Proinflammatory effects of bare and PEGylated ORMOSIL-, PLGA- and SUV-NPs on monocytes and PMNs and their modulation by f-MLP

Aims: We wanted to test the proinflammatory effects of vinyltriethoxysilane-based organically modified silica nanoparticles (ORMOSIL-NPs) *in vitro* on blood leukocytes. **Materials & Methods:** Cell selectivity, cytokines/chemokines and O_2^- production were analyzed using nonpolyethylene glycol (PEG)ylated and PEGylated ORMOSIL-NPs, poly(lactic-co-glycolic acid) (PLGA)-NPs and small unilamellar vesicles (SUV)-NPs. **Results:** ORMOSIL-NPs mostly bound to monocytes while other NPs to all leukocyte types similarly. Cell capture of PEGylated-NPs decreased strongly (ORMOSIL), moderately (PLGA) and weakly (SUV). Bare ORMOSIL-NPs effectively stimulated the production of IL-1β/IL-6/TNF-α/IL-8 by monocytes and of IL-8 by polymorphonuclear leukocytes (PMNs). NP PEGylation inhibited such effects only partially. Formyl-methionine-leucine phenylalanine (f-MLP) further increased the release of cytokines/chemokines by monocytes/PMNs primed with bare and PEGylated ORMOSIL-NPs. PEGylated SUV-NPs, bare and PEGylated ORMOSIL- and PLGA-NPs sensitize PMNs and monocytes to secrete O_2^- upon f-MLP stimulation. **Conclusion:** ORMOSIL-NPs are preferentially captured by circulating monocytes but stimulate both monocytes and PMNs *per se* or by sensitizing them to another agonist (f-MLP). PEG-coating confers stealth effects but does not completely eliminate leukocyte activation. Safe nanomedical applications require the evaluation of both intrinsic and cooperative proinflammatory potential of NPs.

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Nanoparticles (NPs) are innovative therapeutic/diagnostics agents, showing unique properties compared with bulk materials or single small molecules [1,2]. They can perform different roles simultaneously if loaded with suitable chemicals: superparamagnetic NPs can be contrasting agents for MRI [3] or generate heat in hyperthermic therapy and luminescent semiconductors NPs can be used for imaging or photodynamic therapy. In addition NPs may target tumor or other diseased tissues if derivatized with specific ligands [4].

On the other hand, the high surface/volume ratio of NPs increases their tendency to bind and activate host factors compared with bulk materials, thus increasing the risk of severe secondary effects [5,6]. Another problem for the medical application of NPs is their rapid phagocytosis by the reticuloendothelial system macrophages, mediated by plasma opsonines [3,7]. Coating with hydrophilic polymers like polyethylene glycol (PEG) counteracts NPs blood-clearance, prolonging their half-life in the circulation (stealth effect) by preventing NP-opsonin association and functions [8,9]. Blood-injected NPs may also stimulate circulating monocytes and polymorphonuclear leukocytes (PMNs) to release proinflammatory cytokines/chemokines and cytotoxic reactive oxygen species (ROS). This may trigger systemic hypersensitivity reactions or shock/disseminated intravascular coagulation [10,11]. The medical hazard represented by NP–leukocyte interaction is suggested by *in vitro* studies showing that TiO_2 NPs, carbon nanotubes and synthetic amorphous silica-NPs activate human PMNs, macrophages and dendritic cells [12–16].

Recently, we have developed a method to produce organically modified silica nanoparticles (ORMOSIL-NPs) characterized by a high-density covalently-engrafted PEG superficial layer (37% w/w for ~40 nm NPs), obtained by copolymerization of vinyltriethoxysilane (VTES) and suitable PEG-silane derivatives in the hydrophobic core of Brij detergent micelles [17–19]. We showed that PEGylated ORMOSIL-NPs are poorly endocytosed by macrophages and display a reduced procoagulant activity compared with the non-PEGylated version [18,19] and therefore suggested that they can be a valuable drug carrier system.

For this reason we here further investigated the interaction of both non-PEGylated and PEGylated VTES-based ORMOSIL-NPs with human blood leukocytes, to test their proinflammatory potential. ORMOSIL-NPs were compared with bare and PEGylated poly(lactic-co-glycolic acid) (PLGA)-NPs and small unilamellar vesicles (SUV)-NPs, two NPs

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already approved for use in humans [20,21]. We characterized the association of these three types of NPs with the principal subsets of blood leukocytes (PMNs, B and T lymphocytes, monocytes and NK cells) and evaluated:

▪ The induction of markers related to antigen presentation

▪ The secretion of cytokines/chemokines

▪ The generation of $O_2^{\cdot-}$ by the potent phagocyte NADPH–oxidase system

It is well known that exposure of leukocytes to some agents, such as lipopolysaccharide (LPS) or the cytokine TNF-α and IFN-γ causes an increased cell responsiveness to other stimuli, including the microbial chemoattractant formyl-methionine-leucine phenylalanine (f-MLP) [22]. Therefore, we also evaluated whether NPs cooperate with f-MLP in the induction of cytokines and $O_2^{\cdot-}$. We think that this approach may be very valuable in assessing *in vitro* NP biocompatibility, because f-MLP receptors (FPRs) are involved in monitoring not only formylated peptides deriving from microbes, but also peptides generated from tissue damage [23]. The intent of our novel experimental approach was to mimic the possible cooperation of NPs with both exogenous and endogenous peptides, to reveal the possible capability of NPs to sensitize PMNs and monocytes to other agonists.

Results showed that the inflammatory responses triggered by NPs are influenced by their chemical composition, surface PEGylation and f-MLP costimulations and by the cell type engaged. Major conclusions are that:

▪ PEG coating may determine, if dense enough, a marked stealth feature of NPs but nevertheless does not completely abolish NPs proinflammatory effects;

▪ f-MLP co-stimulation strongly improved bioactivities of both non-PEGylated and PEGylated NPs.

Our results also emphasize that the *in vitro* estimation of the inflammatory potential of nanostructured materials is very complex and requires multiple analysis based on:

▪ Different primary cell types

▪ Analysis of a wide range of activation parameters

▪ Cell costimulation protocols using NPs together with other agonists, to uncover possible hidden biological activities

Materials & methods
▪ Nanoparticles
All NPs were prepared using aseptic (0.22-μm membrane filter-sterilized) and endotoxin-free solutions (<0.025 endotoxin units/ml as measured with the Limulus test). Non-PEGylated and PEGylated ORMOSIL-NPs of different diameters were loaded with cyanine dye IR775 as described elsewhere [18,19]. Non-PEGylated or PEGylated (PLGA)- and SUV-NPs, doped with 0.1% phycoerythrin (PE), a derivative of dipalmytoilfosfatidilcholine were prepared as described in [19]. PEG 2000 was used in all NP formulations and PEG incorporation was the maximal ones: 37% (~50 nm ORMOSIL-NPs), 7.5% (PLGA-NPs), 4% (SUV-NPs). The mean diameters (± SE; n = 4) of ORMOSIL-NPs, measured by dynamic light scattering (using a zetasizer Nano S, Malevern Instruments) were: 20 ± 4 nm, polydispersity index (PI): 0.6 ± 0.1; 51 ± 3 nm, PI: 0.07 ± 0.02; 110 ± 14 nm, PI: 0.03 ± 0,01 and 170 ± 10 nm, PI: 0.07 in the case of non-PEGylated NPs and: 25 ± 3 nm, PI: 0.5 ± 0.005; 45 ± 8 nm, PI: 0.15 ± 0.06; 90 ± 7 nm, PI: 0.07 ± 0.01 and 180 ± 14 nm, PI: 0.1 ± 0.05 for PEGylated NPs. The zeta potential values (in phosphate-buffered saline [PBS] buffer, pH 7.4) were -6.2 ± 2.9 mV and -4.3 ± 0.84 mV respectively for the non-PEGylated and the PEGylated NP versions and -9.4 ± 1,6 mV and -5.5 ± 0.37 in 10% (v/v) fetal calf serum (FCS). ORMOSIL-NPs aggregation state/distribution size in 10% FCS did not change significantly [19]. The characteristics of PLGA-NPs were as follows:

▪ PLGA-NP in water: size: 340.0 ± 20.0 nm; PI: 0.34 ± 0.03; zeta potential -25.0 ± 2.4 mV;

▪ PLGA-NP in 10% FCS: size: 609.2 ± 19.4 nm; PI: 0.54 ± 0.06; zeta potential: -5.52 ± 0.43 mV;

▪ PEG-PLGA in water: size: 282.0 ± 11.2 nm; PI: 0.3 ± 0.04; zeta potential: -21.3 ±-2.0 mV;

▪ PEG-PLGA in 10% FCS: size: 445.4 ± 7.9 nm; PI: 0.31 ± 0.04; zeta potential: -2.53 ± 1.06 mV.

