and Tambourgi, 2010). Passive immunization by oral administration of specific antibodies has been an attractive approach against gastrointestinal pathogens in both humans and animals. Immunoglobulin Y is an alternative to antibiotics in the treatment of various infections with antibiotic-resistant pathogens [e.g., *Escherichia coli*, *Salmonella*, *Staphylococcus*, *Coronavirus*, and *Rotavirus* (Mine and Kovacs-Nolan, 2002; Liou et al., 2011)].

The immunity transfer from a mother to the newborn does not only depend on immunoglobulin. Colostrum and milk are rich in proteins and peptides, which play a regulatory role and may stimulate the neonate immune system. Peptides, which are characterized by immunoregulatory properties that accompany IgG_2, have been discovered for the first time in ovine colostrum (Janusz et al., 1974). Because of high proline residue content, they are referred to as proline-rich polypeptide complex (**PRP**), subsequently named Colostrinin. The complexes similar to PRP were also found in human, bovine, and caprine colostra (Piasecki et al., 1997; Sokołowska et al., 2008).

The PRP possesses immunoregulatory properties, including effects on humoral and cellular immune responses, and has regulatory activity over Th1 and Th2 cytokine induction and the ability to inhibit the overproduction of reactive oxygen species and nitric oxide. In addition to its immunoregulatory activity, PRP also shows psychotropic properties, improving cognitive activity and the behavior of old rats, chickens, and humans (for reference see Janusz and Zabłocka, 2010). When administered in the form of sublingual tablets, the PRP can stabilize or improve the health status of patients with Alzheimer's disease (Bilikiewicz and Gaus, 2004).

It was very interesting to find that some immunomodulatory peptides associated with the main immunoglobulin class are present not only in the mammalian, but also in the avian immune system. Given that hen eggs are easily available, it is of interest to determine if the egg contains regulatory substances with potential clinical value. Therefore, the goal of this project was to determine if immunomodulatory peptides are associated with chicken IgY that is derived from egg yolk.

MATERIALS AND METHODS

Materials

The RPMI 1640 medium was obtained from the Laboratory of Biopreparations of the Institute of Immunology and Experimental Therapy. The tissue culture plates were obtained from Costar (USA). Bacterial lipopolysaccharide (**LPS**) from *E. coli* (serotype 055:B5), phytohemagglutinin (**PHA**) from *Phaseolus vulgaris*, L-glutamine, and antibiotics (penicillin/streptomycin mixture) were obtained from Sigma (Germany). Fetal bovine serum (**FBS**) was obtained from Gibco BRL (UK). The ELISA sets for cytokine determination [interleukin (**IL**)-1β and tumor necrosis factor (**TNF**)-α] were purchased from BD Pharmingen (USA).

Blood samples from healthy donors were kindly provided by the Station of Blood Donation, 4th Military Hospital, Wrocław, Poland. The murine macrophage-like cell line, J774.2, was obtained from the American Type Culture Collection.

Chicken IgY was isolated from hen egg yolk by the method described by Ko and Ahn (2007) with modifications. Briefly, the crude fraction of IgY (termed **IgY+**) was further purified with size exclusion chromatography (**SEC**) on a Sephacryl S-100 column to separate IgY (termed **IgY−**) from low molecular mass proteins (termed Yolkin or abbreviated as **Y**). A crude fraction of IgG_2 (termed **IgG+**) derived from ovine colostrum was further purified to separate IgG_2 (termed **IgG−**) from the PRP complex by the method of Janusz et al. (1981). The bovine and equine IgG_2 (termed IgGb and IgGh, respectively) was obtained from a commercial source. All proteins were adjusted to a concentration of 1 mg/mL for use in cytokine induction experiments.

Cytokine Induction in Human Whole Blood Cell Cultures and Cytokine Determination

Cytokine secretion was induced according to the procedure described by Inglot et al. (1996). Blood samples from at least 5 donors were collected into syringes containing sodium heparin. Within 1 h after the collection, the blood was diluted 10-fold with RPMI 1640 medium supplemented with penicillin/streptomycin, L-glutamine, and 2% FBS. One-milliliter portions of the cell suspension were transferred in duplicate to 24-well flat-bottomed tissue culture plates. The inducers, at doses of 1 to 100 μg, were added in the volume of 100 μL of RPMI 1640. As a reference, a positive inducer LPS (4 μg/mL) was used. The control wells containing the nontreated blood cell sample were used to measure the spontaneous production of cytokines (negative control). The plates were incubated at 37°C for 22 h in a 5% CO_2 atmosphere. After incubation, the plates were centrifuged at 200 × *g* at room temperature for 15 min. The supernatants were collected and used for cytokine determination.

