ORIGINAL RESEARCH

Effect of buckminsterfullerenes on cells of the innate and adaptive immune system: an in vitro study with human peripheral blood mononuclear cells

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Abstract: C60 nanoparticles, the so-called buckminsterfullerenes, have attracted great attention for medical applications as carriers, enzyme inhibitors or radical scavengers. However, publications evaluating their immunological mechanisms are still rather limited. Therefore, we aimed to analyze systematically the in vitro influence of polyhydroxy-C60 (poly-C60) and N-ethyl-polyamino-C60 (nepo-C60) on peripheral blood mononuclear cells (PBMC) from healthy individuals, angling their effect on proliferation, expression of surface markers, and cytokine production. We isolated PBMC from 20 healthy subjects and incubated them in a first step only with poly-C60 or nepo-C60, and in a second step together with recall antigens (purified protein derivative, tetanus toxoid, bacillus Calmette-Guérin). Proliferation was determined by ^{3}H-thymidine incorporation, activation of PBMC-subpopulations by flow cytometry by measurement of the activation marker CD69, and secretion of T helper cell type 1 (TH1)- (interferon-gamma [IFN-γ], tumor necrosis factor beta [TNF-β]), TH2- (interleukin-5 [IL-5], -13, -10) and macrophage/monocyte-related cytokines (IL-1, IL-6, TNF-α) into the supernatants by enzyme-linked immunosorbent assay. Both fullerenes did not influence T cell reactivity, with no enhanced expression of CD69 and production of T cell cytokines observed, the CD4/CD8 ratio remaining unaffected. In contrast, they significantly enhanced the release of IL-6 and CD69-expression by CD56 positive natural killer cells. PBMC, which had been cultured together with the three recall antigens were not affected by both fullerenes at all. These data indicate that fullerenes do not interact with T cell reactivity but may activate cells of the innate immune system. Furthermore, they seem to act only on 'naïve' cells, which have not been prestimulated with recall antigens, there are however, large inter individual differences.

Keywords: buckminsterfullerenes, polyhydroxy-C60, N-ethyl-polyamino-C60, natural killer cells, dendritic cells, T cells, cytokines

Introduction

Fullerenes were first described in 1985,[1] and in 1996, three of the authors (Kroto, Curl, and Smalley) shared the Nobel Prize in Chemistry for their discovery. C60 fullerenes are completely composed of carbon, which forms hollow spheres like a football/ soccer ball. They are also known as buckminsterfullerenes or buckyballs.[1–3] This chemical structure together with their physical properties implies that they may be successfully used in many different fields including several technological and medicinal applications, for example as a carrier for genes, antibodies, and drug-delivery systems.[4–6] Thus, these spheres are rather lipophilic, a quality that may allow them to

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interact with the active sites of various enzymes such as the human immunodeficiency virus (HIV) protease or hepatitis C virus RNA-dependent RNA polymerase.[7] It also enables C60 to intercalate into biological membranes and destabilize them, which would explain its antibacterial activity.[4,8–11] On the other hand, their poor solubility in polar solutions and their high potential to form aggregates has several negative aspects, which have, however, been overcome by different methods. Thus, fullerenes can be functionalized and derived in multiple ways in order to enhance their hydrophilicity (eg, coupling with –OH, -COOH, or -NH$_2$).[4,12]

Several studies have looked at the toxicity of fullerenes under different circumstances, with different subtypes of fullerenes,[12–14] the evidence clearly showing that with increasing hydrophilicity their toxicity decreased.[12,15,16] This led to an interest in the analysis of their effects on immunocompetent cells. Thus, antiallergic, tumor-inhibitory, and immunomodulatory properties have been described in vitro,[17–20] with some studies in animal models also showing their safety, even at high doses and their potential effect on inflammatory disorders in vivo.[21,22] However, all studies clearly show that different fullerenes may exhibit differential effects, systematic studies still being required. Furthermore, the individual C60 molecules only have a diameter of 0.7 nm and are therefore, small enough to potentially enter the cell via passive diffusion through ion channels or diffuse through pores in the nuclear membrane.[23] Moreover, the interaction of C60 with several proteins is well known.[24] Hence it is essential to determine the degree of interactions of C60 with immunological pathways before its use becomes widespread.

Therefore, we designed an in vitro study to analyze the effect of the most widely used water-soluble fullerenes, polyhydroxy-C60 (poly-C60) and N-ethyl-polyamino-C60 (nepo-C60), on immunocompetent cells at several levels, but focusing primarily on T cells. In order to avoid any influences of inflammatory processes, underlying diseases or therapies, we used peripheral blood mononuclear cells (PBMC) from healthy individuals and analyzed how the two fullerenes affected the proliferative response, activation of lymphocyte subsets, and secretion of T-helper type 1 (TH1) and T-helper type 2 (TH2) associated cytokines. In the first step we investigated their influence on unstimulated cells, and in the second step we mimicked inflammatory stages by preincubating the PBMC with TH1- (purified protein derivative, [PPD]), and TH2-cell activating antigens (tetanus toxoid [TT]) as well as BCG (bacillus Calmette-Guérin), stimulating mainly macrophages/monocytes.[25–27] It will be shown that these two fullerenes do not influence T cell reactivity but there is some evidence that they may activate cells of the innate immune system such as macrophages, dendritic cells (DC), or natural killer (NK) cells.