ORMOSIL-NPs and SUV-NPs are stable for months at room temperatures, while PLGA-NPs tend to destabilize in solution after more than 1 day.

▪ Purification of whole leukocytes
Venous blood was taken from healthy volunteers and immediately anticoagulated with 3.8% trisodium citrated (9 vol blood + 1 vol citrate). After erythrocytes lysis by hypotonic shock in 155 mM NH_4Cl, 10 mM $KHCO_3$ and 100 mM

Na$_2$EDTA at pH 7.4 for 3 min at room temperature, remaining leukocytes were washed twice with lysis buffer.

■ Purification of monocytes & polymorphonuclear leukocytes

Peripheral blood mononuclear cells were isolated from buffy coats of healthy donors by centrifugation over a Ficoll-Hypaque (Amersham Biosciences, Björkgatan, Sweden) step gradient and a subsequent Percoll (Amersham Biosciences) gradient and resuspended in RPMI-1640 (GIBCO, CA, USA) supplemented with antibiotic and 2% FCS (EuroClone, Milan, Italy). Residual T and B cells were removed from the monocyte fraction by plastic adherence for 1 h at 37°C. The purity of preparations (percentage of CD14$^+$ cells) and cell viability (using the trypan blue exclusion test) were both higher than 98%.

Human PMNs were purified from healthy donors as previously described, using dextran sedimentation, centrifugation through Ficoll-Hypaque gradient, followed by hypotonic lysis of contaminating erythrocytes and washing with PBS. The percentage of contaminating cells was under 5%. Unless otherwise specified, monocytes and PMNs were kept at 37°C in a humidified atmosphere containing 5% (v/v) CO$_2$ in RPMI-1640 supplemented with antibiotic and 10% FCS.

■ Nanoparticle association with leukocyte subpopulation

Whole leukocytes (2 × 10^6) were incubated for 20 h with NPs in RPMI-1640 medium supplemented with 10% FCS at 37°C. Cells incubated with no NPs were used as a negative control. Cells were then washed twice with PBS (pH 7.2), then resuspended in FACS buffer (PBS containing 1% FCS and 0.1% NaN$_3$) and then further incubated with the proper dilution of different anti-CD monoclonal antibodies (CD3, CD16, CD19, CD66b and CD14, Biolegend, CA, USA) conjugated to PE for 30 min on ice, to identify the main leukocyte populations. After a wash with FACS buffer, propidium iodide was added to exclude dead cells and cell fluorescence intensities of the gated populations were measured with a FACS Canto flow cytometer and analyzed with FACSDiva software (Becton Dickinson, NJ, USA).

■ Nanoparticles association with monocytes & PMNs

Monocytes and PMNs (2 × 10^6), seeded onto 24-well plates, were incubated with different concentrations (10–25–50–100 and 200 µg/ml) of fluorescent, bare or PEGylated ORMOSIL-, PLGA- and SUV-NPs, for 3 and 24 h, respectively, in RPMI-1640 medium supplemented with 10% FCS, at 37°C. Cells were then collected, washed with PBS and resuspended in cold FACS buffer (PBS containing 1% FCS). Propidium iodide was added to exclude dead cells. The capture of NPs was evaluated by cell fluorescence intensities (cyanine for ORMOSIL-NPs, PE-dipalmytoilfosfatidilcholine for PLGA- and SUV-NPs) of the gated populations, measured with BD FACSCanto II flow cytometer and analyzed with FACSDiva software.

■ Real-time PCR analysis

A total of 3 × 10^6 PMNs and monocytes were incubated for 5 and 20 h, respectively, at 37°C with different stimuli: bare or PEGylated ORMOSIL-, PLGA- and SUV-NPs at 50 µg/ml, or LPS 0.2 µg/ml. Treated and untreated cells were scraped and total RNAs were isolated using TRIzol solution (Sigma, MO, USA), according to manufacturer's instructions, and resuspended in 12 µl of RNase-free water (Gibco). RNA was quantified by spectrophotometric analysis (NanoDrop® ND-1000 Spectrophotometer, EuroClone). Equal amounts of RNA (300 ng) were retrotranscribed and the cDNA concentration of $IL-1\beta$, $IL-6$, $IL-8$ and $TNF-\alpha$ encoding genes were quantified by real-time quantitative PCR using a iQ^{TM} SYBR Green Supermix (Biorad, CA, USA) with iQ5 2.0 Biorad System according to the manufacturer's instructions (Biorad). The following primers were used: for GAPDH, 5′-AGCAACAGGGTGGTGGAC-3′ and 5′-GTGTGGTGGGGGACTGAG-3′; for IL-1β, 5′-CTGTCCTGCGTGTTGAAAGA-3′ and 5′-TTGGGTAATTTTTGGGATCTACA-3′; for IL-6, 5′-AACCTGAACCTTCCAAAGATGG-3′ and 5′- TCTGGCTT GTTCCTCACTACT-3′; for IL-8, 5′-TTGGCAGCCTTCCTGATT-3′ and 5′-AACTTCTCCACAACCCTCTG-3′ and for TNF-α, 5′- ATGAGCACTGA AAGCATGATC-3′ and 5′-GAGGGGCTGA TTAGAGAGAGGT-3′.

Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. Cycle threshold (C$_T$) values were determined by the GeneAmp 5700 SDS software using fluorescence thresholds manually set and exported into Excel for analysis. After the amplification, data analysis were performed using the second derivative method algorithm. For each sample, the amount of mRNA of the cytokines was expressed as the n-fold of the normalized amount of mRNA in untreated

cells (1 arbitrary unit = cytokine mRNA concentration/GAPDH mRNA concentration [both in fmol/μl]).

■ Detection of IL-1β, IL-6, TNF-α & IL-8 in culture supernatants

Culture supernatants of monocytes and PMNs harvested for mRNA quantification were collected at the same time points, and the amounts of IL-1β IL-6, IL-8 and TNF-α protein were measured by capture ELISA with Ab pairs and cytokine standard. IL-1β assay kit was purchased from Bender MedSystems (CA, USA), IL-6, IL-8 and TNF-α from Peprotech (NJ, USA). The responsivity of cells after isolation was found to be subjected to some variability, depending on the buffy coat.

■ Detection of f-MLP modulation of IL-1β, IL-6 & IL-8 production by monocytes & PMNs

A total of 2×10^6 monocytes were incubated at 37°C for 1 h in presence or absence of different concentrations (5–10–25–50–100 μg/ml) of bare or PEGylated ORMOSIL-NPs, and then stimulated with 0.01–0.1–1–10–100–1000 nM fMLP for 20 h at 37°C. Culture supernatants were collected and the amount of IL-1β, IL-6, TNF-α and IL-8 protein was quantified by ELISA.

A total of 2×10^6 PMNs were preincubated at 37°C for 1 h in the presence or absence of different concentrations (5–10–25–50–100 μg/ml) of bare or PEGylated ORMOSIL-NPs stimulated with 1 nM or 1 μM f-MLP for 20 h at 37°C. Culture supernatants were collected and the amount of IL-8 protein was quantified by ELISA.

■ Superoxide anion production

$O_2^{\cdot-}$ release was estimated by cytochrome C reduction. Briefly, cells were preincubated at 37°C for 1 h in the presence or absence of 50 μg/ml of NPs and then stimulated with 1 nM or 1 μM f-MLP in Hank's-buffered salt solution pH 7.4 containing 80 μM ferricytochrome C type III (Sigma). Cytochrome C reduction was evaluated at 550 nm at different time points by using an automated microplate reader (Biotek® Instruments Inc., VT, USA).

