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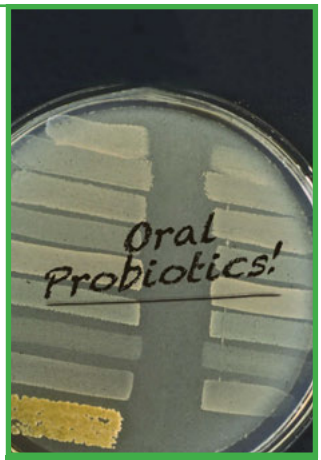
In vitro oxidative burst assay to evaluate the efficacy of Immune-G-matrix™, an innovative yeast-β-glucans, zinc and copper-based formulation

Daniela Buonocore[1], Antonio Seneci[2], Maria Elena Carrabetta[2], Federico Pialorsi[2], Maria Zurlo[2], Maria Grazia Bottone[1], Paola Veneroni[1], Manuela Verri[1], Maurizia Dossena[1]



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Correspondence to:
Daniela Buonocore
daniela.buonocore@unipv.it

Abstract

Oxygen is necessary for the survival of aerobic organisms, while at the same time it is able to acquire electrons generating toxic molecules called reactive oxygen species (ROS) (e.g. superoxide anion, hydrogen peroxide). ROS can generate reactive oxygen metabolites, causing oxidative damage to biological macromolecules. On the other hand, in the higher organisms the immune system uses ROS against pathogens. Infections or other stimuli can induce cell activation (e.g. neutrophil cells) and functional responses, such as the oxidative or respiratory burst and later ROS release. Subjects with deficits in intracellular defence mechanisms have an increased susceptibility to infections by microorganisms. The aim of the present study was to evaluate *in vitro* the capacity of an innovative yeast-β-glucans, zinc (Zn) and copper (Cu) formulation, Immuno-G-Matrix™, based on G-MatrixLipid™ technology, to induce oxidative burst in neutrophil cells in comparison with an activator, phorbol 12-myristate 13-acetate (PMA). Oxidative burst evaluation was based on the capacity of released ROS to change the fluorescent properties of a substrate, dihydrorhodamine 123 (DHR). We measured the intensity of fluorescence compared with a negative control sample (differentiated cells) and a positive control sample (cells incubated with PMA). The results showed that Immuno-G-Matrix™ was able to induce functional responses, such as the neutrophil oxidative burst, as is the activating agent PMA.

1. Introduction

The immune system is a network of cells, tissues and organs that work together to protect the body from infection, acting against invading microbes, infected cells and tumour cells while not attacking itself; it has also the capacity to 'know' its own cells (self) and to 'recognize' as foreign the cells of another organism (non-self). The immune system has three principal functions: (1) protection against pathogens; (2) removal of damaged or dead cells and tissues and aged red blood cells; and (3) recognition and removal of cells with aberrations (e.g. induced neoplastic cell transformations). One of the principal mechanism is the 'oxidative burst', a phenomenon during which there is an increase in oxygen consumption that produces different reactive oxygen species (ROS), such as hydroxyl radical, superoxide and hydrogen peroxide generated by NADPH oxidase after neutrophil activation in response to infection [1]. ROS attack and damage the membranes of pathogens thus inducing their death. Subjects with suboptimal microor-

[1] Department of Biology and Biotechnology, "L. Spallanzani" University of Pavia, Pavia, Italy
[2] Truffini & Reggè Farmaceutici Srl, Milan, Italy

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ganisms/intracellular defence are more susceptible to infection [2,3]. It is well known that a healthy balanced diet is important for the immune system and cell protection against oxidative stress (an unbalanced condition between oxidative species and antioxidant defence) [4]. Food supplements are useful for limiting the damage caused by a deficient and unbalanced diet. In this study we present an innovative formulation, Immuno-G-Matrix™, which contains a special thermo-plastic granulate of β-glucans from yeast [5,6], zinc (Zn) and copper (Cu), each of which plays an important role in maintaining and improving the functionality of the immune system. Recently, the European Food Safety Authority (EFSA) evaluated the immune-modulating effect of these three components (yeast-β-glucans, Zn and Cu) as positive [7-9].