Materials and methods
Subjects
Twenty healthy subjects (No 1–20) were enrolled in the study. They were selected from amongst the laboratory staff of the Department of Internal Medicine II, University of Tübingen, and students (11 females, 9 males; aged 22–38 years). Individuals who suffered from any acute or chronic diseases including acute allergic reactions, or who had been vaccinated (including tetanus) within the last 12 months were excluded from the study. The local ethics committee of the medical faculty of the University of Tuebingen approved the study. The study was in accordance with the tenets of the Declaration of Helsinki, and all subjects gave their written consent to participate.

Antigens
Water-soluble fullerenes (poly-C60: C60(OH)$_n$; and nepo-C60: C60(NHCH$_2$CH$_3$)$_n$) were obtained from Bucky (Houston TX). Their purity had been proven by high-performance liquid chromatography. Both preparations were shown using the limulus amebocyte lysate test (Pyrogent® 5000; Lonza, Walkersville, MD) to be negative for endotoxins.

As recall antigens we used purified protein derivative (PPD) and tetanus toxoid (TT) for the activation of TH1- and TH2- cells, respectively. Furthermore, BCG was used for the activation of macrophages/monocytes.[26,28,29] PPD was purchased from Statens Serum Institute (Copenhagen, Denmark); TT (40 IU) was obtained from Novartis Behring (Marburg, Germany); and BCG (2×10^8 bacteria/50 mL) was obtained from Medac (Wedel, Germany). Pokeweed mitogen (PWM) was purchased from Biochrom AG (Berlin, Germany) and was applied as a positive control nonspecifically stimulating several PBMC subpopulations.

PBMC cultures
From each volunteer, 120 mL of heparinized blood was drawn, and within the next 24 hours, PBMC were isolated by centrifugation through Ficoll-hypaque gradient. In previous studies we showed that within this time interval cells are still viable and reliable, and reproducible results without significant changes were able to be obtained.[25,26,29–32] PBMC were adjusted to 1 Mio cells/mL in RPMI 1640 medium supplemented with 25% decomplemented autologous serum and gentamycin.[25,26,29,32,33] In order to determine the optimal

conditions for the analysis, kinetics were performed prior to the experiments presented in this study. Best results for flow cytometry were observed after incubation of PBMC with the different antigens for 24 hours, incubation for 7 days revealing optimal results for proliferative responses and cytokine release.

First, PBMC were incubated only with poly-C60 and nepo-C60 at concentrations of 0.08, 0.8, 8, 80, and 800 ng/mL ($\cong$0.08–800 nM) according to the literature where 0.1–1000 nM were shown to inhibit the inflammatory response.[22,34] Then, PBMC were cocultured with PPD, TT, and BCG at concentrations of 2.0 TU (tuberculin units)/mL, 1.6 IU/mL, and 5×10^5 bacteria/mL, respectively, as shown in recent studies to provide best results.[26,27]

Proliferation assay

For the determination of the proliferative response, 1×10^6 PBMC/well were incubated in quadruplicates with the antigens for 7 days. H-thymidine (0.74 MBq/mL, 20 µL/well) was added for the last 18 hours following which, cells were harvested onto glass-fiber filters, the incorporated radioactivity then determined by liquid scintillation spectroscopy using a β-counter. Results were given as counts per minute (cpm).[33] For statistical analysis, means of the quadruplicate values were calculated.

Cytokine production

For the release of different cytokines PBMC (5×10^5 cells) were cultured with the different antigens in 24-well culture plates. At day 7, the culture supernatants were collected and frozen at $-20°C$.

Cytokine concentrations were determined in the supernatants by ELISA as recently described.[25–27] Ninety-six-well microtiter plates were coated with a monoclonal antibody to human cytokines (1.75 µg/mL for IL-1, IL-5, IL-6, IL-10, IL-13, TNF-α, IFN-γ, TNF-β, granulocyte/macrophage colony-stimulating factor; Pharmingen, San Diego, CA) in hydrogen bicarbonate buffer, pH 9.6, overnight at 4°C. In order to avoid nonspecific binding, wells were then blocked with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS 60 mmol/L, pH 7.4) for 1 hour at room temperature. Undiluted culture supernatants were added in duplicates and incubated for 2 hours at 37°C. Antibodies to human cytokines coupled with biotin (Pharmingen) were used as secondary antibodies (1.25 µg/mL) and incubated with the plates at 37°C for 2 hours. Afterwards, avidin-peroxidase (2.5 µg/mL) was added for 1 hour, and then 0.5 mg orthophenylenediamine/mL citrate buffer (pH 5.0)

with 0.01% H_2O_2 as substrate solution. After stopping the enzyme reaction with 25% sulphuric acid, optical density was measured in a microtiter plate reader at 450 nm. Recombinant cytokines were used to establish a standard curve to which the results obtained with the culture supernatants were related.