Results
### ■ Distribution of nanoparticles in blood leukocytes

Organically modified silica-NPs are the main object of this study: we therefore initially evaluated their association to human leukocytes as a function of size. The whole leukocyte pool from fresh blood was incubated with fluorescent versions of bare or PEGylated ORMOSIL-NPs with diameters of 20, 50, 100 and 180 nm: larger NPs could not be obtained with our synthesis procedure [18]. Cell-uptake of NPs was determined after an overnight incubation by cytofluorimetry using simultaneous labeling with cell-specific markers (CD3 for T lymphocytes, CD19 for B lymphocytes, CD16 for NK cells, CD66b for PMNs and CD14 for monocytes). Results showed that, within this dimension range, non-PEGylated ORMOSIL-NPs were endocytosed by monocytes much more efficiently than by other leukocytes, regardless of their size (Figure 1A). However, while for diameters below 100 nm the extent of cell capture was similar, a strong increase was observed for diameters greater than 100, monocyte selectivity always being kept. High-density PEGylation of ORMOSIL-NPs always reduced their cell uptake, but with different efficacies according to size. PEGylated ORMOSIL-NPs monocyte capture was in fact approximately 50% (20-nm diameter NPs), 24% (50-nm diameter NPs), 33% (100-nm diameter NPs) and 3.4% (180-nm diameter NPs) of their correspondent non-PEGylated version. In absolute terms, endocytosis reached a minimum with 50-nm diameter non-PEGylated and PEGylated ORMOSIL-NPs. Since our goal was the characterization of PEGylated ORMOSIL-NPs as stealth nanocarriers, we focused on this optimal dimension for further experiments.

Figure 1B shows that PLGA-NPs were captured in a similar way by the different leukocyte types, while SUV-NPs associated to monocytes two- to three-fold more efficiently than to other leukocytes. Moreover, PEG coating determined a small decrease of cell uptake (5–30% inhibition according to cell type).

Given the importance of professional phagocytes in the physiopathology of inflammatory reactions, we decided to focus on the interaction between our different NPs with monocytes and PMNs (Figure 2). The efficacy and selectivity of NP–cell association, and the acquisition of stealth properties by NPs consequent to PEG coating were tested at increasing NP concentrations after 3- and 24-h incubations (37°C). The measurement after 3 h incubation was necessary to detect more accurate differences in NP uptake kinetics, but also to avoid problems derived from the tendency of resting PMNs to undergo significant apoptosis after prolonged culture *in vitro*. In these conditions, although many NPs have been reported to
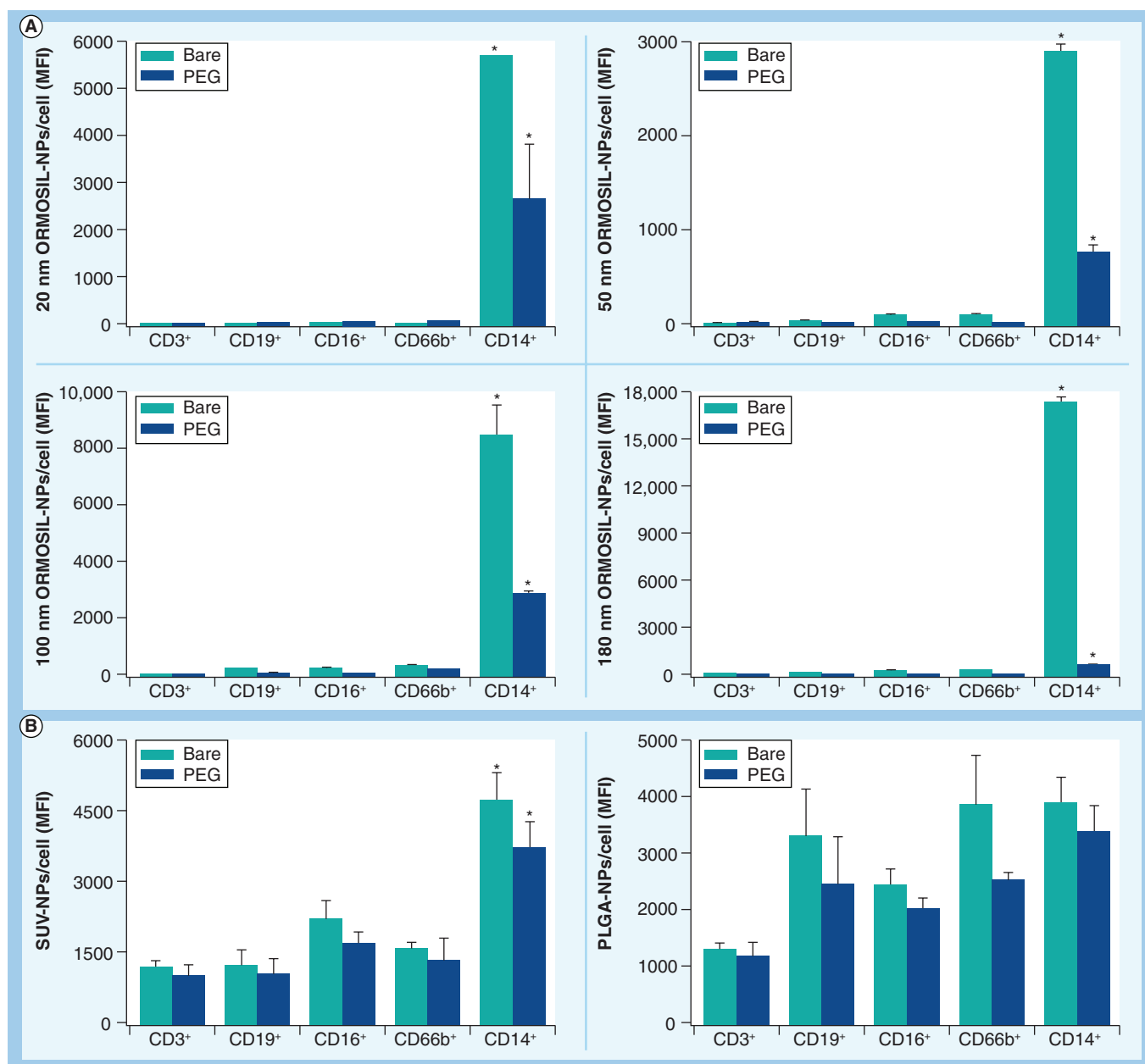
Figure 1. Association of bare or PEGylated nanoparticles to leukocyte subpopulations. (A) Whole leukocytes were incubated for 20 h at 37°C with 50 µg/ml of indicated bare or PEGylated ORMOSIL-NPs of indicated diameters or with **(B)** bare or PEGylated SUV- and PLGA-NPs. Cells were then collected, and nanoparticle association was determined as MFI in different leukocytes, labeled with cell-specific markers (CD3 for T lymphocytes, CD19 for B lymphocytes, CD16 for NK cells, CD66b for PMNs and CD14 for monocytes) by cytofluorimetry. Data are the means from a representative experiment out of three, run in duplicate and error bars represent ± standard error.
*$p < 0.05$, significance between the different uptake of NPs by monocytes with respect to other cell types.
MFI: Mean fluorescence intensity; NP: Nanoparticle; ORMOSIL: Organic modified silica; PEG: Polyethylene glycol;
PLGA: Poly(lactic-co-glycolic acid); PMN: Polymorphonuclear leukocyte; SUV: Small unilamellar vesicles.

determine cell death [24], we found no cytotoxicity in monocytes after 24 h as tested by propidium iodide exclusion and annexin V binding. In the case of PMNs no increased cell death rate was evidenced after 3 h of NPs incubation, given the shorter half-life of these cells (not shown).