β-Glucans are a heterogeneous group of natural polysaccharides, consisting of D-glucose monomers linked by (1,3)-β, (1,4)-β or (1,6)-β glycosidic bonds. Usually, β-glucans present a linear constructed backbone of various lengths with side-chains of D-glucose attached by (1,4)-β or (1,6)-β bonds. Their activity depends on the molecular structure, in particular the primary chemical structure, size, molecular mass, tertiary structure, polymer charge and solution conformation (triple or single helix or random coil, branching frequency, structural modification, conformation and solubility) [10]. The literature shows that β-glucans, (1,3)-β-linked backbones with small numbers of (1,6)-β-linked side chains from yeast and fungi, modulate the immune system and protect against bacteria, viruses and other pathogens. Traditionally, macrophages and dendritic cells are considered the main target cells of β-glucans, although neutrophils, B cells, T cells and natural killer cells are also known to be activated [5,6]. The immune modulating activities of β-glucans are usually studied with reference to the activation of macrophages, which together with dendritic cells have typical cell surface receptors called PRRs that detect pathogen-associated molecular patterns (PAMPs). Since β-glucans cannot directly penetrate cell membranes due to their large molecular size, they might act as PAMPs by recognizing PRRs. The major PRRs for β-glucans might be dectin-1 and the Toll-like receptor (TLR); upon binding with β-glucan, dectin-1 and TLR might induce signalling pathways and activate immune cells [5,6,11]. The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) delivered the scientific opinion that the allergenic risk of the yeast β-glucans is no higher than the risk from other products containing baker's yeast. β-Glucans from other sources have already been evaluated for safety by EFSA [8].

Zn is present in all tissues and has structural, regulatory and catalytic roles. Zn deficiency is associated with a decline in immune function, reduced cell-mediated and antibody-mediated responses, and appears to induce apoptosis, resulting in a loss of B-cell and T-cell precursors within the bone marrow. Moreover, it appears to reduce the production of cytokines by mononuclear cells, increasing susceptibility to infection. Zn supplementation can contribute to maintaining an adequate Zn status [12-14]. In the scientific opinion of the EFSA NDA, the Panel concluded that a cause and effect relationship has been established between the dietary intake of zinc and normal function of the immune system [7]; therefore, zinc achieved the health claim "zinc contributes to the normal function of the immune system" (Commission Regulation (EU) No 432/2012).

Cu in living organisms, including man, plays the principal role of cofactor for specific enzymes and electron transport proteins involved in energy or antioxidant metabolism [15], for example cytochrome C oxidase and superoxide dismutase (SOD), which are involved in electron transport and antioxidant defences, respectively. Moreover, a lack of Cu results in neutropenia in humans receiving parenteral nutrition. Individuals described in case studies [16] who had received long-term parenteral nutrition for months to years were diagnosed as Cu deficient. Neutropenia was reversed upon the reintroduction of Cu [16]. Therefore, Cu deficiency has negative effects on neutrophil and macrophage function [17]. It was also observed that Cu deficiency suppressed various lymphocyte and phagocytic cell activities in both animals [18] and humans [19-21]. In the scientific opinion of the EFSA NDA, the Panel concluded that a cause and effect relationship has been established between the dietary intake of copper and the normal function of the immune system [9]; therefore, a claim concerning copper and the maintenance of the normal function of the immune

system has already been favourably assessed (Commission Regulation (EU) No 432/2012).
The aim of the present study was to evaluate the efficacy of the innovative formulation Immuno-G-Matrix™ to induce cell activation in neutrophils evaluated as oxidative burst. Oxidative burst was evaluated by an *in vitro* experimental system using the activator phorbol 12-myristate 13-acetate (PMA). The oxidative burst induced by Immuno-G-Matrix™ was measured by cytometry and fluorescence microscopy techniques using HL-60 cells differentiated in neutrophils (98%).