Interleukins IL-1β and TNF-α were determined by ELISA test using commercially available ELISA Sets from BD Pharmingen according to the procedure recommended by the manufacturer.

NO Generation

The murine macrophage-like cell line J774.2 was maintained in RPMI 1640 supplemented with 10%

heat-inactivated FBS, 3% L-glutamine, and penicillin/streptomycin. Cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Adherent cells from confluent cultures were detached, centrifuged at 150 × *g* for 10 min, and resuspended in complete culture medium to 1×10^6 cells/mL. Aliquots (1 mL) were placed in individual wells of 24-well cell-culture plates and allowed to adhere to the surface for 1 h. Samples of Y at doses of 1, 10, and 25 μg/mL were added to the wells, and the plates were incubated at 37°C. Lipopolysaccharide from *E. coli* (8 μg/mL) was used as positive control. After 24 h, samples of the culture supernatants were collected and stored at −20°C for nitrite determination.

NO Determination

Nitrite and nitrate levels, indicators of NO synthesis, were measured in supernatants after reduction of nitrate to nitrite with NADPH nitrate reductase as described by Guevara et al. (1998) and Moshage et al. (1995) with some modifications. One hundred microliters of supernatants was incubated for 45 min at 37°C with nitrate reductase (25 mU per sample) in the presence of β-NADPH (final concentration 80 μ*M*) in 20 m*M* Tris buffer, pH 7.5. The total volume of the reaction mixture was 300 μL. After the enzymatic conversion, nitrite concentration in the supernatants was measured by Griess reagent [0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid]. Each sample was treated with an equal volume of the Griess reagent by 10 min at room temperature, and the absorbance at 550 nm was measured. The concentration of nitrite was calculated from $NaNO_2$ standard curve.

Statistical Analysis

Each experiment was repeated 5 times at least in duplicates. Data are presented as the median ± SD.