FIGURE 2A shows all bare NPs associated with monocytes and PMNs with a relatively slow time-course, the cell signal being much reduced after 3 h of NP incubation compared with that observed after 1-day incubation, and that cell uptake was generally maximal around 100–200 µg/ml NPs concentration. The tendency of NPs to associate with monocytes more intensely was clearer after 3 h than after 24 h: not only SUV- and ORMOSIL-NPs, but also PLGA-NPs preferentially bound to monocytes compared with PMNs. Data also confirmed that SUV-NPs
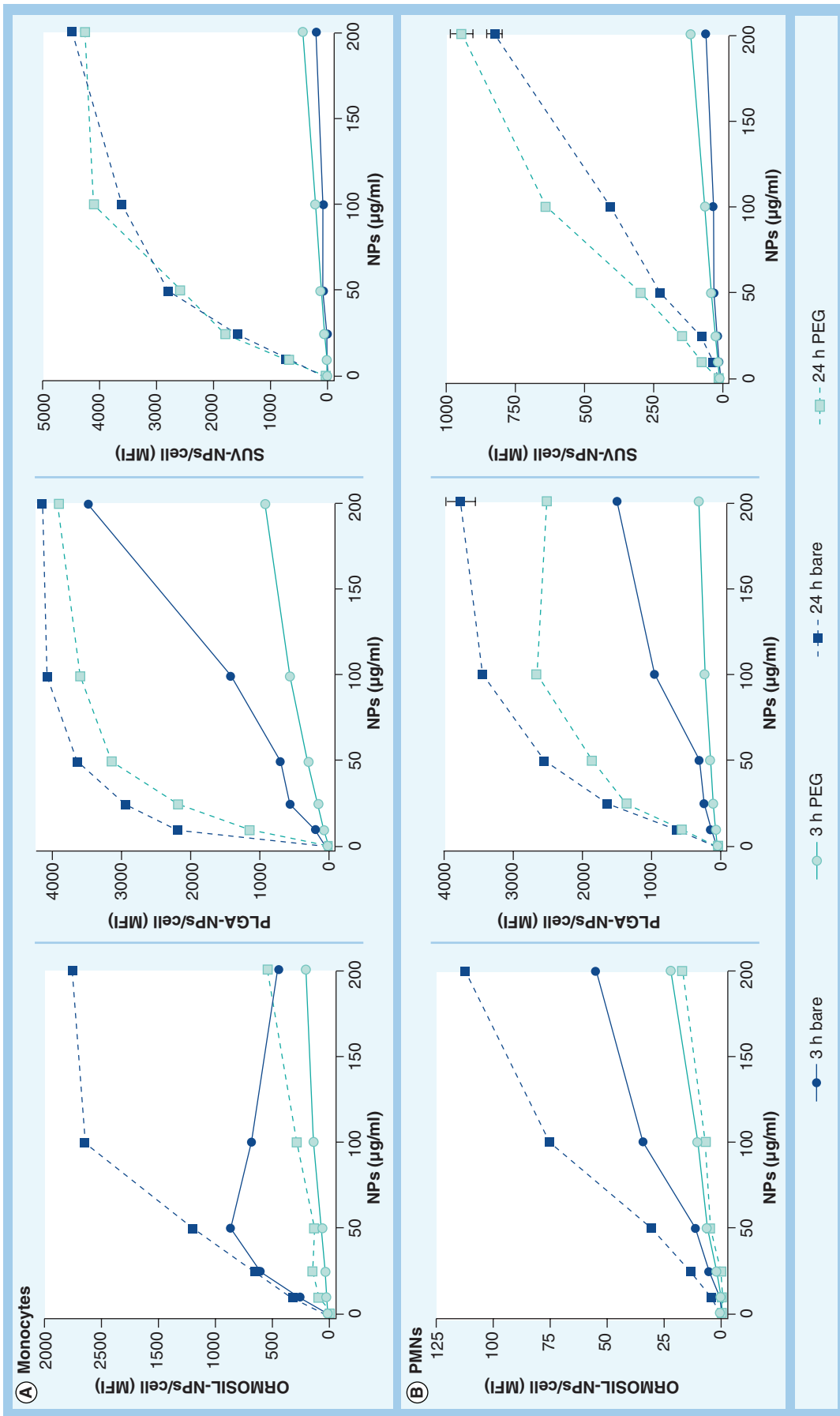
Figure 2. Nanoparticle uptake by monocytes and polymorphonuclear leukocytes. (A & B) Dose-dependent nanoparticle uptake by monocytes and PMNs treated with different concentrations of bare (dark colored circle and square) or PEGylated (light colored circle and square) nanoparticles, for 3 h (circle) and 24 h (square) at 37°C. Nanoparticle capture is expressed as MFI, determined by cytofluorimetry. Data are the means from a representative experiment out of five, run in duplicate and error bars represent ± standard error.
MFI: Mean fluorescence intensity; Mon: Monocyte; NP: Nanoparticle; ORMOSIL: Organic modified silica; PEG: Polyethylene glycol; PLGA: Poly(lactic-co-glycolic acid);
PMN: Polymorphonuclear leukocyte; SUV: Small unilamellar vesicle.
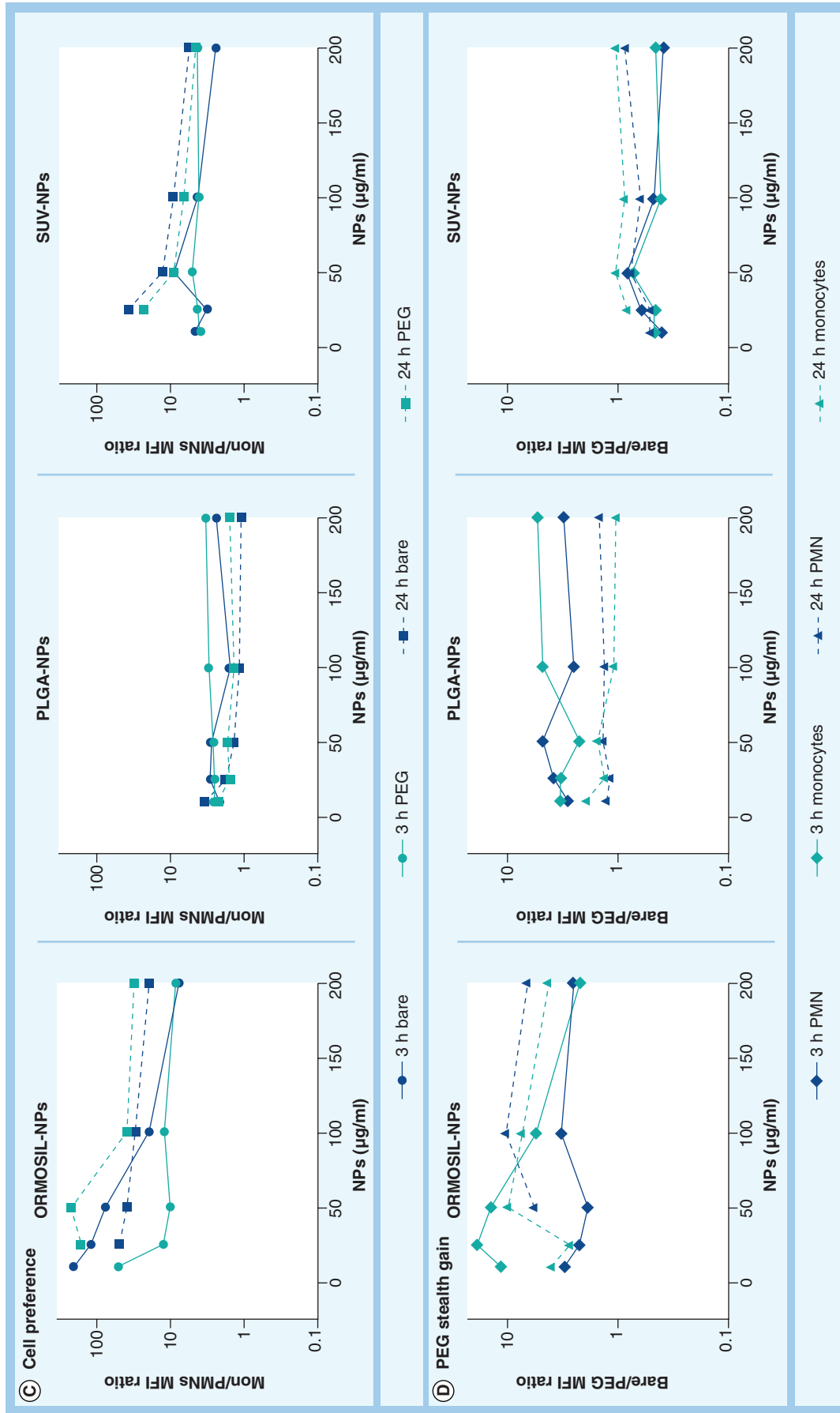
Figure 2 (cont.). Nanoparticle uptake by monocytes and polymorphonuclear leukocytes. (C & D) Quantitative analyses of nanoparticle association to monocytes and PMNs. Ratio between the cell MFI due to nanoparticle association to monocytes and PMNs (Mon/PMNs MFI ratio, **[C]**), showing the cell preference of nanoparticles. Ratio between the MFI due to bare and PEGylated nanoparticles in the same cell type (monocytes or PMNs; bare/PEG MFI ratio, **[D]**), showing the gain of NPs' stealth property. NP uptake was evaluated after 3 and 24 h of cell treatment with different concentrations of bare or PEGylated SUV-, PLGA- and ORMOSIL-NPs.
MFI: Mean fluorescence intensity; Mon: Monocyte; NP: Nanoparticle; ORMOSIL: Organic modified silica; PEG: Polyethylene glycol; PLGA: Poly(lactic-co-glycolic acid); PMN: Polymorphonuclear leukocyte; SUV: Small unilamellar vesicle.

and PLGA-NPs uptake were not significantly reduced by PEGylation after 24 h incubation. However, PEGylated PLGA-NP uptake was significantly lower after a 3-h incubation compared with bare PLGA-NPs. PEGylation of ORMOSIL-NPs determined a strong reduction of both monocytes and PMNs uptake at any time, in agreement with whole leukocyte population studies.