Flow cytometry

As mentioned above, for the determination of the activation marker CD69, the incubation time of PBMC (5×10^5 cells/well) with antigens was only 24 hours.[27] Antibody cocktails were used for the demonstration of activated PBMC subpopulations (activated CD4+ T cells: FastImmune™ CD4/CD69/CD3, activated, CD8+ T-cells: FastImmune™ CD8/CD69/CD3; activated CD19+ B-cells: FastImmune™CCD19/CD69/CD45); activated CD56+ NK-cells: FastImmune™ CD56/CD69/CD45; Becton-Dickinson [San Jose, CA]). Furthermore, an immunoglobulin G (IgG) isotype control antibody was used (BD Biosciences San Jose, CA; Pharmingen). At least 10,000 PBMC were counted. Quadrants were set based upon the isotype controls for each antibody. Results were expressed as the percentage of CD69 expressing cells of the respective cell types. Furthermore, the percentage of CD4+/CD3+ and CD8+/CD3+ T-cells, CD16+/CD45+ B- and CD56+/CD45+ NK-cells was determined.

Investigation of the viability of PBMC

Pokeweed mitogen (PWM; 10 µg/mL) was used to prove the viability of PBMC in all experiments. An absence of proliferative response, cytokine production or a failure of CD69 expression towards this mitogen were indicative of nonviability of the cells or other technical problems, and these experiments were then excluded from the analysis and repeated.

Statistics

SPSS (version 19.0; SPSS Inc, Chicago, IL) was used for statistical analysis. For paired parameters, the nonparametric Wilcoxon signed-rank test was applied. P-values < 0.05 were considered statistically significant. PBMC already showing a spontaneous reaction, ie, without adding poly-C60 or nepo-C60 to the cultures, were excluded from the statistical analysis.

Results

The influence of poly-C60 and nepo-C60 on 'naïve' PBMC

First, we investigated the effects of poly-C60 and nepo-C60 on naïve PBMC. For this purpose, PBMC were isolated from

20 healthy individuals and incubated with the two substances without costimulation with other antigens. Their effect on proliferative response, induction of cytokine production, and expression of CD69 was analyzed.

Effect on proliferative response

A significant enhancement of the proliferative response with a stimulation index > 3 was observed with PBMC from five individuals (no 7, 8, 9, 13, 20). This increase was statistically significant on the whole group level with poly-C60 at concentrations of 8–800 ng/mL and nepo-C60 at concentrations 0.08–800 ng/mL (Figure 1).

Effect on cytokine secretion

The production of the TH1-cytokine IFN-γ, but not TNF-β, was enhanced by poly-C60 in PBMC from four individuals (no 3, 8, 9, 18) and by nepo-C60 in PBMC from two individuals (no 7, 9). However, there were no statistically significant differences at the whole group level comparing

the spontaneous and the antigen-induced cytokine production (Figure 2).

The secretion of the TH2-cytokines IL-5 and IL-13 was not affected. PBMC from two individuals (no. 8, 9) showed a strong release, and from four individuals (no. 1, 6, 18, 13) a weak release of IL-10 after incubation with poly-C60. Nepo-C60 induced in two individuals (no. 2, 9) a strong production, and in a further two individuals (no. 6, 7) a weak production of IL-10. However, at the whole group level, the differences as compared to the spontaneous production were, again, statistically not significant (Figure 3).