2. Materials and Methods

2.1 Sample

Immuno-G-Matrix™ was tested. This formulation contains a special thermo-plastic granulate of β-glucans from yeast Saccharomyces cerevisiae [5,6], zinc oxide and copper gluconate. The β-glucans are characterized by a backbone of β-(1-3)-linked β-D-glucopyranosyl units with β-(1-6)-linked side chains of varying distributions and lengths. This thermo-plastic granulate maximizes the immune stimulating activity of β-glucans [5] using a Zn and Cu combination; the modulating activity and efficacy on the immune system of Zn and Cu has been positively evaluated by EFSA [7,9]. The innovative technology G-MatrixLipid™ allowed granulates to be produced for food use by mixing the active ingredients with a food grade lipid-matrix in order to obtain better enteric bioavailability. The analyzed concentration of Immuno-G-Matrix™ was 0.06 mg/ml, chosen according to the established daily intake per person (300 mg/capsule/day).

2.2 Cell culture and experimental procedure

The HL-60 cell line used for this study (accession number ICLC HTL95010) was obtained from the European Collection of Authenticated Cell Cultures (ECACC); species: human, Caucasian; tissue: peripheral blood; tumour: leukaemia, promyelocytic; lymphoblast-like morphology [22]. Other HL-60 cell line properties are reported in Table 1.
HL-60 cells were cultured in complete medium RPMI 1640 with the addition of 1.25% DMSO for 7 days to differentiate them along the granulocytic line [23]; HL-60 cells differentiated in neutrophils at 98%. Cell differentiation was monitored using two monoclonal antibodies, purified mouse anti-human CD123 and CD63 (BD Pharmingen™) [24-26], conjugated to fluorescein isothiocyanate FITC and recognizing, respectively, the two membrane markers: CD 123 [27] and CD 63 [28]. BD Pharmingen™ technical protocols were followed.
Subsequently, differentiated cells were incubated with: (1) PMA (0.5 nM; Sigma p8139); (2) Immuno-G-Matrix™ (0.06 mg/ml); and (3) PMA (0.5 nM) + Immuno-G-Matrix™ (0.06 mg/ml).
Incubation time to induce cell activation was 20 min [24, 29]. The negative control sample was not incubated with PMA or Immuno-G-Matrix™.
The tested samples were as follows:

- negative control sample: ctr (–) (differentiated cells)
- positive control sample: ctr (+) PMA (differentiated cells incubated with PMA)
- immuno-G-Matrix™: differentiated cells incubated with Immuno-G-Matrix™
- PMA + Immuno-G-Matrix™: differentiated cells incubated with PMA + Immuno-G-Matrix™.

Cell line name	HL-60
Culture conditions	Medium RPMI 1640 + 10% fetal bovine serum (FBS) + 2 mM L-glutamine + 1% antibiotics penicillin/streptomycin (10,000 U/ml); split confluent cultures 1:5–1:10; maintain cultures 5–9×10^5 cells/ml; 37°C; 5% CO_2
Properties	Phagocytosis; antitumor testing; differentiation; pharmacodynamics; cloning; susceptible to: HIV-1, HTLV-1
Species validation	Validated by isoenzymes: confirmed as human with malate dehydrogenase (MDH), mannose phosphate isomerase (MPI), nucleoside phosphorylase (NP)
Tumorigenicity	Tumorigenic in nude mice
Karyology	Pseudodiploid
Freezing medium	Culture medium + 50% FBS + 10% dimethyl sulfoxide (DMSO)
Sterility	Mycoplasma negative, HOECHST and PCR
Differentiation conditions	After treatment with DMSO, the cells differentiate along the granulocytic line (after 4 days in 1.25% DMSO, 72% of the cells become CD11b positive)