The different behavior of the diverse NP formulation is better exemplified by a quantitative analysis based on the calculation of the ratio between the cell mean fluorescence intensity (MFI) due to particle association in monocytes and in PMNs (mon/PMN MFI ratio) (Figure 2B). Such an analysis confirmed that all NPs associate more selectively with monocytes, but with different intensities: it was very strong in the case of ORMOSIL-NPs (mon/PMN MFI ratio ranging from 10 to 100 according to NPs dose and incubation time), moderate in the case of SUV-NPs (4–10) and relatively small, although significant, in the case of PLGA-NPs (1–3). With a similar approach we could analyze the gain of NP stealth property due to surface PEGylation, by plotting the ratio between the bare and PEGylated-NPs MFI, after uptake in the same cell type at the same concentration (bare/PEG MFI ratio). Data (Figure 2B) show that bare/PEG MFI ratios followed this order: ORMOSIL-NPs (5–14 in monocytes and 2.6–6.5 in PMN) > PLGA-NPs (1.2–3.6 in both monocytes and PMNs) > SUV-NPs (0.5–0.95 in both monocytes and PMNs). These data confirmed that PEGylation strongly decreases the uptake of ORMOSIL-NPs by PMNs and monocytes and has a moderate reduction effect on PLGA-NPs cell association. In this latter case, uptake was significantly lower after 3 h incubation compared with bare PLGA-NPs, while after 24 h incubation the PLGA-NPs were at least partially degraded and the difference was not obvious. PEGylation was virtually irrelevant for SUV-NP cell capture.

■ Monocytes & polymorphonuclear leukocytes activation by nanoparticles

Cytokine/chemokine secretion is a hallmark of inflammation and their generation in the blood is a dangerous condition that may lead to a generalized shock. Monocytes may produce significant amounts of proinflammatory cytokines and chemokines when appropriately stimulated by microbial agonists, such as LPS, while PMNs secrete a more restricted panel of immune mediators, among which the most

abundant is the chemokine IL-8. We therefore decided to analyze the activation of the transcription of the gene encoding the main cytokines/chemokines in monocytes (after 24 h) and in PMNs (after 5 h due to the high mortality of these cells after prolonged incubation) by real-time PCR (Figure 3). In addition, we also analyzed the amount of the secreted cytokines/chemokines (in this case both monocytes and PMNs were incubated for 24 h with agonists). The effects induced by our ORMOSIL-, PLGA- and SUV-NPs in bare or PEGylated state, were compared with those induced by a maximally active LPS dose (0.2 μg/ml). We found that only a few of them were reproducibly and consistently induced by bioactive NPs: IL-1β, IL-6, TNF-α and IL-8 in monocytes and IL-8 in PMNs. We therefore concentrated our attention on these mediators in the rest of our study.

Bare ORMOSIL-NPs induced a relevant secretion of IL-1β, IL-6, TNF-α and IL-8 by monocytes and of IL-8 by PMNs. We documented in the previous paragraph that PEGylation of ORMOSIL-NPs resulted in an approximately 80–90% reduced capture by both monocytes and PMNs. However, we found that coating with this stealthing agent did not reduce all cytokine/chemokine production with parallel efficacy. In fact, while IL-6 and IL-8 production were strongly affected by PEGylation (70–80% inhibition) in monocytes, IL-1β and TNF-α were only 40 and 30% reduced, respectively, compared with bare ORMOSIL-NPs effects. In PMNs, PEGylated ORMOSIL-NPs showed a strong reduction in their IL-8 inducing activity. Bare and PEGylated SUV- and PLGA-NPs did not stimulate cytokine/chemokine production by either monocytes or PMNs. Parallel real-time PCR analysis confirmed these data and demonstrated that active NP effects are mediated by an increased transcription rate of IL-1β, IL-6, IL-8 and TNF-α encoding genes.