Analysis of the production of macrophage related cytokines after incubation of PBMC with poly-C60 or nepo-C60, showed an enhancement of the IL-6 secretion by PBMC from five individuals by poly-C60 (no. 6, 7, 8, 12, 13) and from five individuals by nepo-C60 (no. 1, 6, 7, 8, 10) resulting in significant differences as compared to the spontaneous production (Figure 4, PBMC from three subjects already produced IL-6 spontaneously and were, therefore excluded
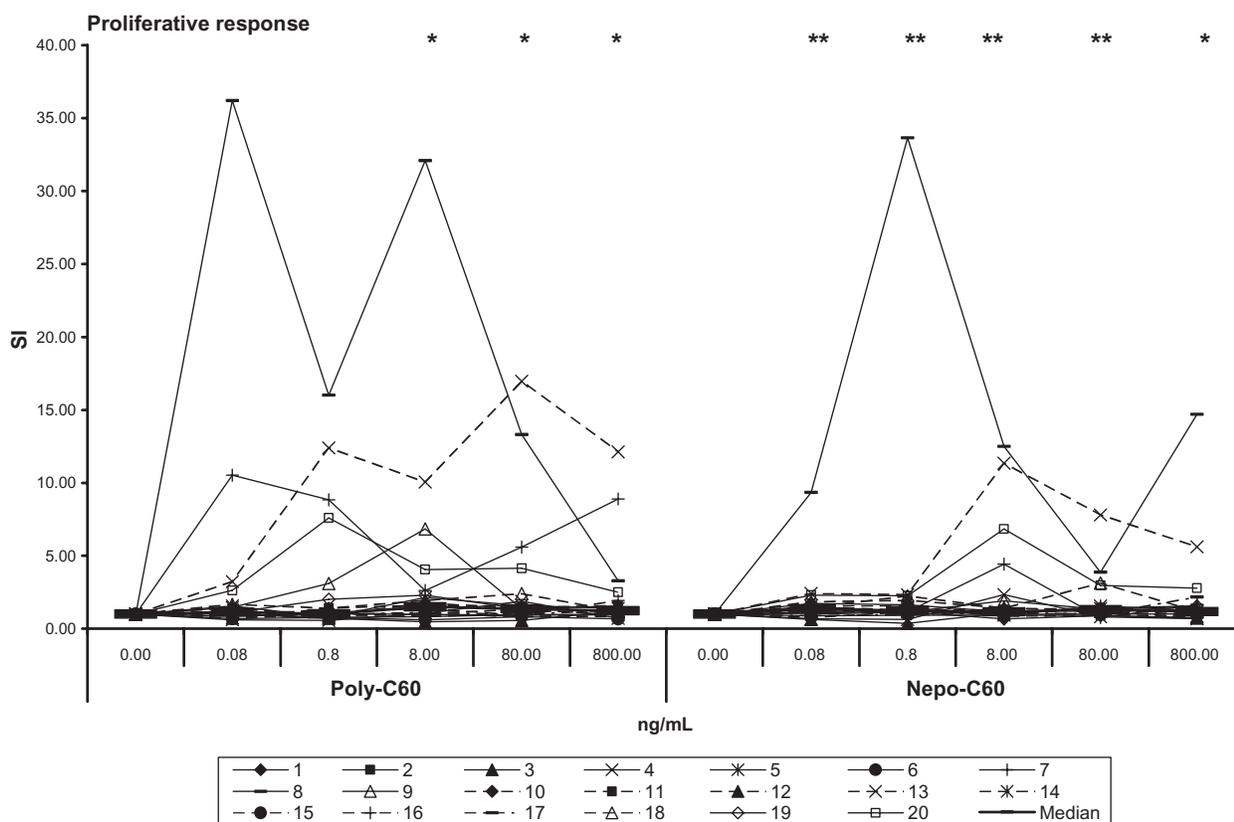

Figure 1 The effect of varying concentrations (0.08–800 ng/mL = 0.1–100 nM) of poly-C60 and nepo-C60 on the proliferative response of PBMC from 20 healthy volunteers measured by the lymphocyte transformation test.
Notes: Individuals values are given. Statistically significant differences on the whole group level comparing the spontaneous and the fullerene-induced proliferation are indicated: *$P < 0.05$; **$P < 0.01$.
Abbreviations: SI, stimulation index; 0,00, spontaneous proliferation without antigen; –, median.
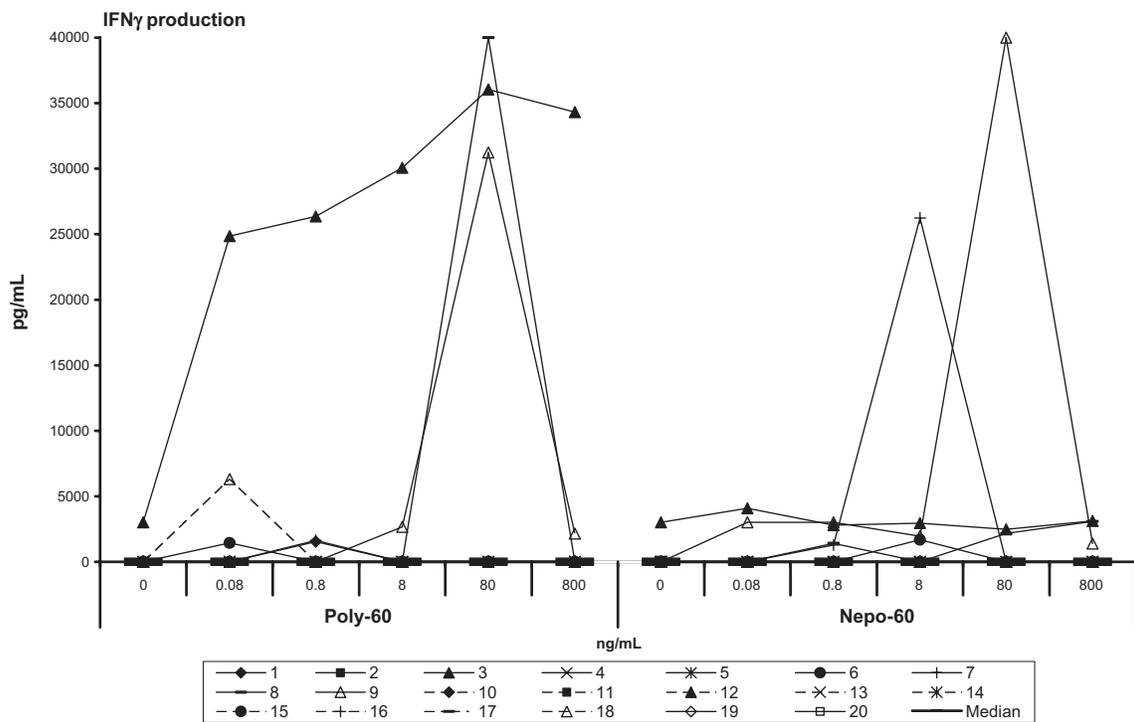
IFNγ production