Table 1 - Cell line properties (ICLC HTL95010 from the European Collection of Authenticated Cell Cultures)

2.3 Oxidative burst assay

Intracellular ROS generation by activated neutrophils was detected by a cytofluorometric assay using dihydrorhodamine (DHR) 123, a cell-permeant non-fluorescent probe/substrate that becomes a brightly fluorescent lipophilic cation, rhodamine 123 (Rh123), when oxidized by the action of hydrogen peroxide. For this study, samples were stained with DHR123 (0.45 µM). For neutrophil ROS production, samples (differentiated cells incubated with PMA, Immuno-G-Matrix™ and both PMA and Immuno-G-Matrix™) containing about 0.5×10^6 leukocytes (volume range, 50–150 µl) were incubated in a polypropylene tube for 20 min at 37 °C, along with 0.45 µM DHR123. Phosphate buffered saline (PBS) 1× was added to the mixture to achieve a final volume of 1 ml [30]. Following incubation with DHR, all samples were fixed with formalin 1 % in PBS 1× for 5 min, washed with PBS 1× and read immediately with a flow cytometer (Partec Pas, Münster, Germany) equipped with an argon laser (λ 488 nm). At least 20,000 cells/sample were considered and four independent experiments were carried out to calculate the mean of the score values. Green fluorescence (due to FITC) was measured using an interference filter (λ 545 nm). The intensity of fluorescence found for Immuno-G-Matrix™ was compared with a negative control (differentiated cells) and a positive control (cells incubated with PMA). Data were displayed on a graph and expressed in (%) of fluorescence intensity (FI). The computerized data provided, in real time, histograms showing the percentages of DHR fluorescence for each sample. Moreover, samples after being stained with DHR were photographed using a fluorescence microscope (Olympus BX50), under excitation conditions of filter BP 450–480 nm, dichroic mirror DM 500 nm and barrier filter BA 515 nm.

2.4 Data Analysis

Descriptive statistics were expressed as means and standard deviation of the mean (SD). Statistical analysis for the difference between samples was performed using a univariate Student's unpaired *t* test. The level of statistical significance was set at a p value of <0.05 (*p<0.05; **p<0.01; ***p<0.001; NS, not statistically significant). Student's *t* test was applied after evaluating data assumptions of normality with the Shapiro-Wilk test.

3. Results

The *in vitro* oxidative burst assay was used to evaluate immune stimulation by Immuno-G-Matrix™ by measuring the ability to induce ROS production in differentiated cells (neutrophils 98%). The results of DHR oxidation determined as Rh123-induced green fluorescence, revealed with flow cytometry, are reported in Table 2 and Fig.1 as the percentage (%) of FI or number of cells related to fluorescence emission, as mean ± SD and *p* value. FI correlated directly with the power of the existing and/or induced oxidative burst and with the number of activated cells. There was an increase in oxidative burst in the cell fraction for the ctr (+) PMA, Immuno-G-Matrix™ and PMA + Immuno-G-Matrix™ samples as shown in Fig. 1.

The fluorescence microscope showed green fluorescence after the different treatments with ctr (+) PMA and Immuno-G-Matrix™ compared with ctr (–) (Fig. 2), as shown also by the values in Fig. 1B and Table 2. The sample incubated simultaneously with PMA + Immuno-G-Matrix™ showed a fluorescence intensity greater than that of the ctr (+) PMA and Immuno-G-Matrix™ samples, in particular, its value was double that of the single treatments (Table 2, Figs. 1 and 2).

	ctr (–) (% FI)	ctr (+)PMA (% FI)	Immuno-G-Matrix™ (% FI)	ctr (+)PMA + Immuno-G-Matrix™ (% FI)
Mean	5.67	10.77	11.36	19.89
SD	0.23	0.86	1.12	1.01
***p* Value**	***$p\leq0.001$			
	***$p\leq0.001$			
	***$p\leq0.001$			
		(NS)		
		***$p\leq0.001$		
			***$p\leq0.001$	

Table 2 - Oxidative burst assay: results obtained with flow cytometry. Data are expressed as mean ± SD fluorescence intensity (FI) (%). Statistical analysis: Student's unpaired *t* test.