Owing to our primary interest in silica-based NPs, cytokine/chemokine secretion was further analyzed using different doses of ORMOSIL-NPs. For simplicity, data concerning IL-1β and IL-6 secretion are shown in the case of monocytes, while data concerning IL-8 secretion are only shown in the case of PMNs (Figure 4). Bare ORMOSIL-NPs effectively induced IL-1β at doses as low as 5 μg/ml in monocytes and reached a plateau at approximately 20 μg/ml. Again, PEGylated ORMOSIL-NPs were less effective than their bare version but not fully inactive: at 50 μg/ml and 100 μg/ml IL-1β production was approximately 50% and approximately 80%, respectively, of the effects
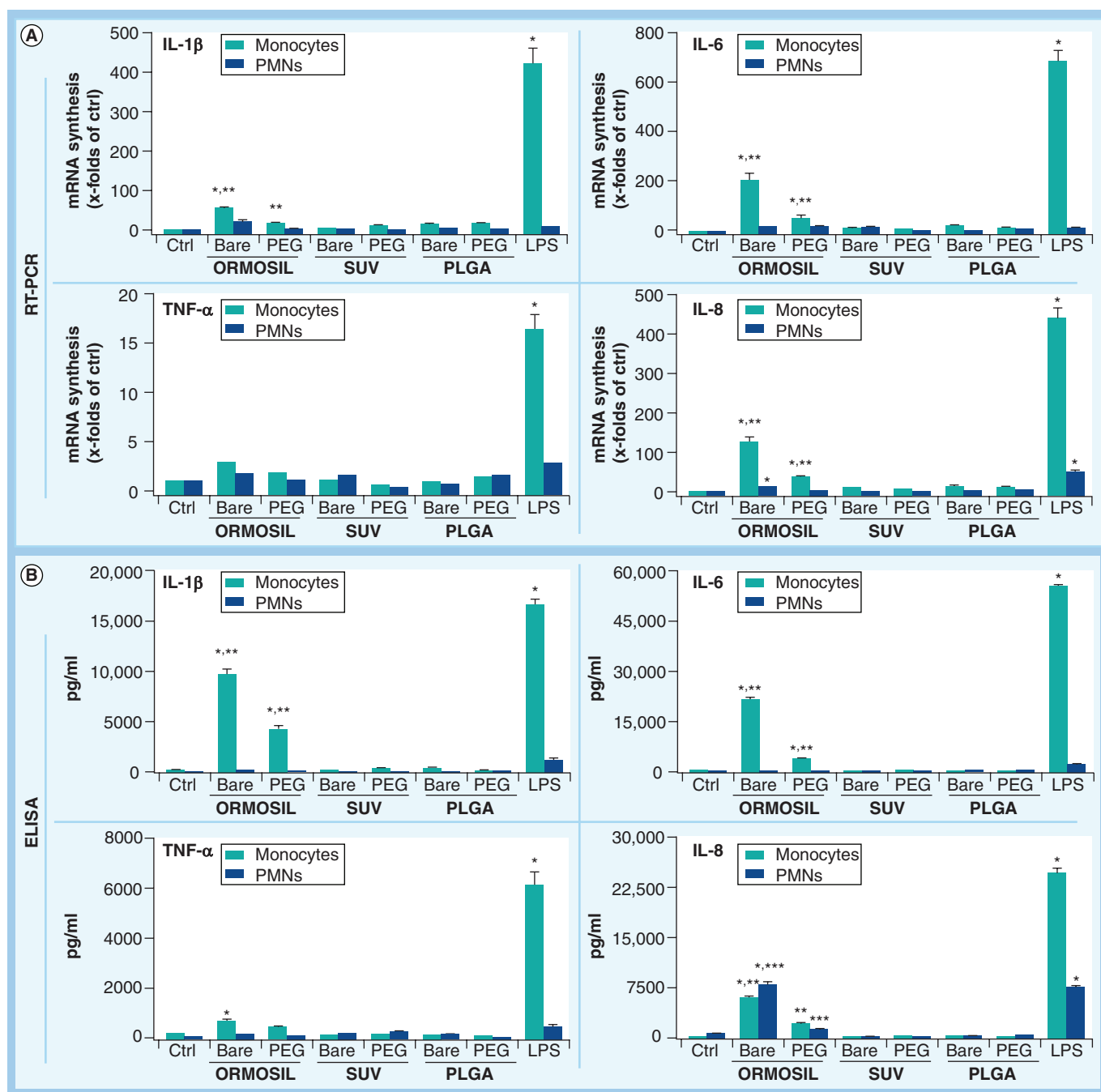
Figure 3. mRNA expression and protein production of IL-1β, IL-6, IL-8 and TNF-α in monocytes and polymorphonuclear leukocytes treated with different nanoparticles. Total RNA was extracted from monocytes and PMNs treated with 50 µg/ml of different NPs (bare or PEGylated) for 20 h at 37°C. **(A)** IL-1β, IL-6, IL-8 and TNF-α expression were analyzed by RT-PCR. Expression levels are depicted as n-folds of the normalized amount of mRNA from untreated cells (1 AU = mRNA cytokine concentration [fmol/µl]/mRNA GAPDH [fmol/µl]) of triplicate reactions for each sample. **(B)** Culture supernatants of monocytes and PMNs treated with nanoparticles for 20 h were used to measure the amount of IL-1β IL-6, IL-8 and TNF-α protein by ELISA. The figure shows the mean value of duplicate assays for each treatment, obtained from a representative experiment out of four and error bars represent ± SE. Cytokine expression was compared with those induced by untreated cells or cells treated with LPS (0.2 µg/ml). Absolute cytokine/chemokine production due to non-PEGylated NPs (50 µg/ml) was found to be subjected to some variability according to buffy coats; the approximate range being 3000–13,000 pg/ml (IL-1β), 6000–45,000 pg/ml (IL-6), 1000–6000 pg/ml (TNF-α), and 6000–15,000 pg/ml (IL-8) for monocytes, and 5000–9000 pg/ml (IL-8) for PMNs.
*p < 0.05, significance of cytokine/chemokine expression with respect to the control.
**p < 0.05, significance of cytokine production in cells treated with bare-NPs with respect to PEGylated-NPs in monocytes.
***p < 0.05, significance of cytokine production in cells treated with bare-NPs with respect to PEGylated-NPs in PMNs.
Ctrl: Cells with no nanoparticles; LPS: Lipopolysaccharide: NP: Nanoparticle; ORMOSIL: Organic modified silica; PEG: Polyethylene glycol; PLGA: Poly(lactic-co-glycolic acid); PMN: Polymorphonuclear leukocyte; RT: Real-time; SUV: Small unilamellar vesicle.
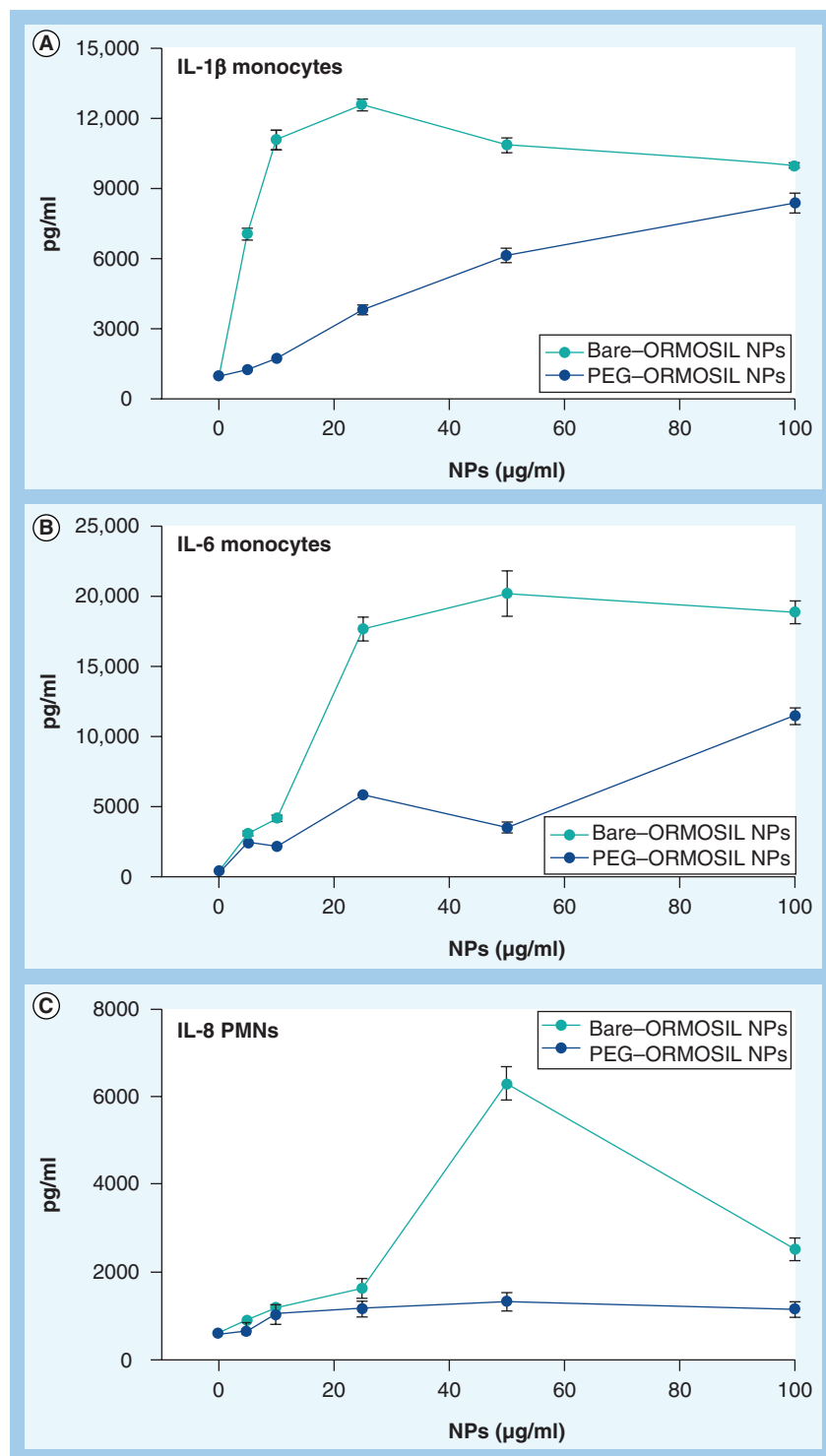
Figure 4. Secretion of IL-1β and IL-6 by monocytes and of IL-8 by polymorphonuclear leukocytes treated with organic modified silica nanoparticles. IL-1β and IL-6 production by isolated monocytes incubated with different concentrations of bare or PEGylated ORMOSIL nanoparticles for 20 h at 37°C. IL-8 production by isolated PMNs incubated with different concentrations of bare or PEGylated ORMOSIL-NPs for 20 h at 37°C. The figure shows the mean value ± standard error of duplicate assays for each NP concentrations, obtained from a representative experiment out of five performed under the same conditions.
NP: Nanoparticle; ORMOSIL: Organic modified silica; PEG: Polyethylene glycol; PMN: Polymorphonuclear leukocyte.

induced by bare ORMOSIL-NPs. In the case of IL-6 secretion we observed the same tendency: PEGylated ORMOSIL-NPs had a reduced but still significant (30–60%) cytokine stimulating activity at high (50–100 μg/ml) doses. It is worth reminding that in these conditions PEGylated ORMOSIL-NPs association to monocytes was ≤5% of that reached by their bare version. On the contrary, in PMNs, PEGylation resulted in a clear ablation of IL-8 inducing activity of ORMOSIL-NPs, possibly because of their association to these cell types was virtually absent in absolute terms (~20 MFI in PMNs compared with ~600 MFI in monocytes (NPs: 200 μg/ml) (FIGURES 1 & 2).

■ **f-MLP modulation of cytokine/chemokine production by nanoparticle-treated monocytes & PMNs**

It is known that some agents induce a hyper-responsiveness to other subsequently added stimuli, such as f-MLP [22]. f-MLP is a model tripeptide for bacterially released formylated leader peptides that bind to special receptors enriched in monocytes and PMNs (FPRs). We then assessed whether NPs induce and/or enhance the cell responsiveness to f-MLP in terms of cytokine production. It is worth mentioning that f-MLP binds to and activates high affinity (Kd < 1 nM) and low-affinity FPRs (Kd 1.5 μM), monitoring the presence of peptides that could be released by bacteria, but also by damaged tissues [23]. Therefore, our experiments could mimic the cooperative effects of NPs and both exogenous and endogenous compounds. FIGURE 5 shows that addition of f-MLP to monocytes preincubated (60 min) with bare ORMOSIL-NPs (50 μg/ml), positively modulates the secretion of IL-1β, IL-6 IL-8 and TNF-α. While f-MLP was without effects *per se* at concentrations up to 1 μM, it synergized the NPs ability to release IL-1β and IL-6, reaching a maximum at a concentration of approximately 1 nM and then decreasing at higher concentrations, bringing cytokine productions back to values equal or lower to the ones induced by NPs alone at a concentration of approximately 1 μM. At variance, TNF-α and IL-8 secretion were only synergized by subsequent f-MLP stimulation at a concentration of approximately 1 μM. In the case of PEGylated ORMOSIL-NPs, f-MLP induced a similar bell-shaped synergic curve, which determined an IL-1β, IL-6 and IL-8 production significantly closer to those induced by bare ORMOSIL-NPs in the same conditions. These data suggest that the activation of high-affinity FPRs potentiate

NP-induced IL-1β and IL-6 release by mono-cytes. The engagement of low-affinity recep-tors counteracts the high-affinity stimulation of IL-1β/IL-6, annihilating the synergy with NPs, but at the same time induced an enhanced release of TNF-α and IL-8.