Figure 2 The effect of varying concentrations (0.08–800 ng/mL = 0.1–100 nM) of poly-C60 and nepo-C60 on the IFN-γ production by PBMC from 20 healthy volunteers measured by ELISA.
Notes: Individual values are given. There were no statistical differences on the whole group level comparing the spontaneous and the fullerene-induced IFN-γ production.
Abbreviations: 0, spontaneous IFN-γ production without antigen; –, median.
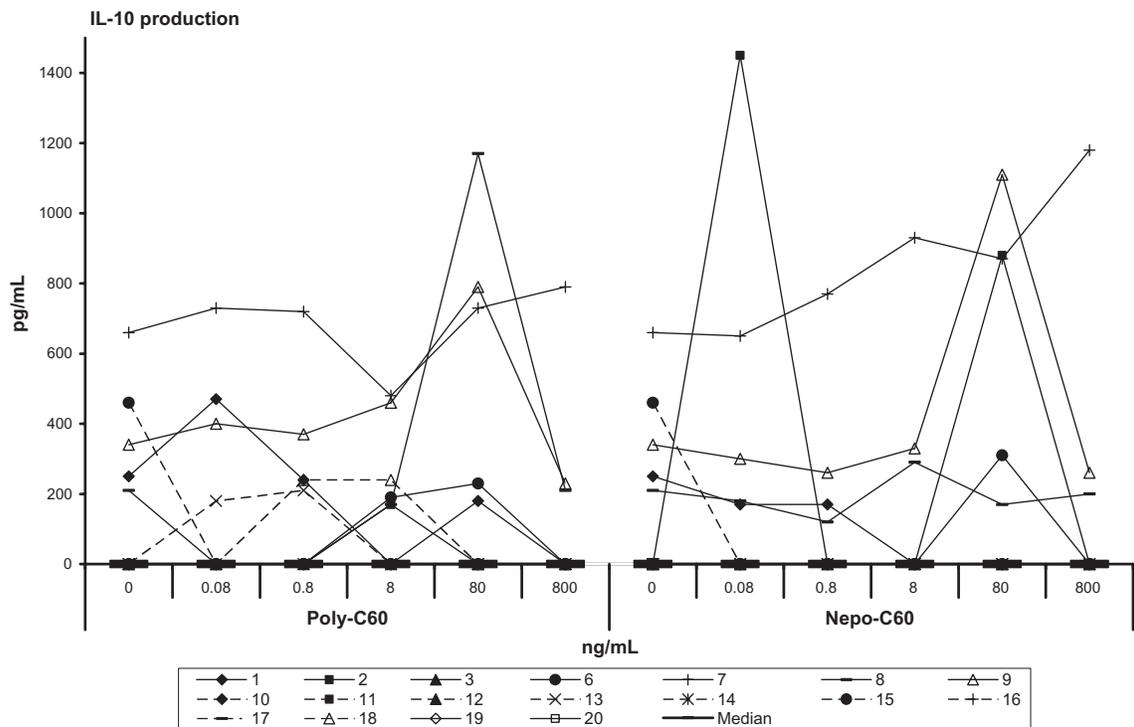
IL-10 production