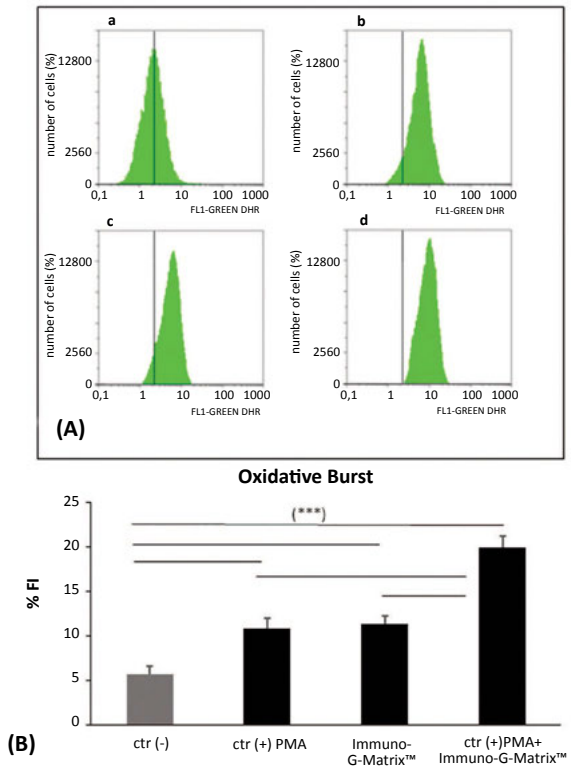

Figure 1 - Oxidative burst by dihydrorhodamine (DHR) oxidation in cells. Human HL-60 cells differentiated in neutrophils were incubated in medium with or without phorbol 12-myristate 13-acetate (PMA) or Immuno-G-Matrix™ or with both together and then treated with DHR. Rhodamine 123 (Rh123) generation, correlated with the number of activated neutrophils, was then assessed by flow cytometry measuring the generated green fluorescence. A typical frequency histogram (**A**) and the summary of four similar experiments (**B**) are presented. The oxidative burst histograms represent the (%) cell fluorescence intensity (FI) for: **a** ctr (–) (differentiated cells); **b** ctr (+) PMA (differentiated cells incubated with PMA); **c** Immuno-G-Matrix™ (differentiated cells incubated with Immuno-G-Matrix™); and **d** PMA + Immuno-G-Matrix™ (differentiated cells incubated with PMA + Immuno-G-Matrix™). Data are expressed as mean ± SD. Statistical analysis: Student's unpaired ± test (***$p \leq 0.001$).