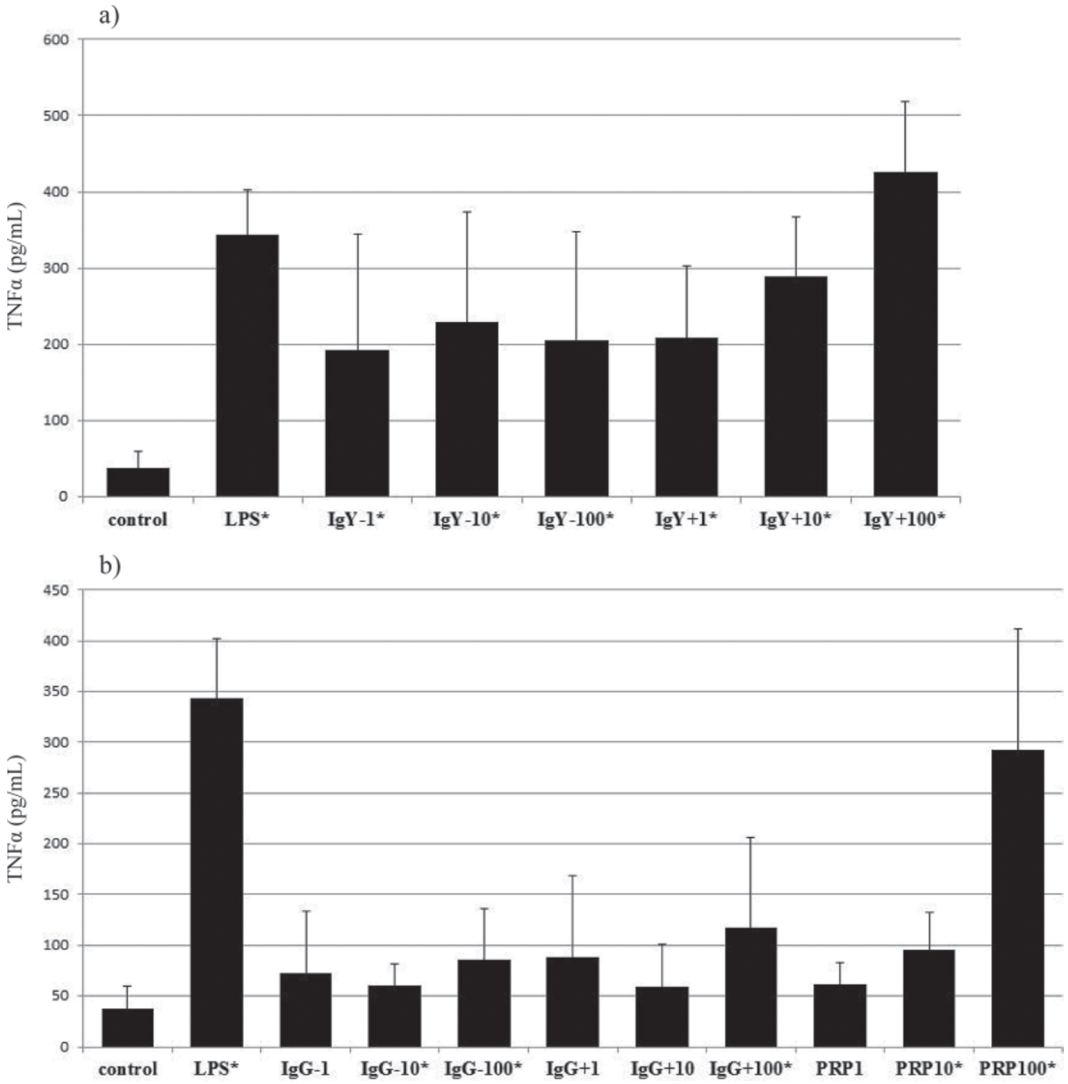

Figure 1. Tumor necrosis factor (TNF)-α-inducing activity: a) IgY, b) ovine IgG_2 (control, untreated cells; LPS, lipopolysaccharide from *Escherichia coli* as positive control; IgY+, isolated IgY; IgY−, purified IgY fraction; IgG−, IgG_2 fraction; IgG+, IgG_2 + PRP complex; PRP, proline-rich polypeptide complex). Data are presented as the median ± SD. The results were considered significant by the nonparametric Wilcoxon test when $P \leq 0.05$ (*) versus control (untreated whole blood cell sample).

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RESULTS AND DISCUSSION

It was shown in newborn Holstein male calves that oral administration of egg yolk enriched in bovine rotavirus-specific IgY modulated the immune response against BVR infection at the mucosal level (Xu et al., 2006) It is not clear whether this immunomodulation was exclusively due to the antibodies themselves or to the presence of other bioactive factors as well (Nelson et al., 2007; Vega et al., 2011).

The results obtained in our earlier studies show that ovine colostral IgG_2 is accompanied by immunoregulatory PRP (Janusz et al., 1974, 1981). It was interesting to study if the IgY, the main chicken immunoglobulin, shows some immunoregulatory properties, similarly to ovine IgG_2. For this reason, we compared the cytokine-inducing activity of a particular fraction obtained after isolation and purification of PRP from ovine colostrum: IgG_2 containing PRP (named IgG+), PRP deprived IgG_2 (named IgG−) and isolated PRP with that of crude and additionally purified IgY fraction (IgY+ and IgY−, respectively), and low molecular fraction after SEC (Y). The cytokine induction was determined in ex vivo stimulated human whole blood cell cultures. The use of unseparated whole blood cultures mimicked the natural microenvironment in which the different leukocyte populations can cooperate. This concept was validated by the use of LPS-stimulated cultures as positive