To better characterize the synergy between NPs and f-MLP, monocytes and PMNs were incubated with different NP concentrations in the presence of f-MLP, 1 nM (to activate only high-affinity FRPs) or 1 µM (to activate both high- and low-affinity FRPs). It is worth noting, that in mono-cytes the bare-ORMOSIL effects in the presence of 1 nM costimulation reaches its maximum already at 5 µg/ml and then tends to decrease. In both cases, in agreement with previously reported evidence, a higher f-MLP dose (1 µM) is accompanied by a decrease of cytokine produc-tion compared with the action of NPs alone. Data show that the potentiation of cytokine/chemokine releasing effects of f-MLP is more evident at low concentrations of bare ORMOSIL-NPs (FIGURE 6).

In the case of PMNs, the bare ORMOSIL-NP effect was synergized by 1 µM f-MLP, but not by 1 nM f-MLP. Concerning PEGylated ORMOSIL-NPs, it was confirmed that their effects were reduced at low concentrations com-pared with bare versions (below 50 µg/ml). However at high doses (100 µg/ml) the effects of PEGylated ORMOSIL-NPs were not disting-uishable from the effects induced by the bare NPs, with the already mentioned exception of PMN IL-8 production, which remained at the background level (FIGURE 6). We confirmed that in monocytes, low f-MLP doses (1 nM) synergized PEGylated ORMOSIL-NPs dependent IL-1β and IL-6 secretion, while in PMNs, high f-MLP doses were necessary to unravel IL-8 secretion activity by PEGylated ORMOSIL-NPs, not far from the one induced in the same conditions by bare versions.

Since cytokine induction may be associated with cytotoxic effect, we analyzed monocyte and PMN cell permeabilization in the absence and in the presence of the different NPs, with or without f-MLP costimulation (not shown). No increase in cell toxicity was observed in any condition. On the other hand, the enhancing effect of silica NPs due to f-MLP may be due to an increased cell association/uptake of the NPs. However, this was found not to be the case, since NP associa-tion to monocytes and PMNs were not affected by f-MLP (not shown). Monocyte activation markers, related to antigen presentation (CD80, CD86, HLA-DR and ICAM-1) were analyzed after treatment with bare and PEGylated NPs as above in the absence or in the presence of f-MLP.
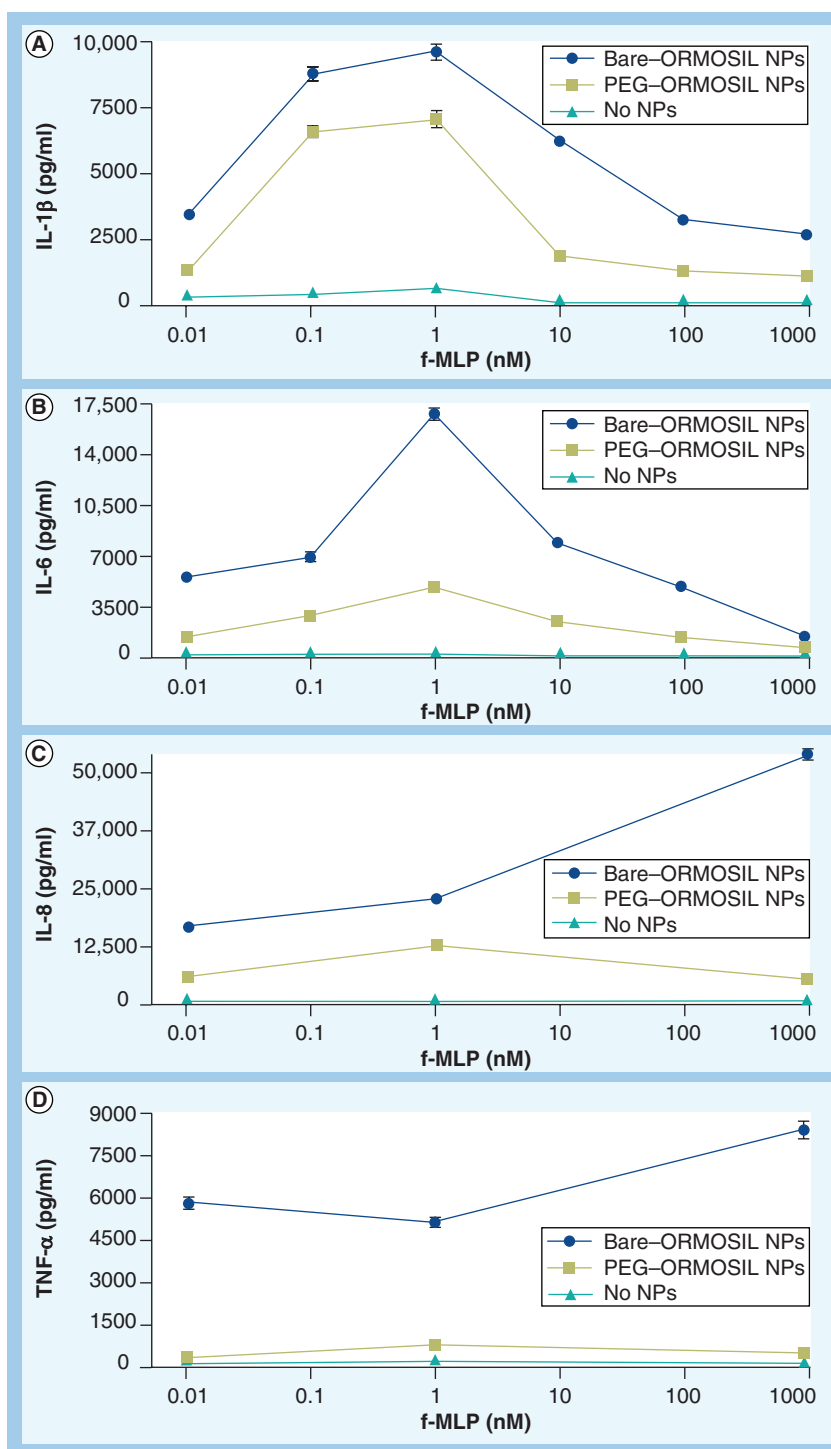
Figure 5. Secretion of IL-1β, IL-6, IL-8 and TNF-α in monocytes pretreated with organic modified silica nanoparticles, is modulated by formyl-methionine-leucil phenylalanine. Monocytes were cultured at 37°C for 1 h in the presence or absence of 50 µg/ml of bare or PEGylated ORMOSIL-NPs and then stimulated with different concentrations of f-MLP for 20 h at 37°C. The graphs show the amount of IL-1β, IL-6, IL-8 and TNF-α protein secreted (pg/ml) in the different treatments, quantified by ELISA. The figure shows the mean value ± standard error of duplicate assays for each f-MLP concentrations, obtained from a representative experiment out of four, performed under the same conditions.
f-MLP: Formyl-methionine-leucil phenylalanine; NP: Nanoparticle; ORMOSIL: Organic modified silica; PEG: Polyethylene glycol; PMN: Polymorphonuclear leukocyte.
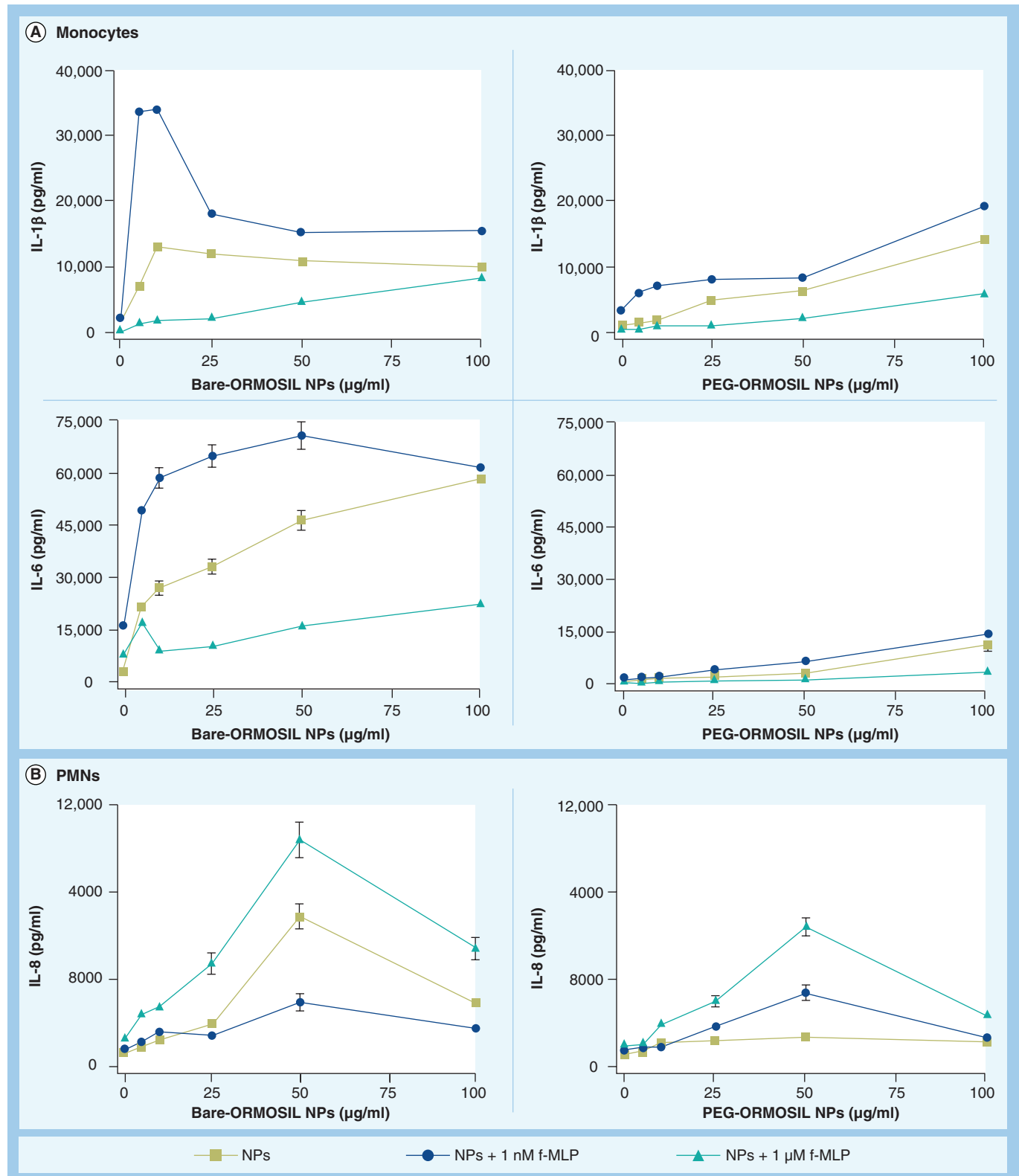
Figure 6. Dose-dependent production of IL-1β and IL-6 in monocytes, and IL-8 in polymorphonuclear leukocytes, treated with organic modified silica nanoparticles and stimulated with formyl-methionine-leucil phenylalanine. Monocytes were incubated at 37°C for 1 h with different concentrations of bare or PEGylated ORMOSIL-NPs, and then stimulated with 0–1–1000 nM of f-MLP for 20 h at 37°C. The graphs show the IL-1β, IL-6 and IL-8 proteins secreted in the different treatments, quantified by ELISA. Data are the means from a representative experiment out of four, run in duplicate and error bars represent ± standard error.
f-MLP: Formyl-methionine-leucil phenylalanine; NP: Nanoparticle; ORMOSIL: Organic modified silica; PEG: Polyethylene glycol; PMN: Polymorphonuclear leukocyte.