Figure 3 The effect of varying concentrations (0.08–800 ng/mL = 0.1–100 nM) of poly-C60 and nepo-C60 on the IL-10 production by PBMC from 18 healthy volunteers measured by ELISA.
Notes: Two individuals producing already spontaneously high levels of IL-10 were omitted. Individual values are given. There were no statistical differences on the whole group level comparing the spontaneous and the fullerene-induced IL-10 production.
Abbreviations: 0, spontaneous IL-10 production without antigen; –, median.
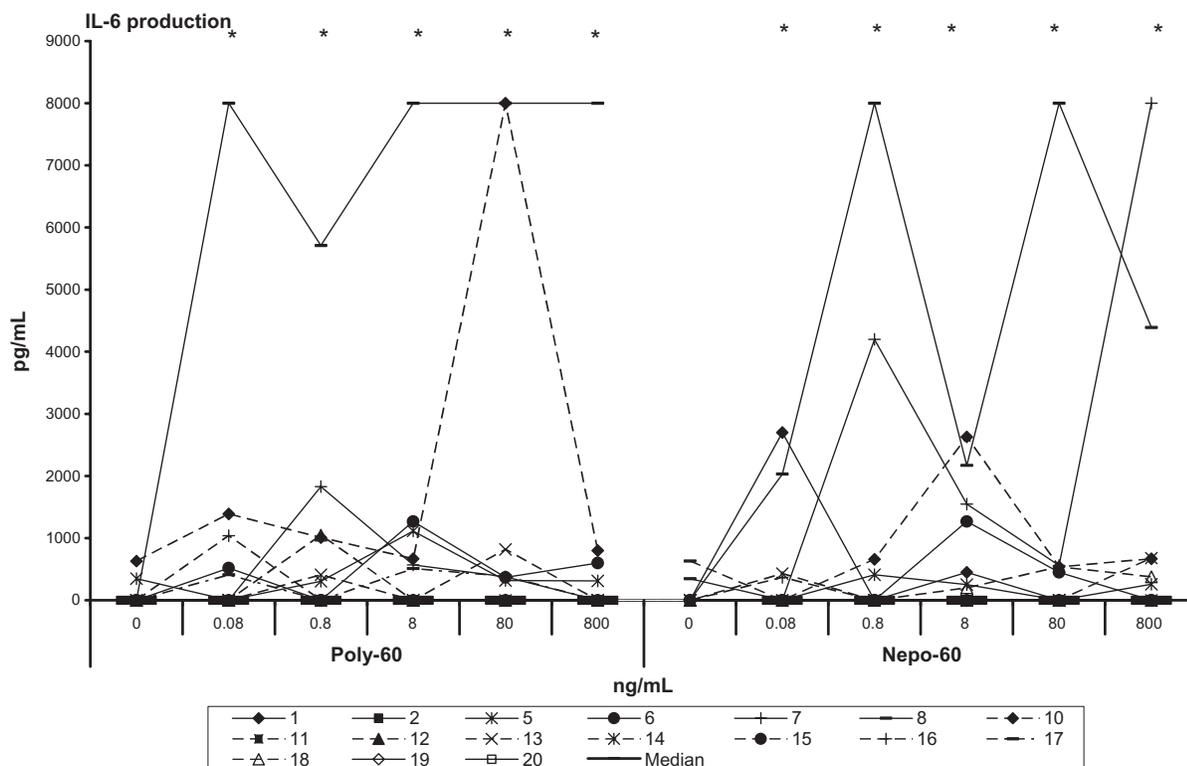
Figure 4 The effect of varying concentrations (0.08–800 ng/mL = 0.1–100 nM) of poly-C60 and nepo-C60 on the IL-6 production by PBMC from 17 healthy volunteers measured by ELISA.
Notes: Three individuals producing already spontaneously high levels of IL-6 were omitted. Individual values are given. Statistically significant differences on the whole group level comparing the spontaneous and the fullerene-induced IL-6 production are indicated: *$P < 0.05$.
Abbreviations: 0, spontaneous IL-6 production without antigen; –, median.

from the statistical analysis). The release of IL-1 and TNF-α was not affected (not shown).

Effect on the activation of PBMC subpopulations

Poly-C60 and nepo-C60 had no effect on the CD4/CD8 ratio or the percentages of CD19+ B or CD56+ NK-cells in the cell cultures of PBMC from the 20 individuals (data not shown).

Analysis of the expression of CD69 as an indicator for activation by flow cytometry showed that both fullerenes had no effect on its expression by CD4+ and CD8+ T cells. A slight activation of CD19 positive B cells was observed with PBMC from eleven individuals, but the differences were not significant at the whole group level. In contrast, both fullerenes significantly enhanced the percentage of activated (CD69+) CD56+ NK cells (Figure 5).

The effect of poly-C60 and nepo-C60 on PBMC stimulated with recall antigens

We were further interested in whether poly-C60 or nepo-C60 had any effect on PBMC costimulated either with the TH1-antigen PPD, the TH2-antigen TT or the monocyte/

macrophage-activating BCG. There was neither an inhibitory nor a stimulatory effect by both fullerenes on these pre-activated PBMC by measurement of the proliferative response, the production of TH1-, TH2- or macrophage-associated cytokines or the CD69 expression by the different subpopulations (data not shown).