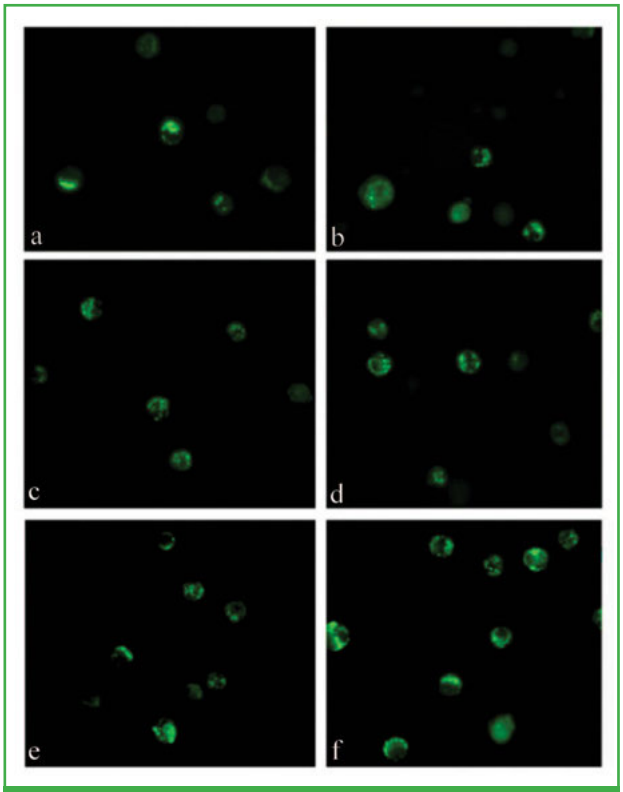

Figure 2 - Intracellular accumulation of rhodamine 123 (Rh123) in cells incubated with dihydrorhodamine (DHR). Human HL-60 cells differentiated in neutrophils after 7 days and cultured in complete medium with the addition of DMSO were incubated with 0.45 µM DHR for 20 min at 37 °C. The cells were fixed in formalin 1 % in PBS 1× and plated on coverslips for microscopy observation. The observations are representative of 10 experiments: **a** ctr (–); **b** ctr (+) PMA; **c–e** Immuno-G-Matrix™; and **f** PMA + Immuno-G-Matrix™. The DHR green fluorescence increased after the different treatments with PMA and Immuno-G-Matrix™ compared with ctr (–). Image photographs by fluorescence microscope (Olympus BX50).

4. Conclusions

Under the experimental conditions used to evaluate the efficacy of Immuno-G-Matrix™, our data show that this innovative formulation is able to induce *in vitro* functional responses in neutrophils, such as the oxidative burst, as is the activator PMA. In particular, Immuno-G-Matrix™ shows better oxidative burst induction when it acts in combination with PMA, showing synergistic action. It is reported in the literature that β-glucans could be good immune modulators *in vivo* but oral β-glucans preparations, due to their low systemic availability, can be effective for modulating the immune system but only when administered parenterally [5]. The tested formulation of Immuno-G-Matrix™ is able to bypass this problem by maximizing the effect of β-glucans in combination with Zn and Cu. This thermo-plastic granulate has been realized using an innovative food grade lipid matrix technology, G-MatrixLipid™. The application of this technology ensures a gradual and prolonged release of the active ingredients at the enteric level, optimizing their bioavailability and gastric tolerability.

We suggest that an oral intake of 300 mg/capsule/day of Immuno-G-Matrix™, containing a combination of β-glucans, Zn and Cu in a lipid matrix, can activate the immune system *in vivo*.

Acknowledgments

With this work, the Authors remember and would like to thank the late Professor Fulvio Marzatico.

Conflict of Interest

Antonio Seneci is an owner of Truffini & Reggè Farmaceutici. Maria Elena Carrabetta, Federico

Pialorsi and Maria Zurlo are employees of Truffini & Reggè Farmaceutici. The other Authors declare that they have no conflicts of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