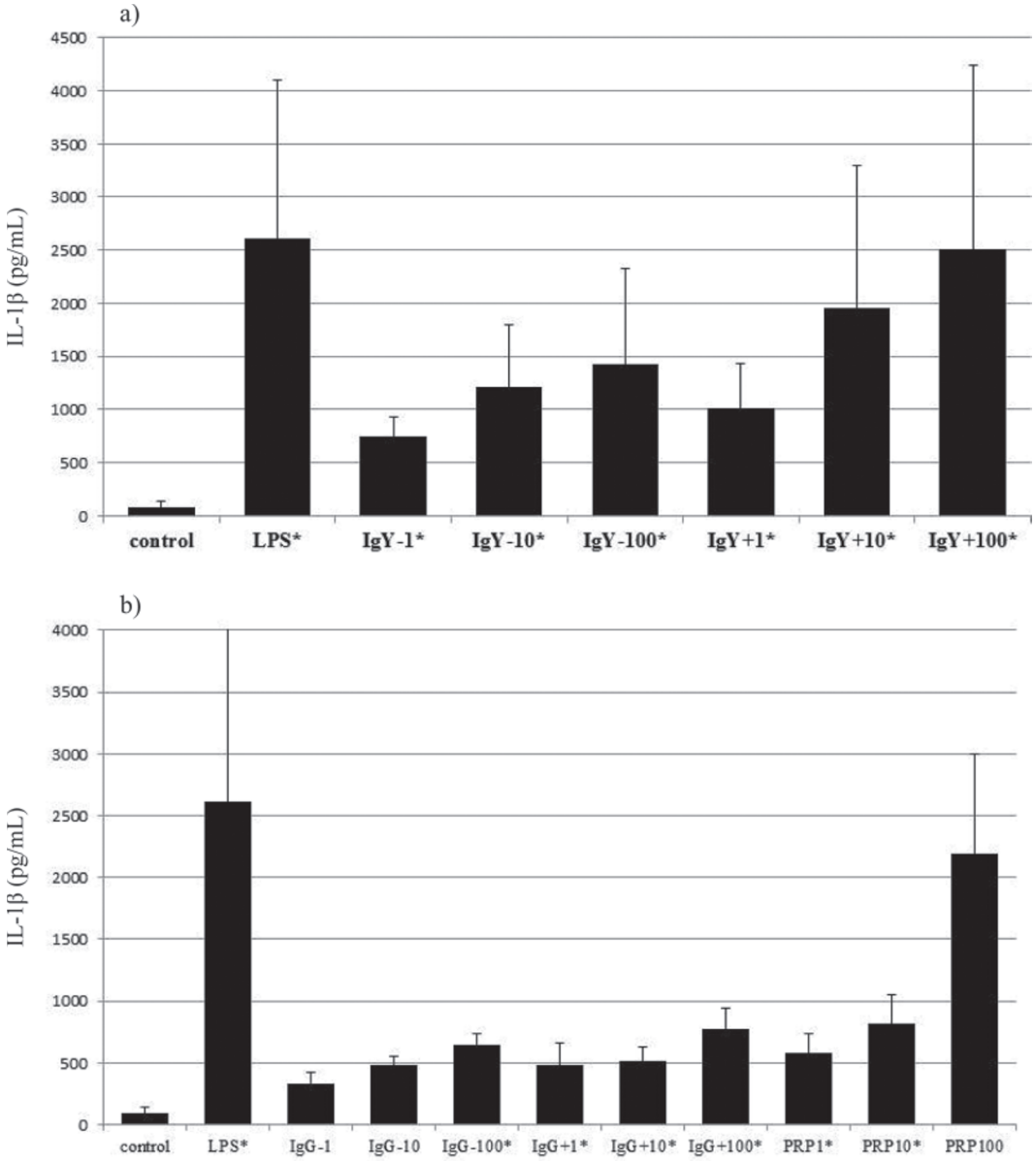

Figure 2. Interleukin (IL)-1β inducing activity: a) IgY, b) ovine IgG_2 (control, untreated cells; LPS, lipopolysaccharide from *Escherichia coli* as positive control; IgY+, isolated IgY; IgY−, purified IgY; IgG−, IgG_2 fraction; IgG+, IgG_2 + PRP complex; PRP, proline-rich polypeptide complex). Data are presented as the median ± SD. The results were considered significant by the nonparametric Wilcoxon test when $P \leq 0.05$ (*) versus control (untreated whole blood cell sample).

controls. The LPS-treated controls revealed a statistically significant increase in TNF-α and IL-1-β in all experiments.

It was found that IgG+ fraction, at doses of 1 to 100 μg/mL, and PRP at doses of 1 to 100 μg/mL, stimulated the whole blood cells to release TNF-α and IL-1β cytokines. It is worth noting that the cytokine-inducing activity of IgG+ is higher than purified IgG (IgG−; Figure 1b, Figure 2b). This confirms that immunoglobulin plays a role of a transporter of PRP, a biologically active substance important for the modulation of cellular signaling pathways. To find the presence of IgY-associated peptides with biological activities similar to PRP, we compared the cytokine inducing activity of IgY isolated from hen's eggs (IgY+), IgY purified using SEC chromatography (IgY−), and IgY-associated peptides (Y) with the activities observed in PRP.