Discussion

This is the first report that systematically analyses the effect of two water-soluble fullerenes, poly-C60 and nepo-C60, on immunocompetent cells from humans in vitro. Four major conclusions can be drawn from the presented data: (1) PBMC from individual subjects strongly differed in their response towards the two fullerenes; (2) both C60 molecules did not influence the T cell reactivity by means of activation (CD69 expression or cytokine production), and only in a few individuals was there an induction of IFN-γ production; (3) both seem to activate cells of the innate immune system as shown by the enhanced production of IL-6 which may be related to macrophages or dendritic cells,[19,35] and an activation of NK-cells (which may also be responsible for the observed IFN-γ release[36] mentioned under point 2); (4) The two fullerenes influenced only 'naïve',
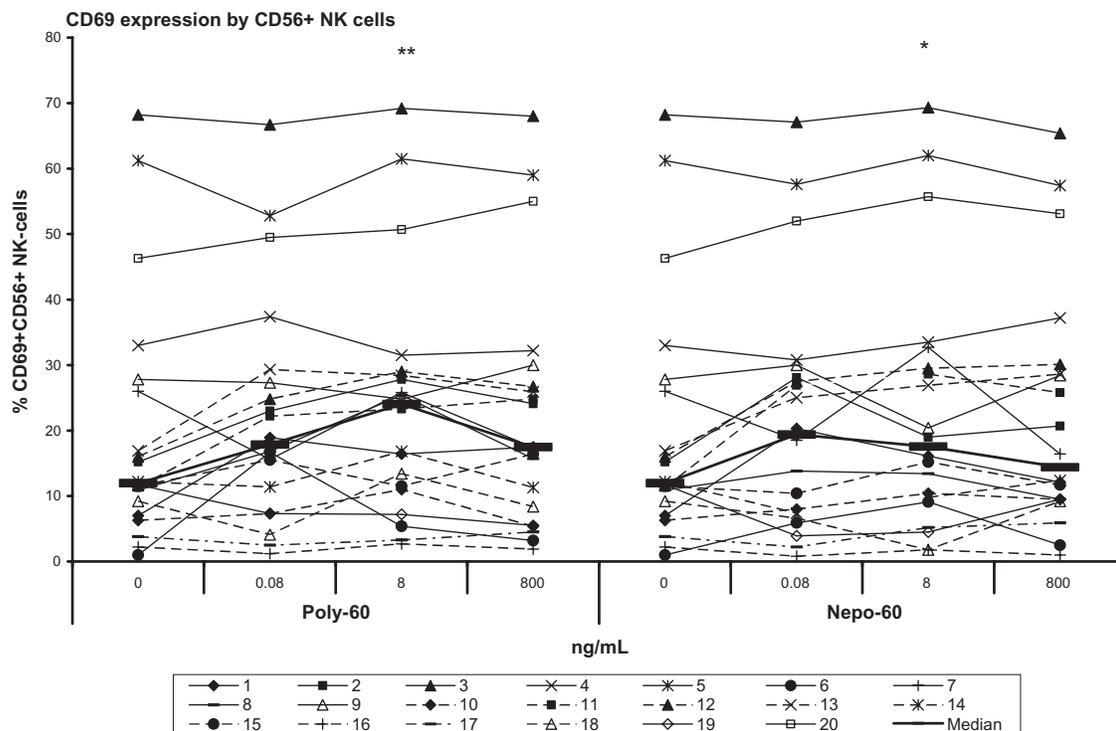
Figure 5 The effect of varying concentrations (0.08–800 ng/mL = 0.1–100 nM) of poly-C60 and nepo-C60 on activation of NK-cells measured by CD69 expression by flow cytometry with PBMC from 20 healthy volunteers.
Notes: Individual values are given. Statistically significant differences on the whole group level comparing the spontaneous and the fullerene-induced CD69 expression are indicated: *$P < 0.05$; **$P < 0.01$.
Abbreviations: 0, spontaneous CD69 expression without antigen; –, median.

resting cells, ie, cells that have not been cocultured with antigens activating TH1 or TH2 cells or macrophages.

A nonspecific activation of the cells of the innate immune system by a contamination of the fullerene preparations with endotoxins could be excluded, as we did not detect any significant endotoxin levels in the limulus amebocyte lysate test. The effect of fullerenes on the innate immune system has already become evident in studies analyzing their effect on mast cells. Thus, it is has been shown that incubation of human mast cells and blood basophils with C60 fullerenes led to a significant inhibition of IgE-dependent mediator release, which was related to an inactivation of signaling molecules, especially the tyrosine phosphorylation of Syk.[34] Using C70 fullerenes, Dellinger et al showed that they exhibit strong anti-inflammatory properties, endocytosed into the cell and trafficked to different organelles such as lysosomes, mitochondria, endoplasmic reticulum, but not nuclei at different time points.[37,38]