References

1. Chanock SJ, el Benna J, Smith RM, Babior BM (1994) The respiratory burst oxidase. J Biol Chem 269:24519–24522
2. Abbas AK, Lichtman MD, Pillai S (2015) Cellular and molecular immunology, 8th edn. Elsevier Saunders, Philadelphia
3. Dahlgren C, Karlsson A (1999) Respiratory burst in human neutrophils. J Immunol Methods 232:3–14
4. Tan HB, Danilla S, Murray A *et al* (2014) Immunonutrition as an adjuvant therapy for burns. Cochrane Database Syst Rev 12:CD007174
5. Stier H, Ebbeskotte V, Gruenwald J (2014) Immune-modulatory effects of dietary yeast beta-1,3/1,6-D-glucan. Nutr J 13:38
6. Kim HS, Hong JT, Kim Y, Han B (2011) Stimulatory effect of β-glucans on immune cells. Immune Netw 11:191–195
7. Scientific Opinion on the substantiation of health claims related to zinc and function of the immune system (2009) (ID 291, 1757). EFSA J 7:1229
8. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) (2011) Scientific Opinion on the safety of 'yeast beta-glucans' as a Novel Food ingredient. EFSA J 9:2137 [22 pp.]
9. Scientific Opinion on the substantiation of health claims related to copper and maintenance of the normal function of the immune system (2011) (ID 1725). EFSA J 9:2079
10. Demleitner S, Kraus J, Franz G (1992) Synthesis and antitumour activity of sulfoalkyl derivatives of curdlan and lichenan. Carbohydr Res 226:247–252
11. Grünebach F, Weck MM, Reichert J, Brossart P (2002) Molecular and functional characterization of human Dectin-1. Exp Hematol 30:1309–1315
12. Institute of Medicine (IoM) (2001) Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. National Academies Press, Washington, DC
13. Freake HC (2006) Zinc: physiology. In: Prentice A (ed) Encyclopedia of human nutrition. Academic Press, San Diego, pp 447–454
14. King JC, Cousins RJ (2006) Zinc. In: Shils M, Shike M, Ross C, Caballero B, Cousins R (eds) Modern nutrition in health and disease. Lippincott Williams & Wilkins, Baltimore, Philadelphia, pp 271–285
15. Linder MC, Hazegh-Azam M (1996) Copper biochemistry and molecular biology. Am J Clin Nutr 63:797S–811S
16. Percival SS (1995) Neutropenia caused by copper deficiency: possible mechanism of action. Nutr Rev 53:59–66
17. Karimbakas J, Langkamp-Henken B, Percival SS (1998) Arrested maturation of granulocytes in copper deficient mice. J Nutr 128:1855–1860
18. Koller LD, Mulhern SA, Frankel NC, Steven MG, Williams JR (1987) Immune dysfunction in rats fed a diet deficient in copper. Am J Clin Nutr 45:997–1006
19. Heresi G, Castillo-Duran C, Munoz C, Arevalo M, Schlesinger L (1985) Phagocytosis and immunoglobulins levels in hypocupremic infants. Nutr Res 5:1327–1334
20. Danks DM (1988) Copper deficiency in humans. Annu Rev Nutr 8:235–257
21. Bonham M, O'Connor JM, Hannigan BM, Strain JJ (2002) The immune system as a physiological indicator of marginal copper status? Br J Nutr 87:393–403
22. Collins SJ, Gallo RC, Gallagher RE (1977) Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature 270:347–349
23. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC (1987) Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci U S A 75:2458–2462
24. Vatrella A, Perna F, Pelaia G *et al* (2010) T cell activation state in the induced sputum of asthmatics treated with budesonide. Int J Immunopathol Pharmacol 23:745–753
25. Vitiello L, Masci AM, Montella L et al (2010) Thymoma-associated immunodeficiency: a syndrome characterized by severe alterations in NK, T and B-cells and progressive increase in naïve CD8+ T Cells. Int J Immunopathol Pharmacol 23:307–316
26. Chan JL, Matarese G, Shetty GK *et al* (2006) Differential regulation of metabolic, neuroendocrine, and immune function by leptin in humans. Proc Natl Acad Sci U S A 103:8481–8486
27. Sun Q, Woodcock JM, Rapoport A *et al* (1996) Monoclonal antibody 7G3 recognizes the N-terminal domain of the human interleukin-3 (IL-3) receptor alpha-chain and functions as a specific IL-3 receptor antagonist. Blood 87:83–92
28. Kishimoto T, von dem Borne AEG, Goyert SM *et al* (eds) (1997) Leucocyte typing VI: white cell differentiation antigens. Garland Publishing, London
29. Mollinedo F, Borregaard N, Boxer LA (1999) Novel trends in neutrophil structure, function and development. Immunol Today 20:535–537
30. Avendaño A, Sales-Pardo I, Marin L, Marin P, Petriz J (2008) Oxidative burst assessment and neutrophil-platelet complexes in unlysed whole blood. J Immunol Methods 339:124–131