It was found that IgY+ shows a higher cytokine inducing activity than purified IgY (IgY−) and was dose dependent (Figures 1a and 2a). The IgY+ at concentration of 100 μg/mL showed the same or even higher activity than the LPS-treated control in stimulating both IL-1β and TNF-α production. Bovine and horse IgG were used as reference samples at concentrations of 1, 10, and 100 μg/mL did not activate (or activated very weekly) a release of TNF-α and IL-1β cytokines by human whole blood cells (data not shown). It was also found that the low molecular mass fraction Y showed high cytokine-inducing activity (Figure 3a,b), and also stimulated nitric oxide release from the macrophage cell line J774.2 (Figure 3c).

The results obtained in the present study suggest that IgY, the main chicken immunoglobulin fraction, is accompanied by an additional polypeptide or polypeptide complex, similarly to the results observed in colostral IgG. It is the first demonstration of immunomodulatory low molecular mass substances associated with hen egg-derived chicken IgY. Taking this into account, additional studies to isolate and characterize IgY-associated peptides will be required to understand their structure and biological properties and to identify an immunomodulatory substance possessing the properties similar to those of the PRP from ovine colostrum.

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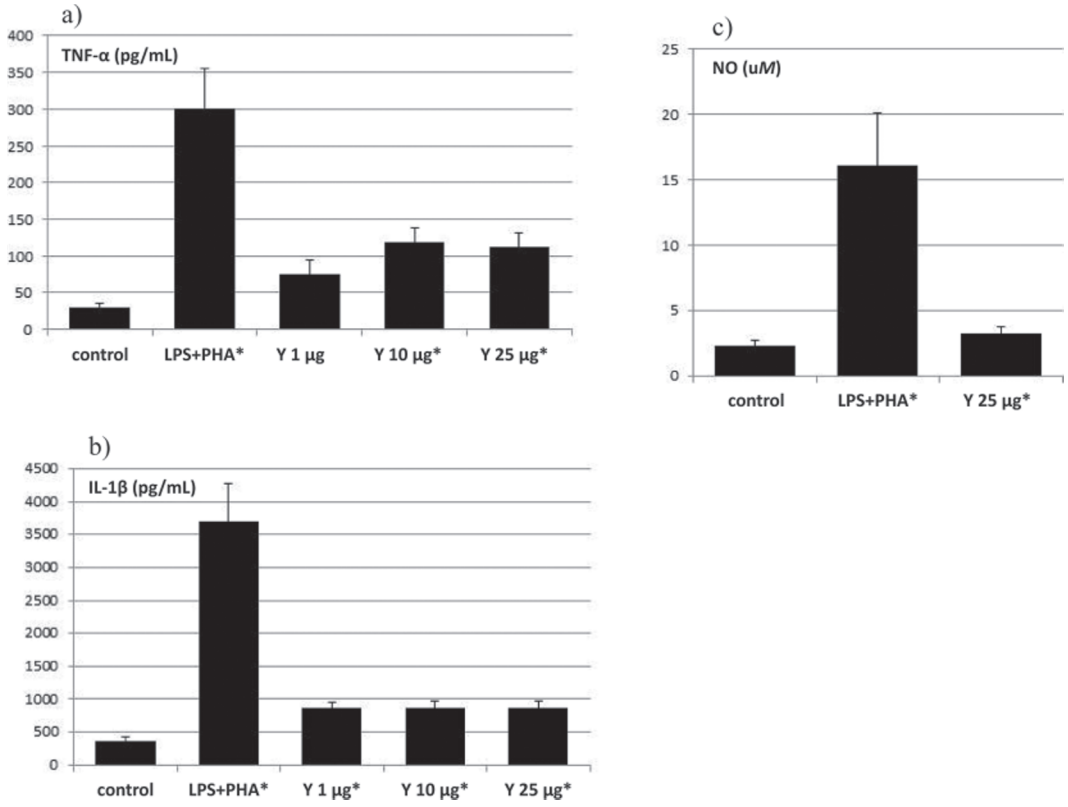

Figure 3. Activity of Yolkin (Y) sample: a) tumor necrosis factor (TNF)-α, b) interleukin (IL)-1β, c) nitric oxide (NO; control, untreated cells; LPS, lipopolysaccharide from *Escherichia coli* as positive control; PHA, phytohemagglutinin as positive control; Y, low molecular fraction after size exclusion chromatography). Data are presented as the median ± SD. The results were considered significant by the nonparametric Wilcoxon test when $P \leq 0.05$ (*) versus control (untreated whole blood cell sample).

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