Our observation of an induction of IL-6 (and in some instances of IL-10) but not of IL-1 and TNF-α-production is in accordance with these data as it may indicate the activation of type 2 macrophages which are considered to be anti-inflammatory.[39] Furthermore, IL-6 is also secreted by

semi mature dendritic cells which are tolerogenic because they express only small amounts of MHC class II and costimulatory molecules and are induced by activation of Toll-like receptors 2 (TLR2) or TLR4.[40] IL-6 inhibited the DC-driven allogeneic T-cell proliferation in mixed lymphocyte reactions,[40,41] and this would explain why we did not observe an influence on T cells in our experiments. The increased production of IFN-γ by PBMC from some individuals after incubation with fullerenes may be derived from NK cells rather than from TH1 cells. The activation of NK cells can then be explained by the bidirectional crosstalk between DC and NK cells, considering the fact that DCs efficiently enhance CD69 expression, proliferation and IFN-γ-secretion of NK cells.[42] An inhibition of proinflammatory cytokine production with C60 particles in similar concentrations as ours (100–1000 nM = about 80–800 ng/mL) has also been shown by Yudoh et al in a rat model of arthritis.[22] Since there is evidence that nanoparticles may activate complement,[43] although this has not yet been analyzed for fullerenes, a direct activation of NK-cells via complement receptors is conceivable. However, the expression of complement receptors by NK-cells is still controversial, as discussed in the literature.[44,45]

In contrast to our data, Liu et al[17] reported an activation of TH1 cells and a strong increase in TNF-α as well as IL-6 production in vivo and in vitro in tumor-bearing and healthy mice using $C_{60}(OH)_{20}$ nanoparticles, and similar effects were also observed by Zhu et al.[20] Furthermore, a C82 fullerene entrapped with a gadolinium derivative atom in the core of the carbon cage[19], used as a new generation of MRI contrast agent[46] has rather proinflammatory properties. Thus, in their animal model the authors found a TH1-polarizing effect, which was also obtained when they used human dendritic cells. This was also confirmed by Liu et al.[47]

An effect on immunocompetent cells has also been analyzed for other nanomaterials as for instance, carbon nanotubes with a one-dimensional structure being functionalized by several noncovalent and covalent methods (f-CNTs).[48] These f-CNTs may activate macrophages, and there is strong evidence that they are actively taken up by these phagocytic cells. They can enter primary immune cells without exerting cytotoxicity, and this would fit our observations that poly-C60 and nepo-C60 also did not exhibit any cytotoxic action on PBMC, even when fullerene concentrations 100 times higher than those presented in this study were used (data not shown). There is, however another study indicating that different concentrations of f-CNT had no significant effect on the proliferation of activated or nonactivated splenic lymphocytes as well as the IL-6 production from mice, the authors also observing a modest increase in immunostimulatory activity when mouse splenocytes were cocultured with synthetic oligodeoxynucleotides containing CpG motifs known to react with TLR9.[49]

All of this contradictory data in the literature may be due to the application of different types and concentrations of nanoparticles, different models (cell cultures, animals) and different disease conditions (tumor-bearing mice), emphasizing the necessity for standardized systems in order to obtain reliable and comparable results.

Our results, that poly-C60 and nepo-C60 hardly activate T cells, especially (allergic) TH2 responses, may be important in rendering them ideal agents as carriers for gene and drug-delivery systems or for diagnostic application.[4,5] Considering the fact that they have been shown to diffuse through cell membranes and also enter the nucleus,[23] and that they can easily interact with signal transduction pathways by their affinity to various proteins and their ability to generate free radicals,[23,24,50] the rather weak interaction of the two water-soluble fullerenes with immunological reactions as shown in this study is rather surprising. However, the data also underline that all types of nanoparticles may have different immunological properties which have to be carefully analyzed before they are applied

in humans. In addition, the strong inter individual differences in reactivity towards the two fullerenes have to be taken into account. In the present study, PBMC from three individuals (no 7, 8, 9) were preferentially influenced, however we did not notice any difference between these three and the remaining 27 other individuals with respect to allergic disposition, prior vaccinations, or infectious disorders (not shown). Of course, one has to keep in mind that individual reactivity is influenced by the personal momentary milieu including nutritional status, physical exercise, infections, and psychological stress. However, it is our experience that those factors do not have a lasting influence on the individual's immunoreactivity with intraindividual fluctuations over a long interval being rather low.[26]

These different behaviors of individuals' PBMC towards fullerenes in vitro may indicate that those differences may also occur in vivo, predisposing some individuals to develop adverse reactions to these substances after in vivo application. To identify those subjects prior to therapy and to better calculate the risk, it is therefore important to analyze in more depth the influence of buckminsterfullerenes on immunocompetent cells in further studies, especially to elucidate their effect on cells of the innate immune system with respect to interaction with receptors, surface, or intracellular molecules as well as signal transduction pathways.

Acknowledgment

Disclosure
The authors report no conflicts of interest in this work.

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