Results showed that none of NPs tested were able to stimulate the upregulation of the main activation markers (not shown).

■ Effect of nanoparticles on the activation of the $O_2^{\bullet-}$-generating NADPH oxidase in monocytes & polymorphonuclear leukocytes

Another relevant aspect shared by monocytes and PMNs is the presence of the microbicidal superoxide-generating NADPH oxidase. This enzyme can generate a large amount of O_2^- and secondary ROS upon proper stimulation: the so called respiratory burst. PMNs constitutively express a higher amount of the NADPH oxidase complex compared with monocytes and are therefore capable of generating more $O_2^{\bullet-}$. ROS production, important in innate defences, may become an important cause of cell and tissue damage. Activation of phagocyte NADPH oxidase is a very dangerous situation characterizing acute and chronic inflammatory conditions.

For this reason we measured the capability of our NPs panel to induce NADPH oxidase activation in monocytes and PMNs. The amount of O_2^- released in the extracellular medium was measured and found to be negligible in both monocytes and PMNs (Figure 7A & 7B) subjected to incubation with all our NP types for up to 5 h, with the exception of PEGylated PLGA-NPs, which induced a significant respiratory burst in PMNs (Figure 7A). This initial screening indicated that our NPs had a poor tendency to activate the NADPH oxidase in PMNs. However, the addition of 1-μM f-MLP determined a significant respiratory burst in PMNs preincubated with bare ORMOSIL-NPs, compared with the effect of 1 nM f-MLP. Such synergy between ORMOSIL-NPs and f-MLP doses occupying low-affinity FPRs was totally absent when PMNs were incubated with PEGylated ORMOSIL-NPs. It is worth noticing that 1 nM f-MLP failed to synergize with NPs in terms of NADPH oxidase activation in PMNs (Figure 7A).

Although in monocytes the O_2^- production activity is, as expected, approximately one order of magnitude reduced compared with that observed in PMNs, we observed a stimulation pattern completely differentiated. In these cells after priming with ORMOSIL-NPs, bare and PEGylated PLGA-NPs and PEGylated SUV-NPs, f-MLP determined activation of the NADPH oxidase. It is hence interesting to note that all PEGylated NPs were able to prime f-MLP effects in monocytes. Moreover, priming was observed at low f-MLP doses (1 nM) and also at high f-MLP doses, with the only exception represented by SUV-NPs priming, which interested only high-affinity f-MLP receptors (Figure 7B).

Discussion

The systemic activation of blood leukocytes by injected NPs for medical use is expected to induce an acute generalized inflammatory condition similar to septic or allergic shock. Consequently, an important nanomedical issue concerns the possibility that NPs are recognized by the immune system receptors. It is already known that innate signaling receptors bind to specific molecules released by microbes (pathogen-associated molecular patterns) or by damaged tissues (damage-associated molecular patterns) [25]. In addition, microcrystalline structures derived from endogenous metabolites, such as uric acid [26] and exogenous bulk or nanostructured crystalline/amorphous materials can induce undesired inflammatory reactions [14,15,27].

In this study we engaged in the *in vitro* characterization of the possible proinflammatory effects of the three main classes of NPs, using human blood leukocytes. We were prompted to do so because in our previous studies we initiated to characterize new drug-delivery formulations based on PEGylated ORMOSIL-NPs [18]. The synthetic procedure devised was very attractive because it allowed obtaining a thick and unprecedented dense (67–36 w/w%) coating formed by PEG molecules firmly attached on the NPs surface by covalent engraftment. We have already shown that these PEGylated ORMOSIL-NPs, differently from the non-PEGylated-forms, largely escape macrophage phagocytosis *in vitro* and poorly activate plasma coagulation [18,19]. However, other studies in animal models have shown that bare ORMOSIL-NPs are efficiently accumulated in the liver, producing clear signs of tissue additional infiltration of inflammatory cells [28]. Consistently, synthetic amorphous silica-NPs induced oxidative stress and IL-1β secretion in murine macrophages [15], suggesting that these, and possibly other related NPs, may activate the inflammosome-mediated IL-1β secretion, similarly to asbestos, silica and urate crystals [27]. An elegant study has also demonstrated that cytokine and ROS release induced by combustion-derived (diesel exhausted particles) and manufactured (CNT, TiO_2) NPs were influenced by an interplay between lung epithelial cells, macrophages and dendritic cells [14], suggesting that nanostructure effects may be modulated by other signals.
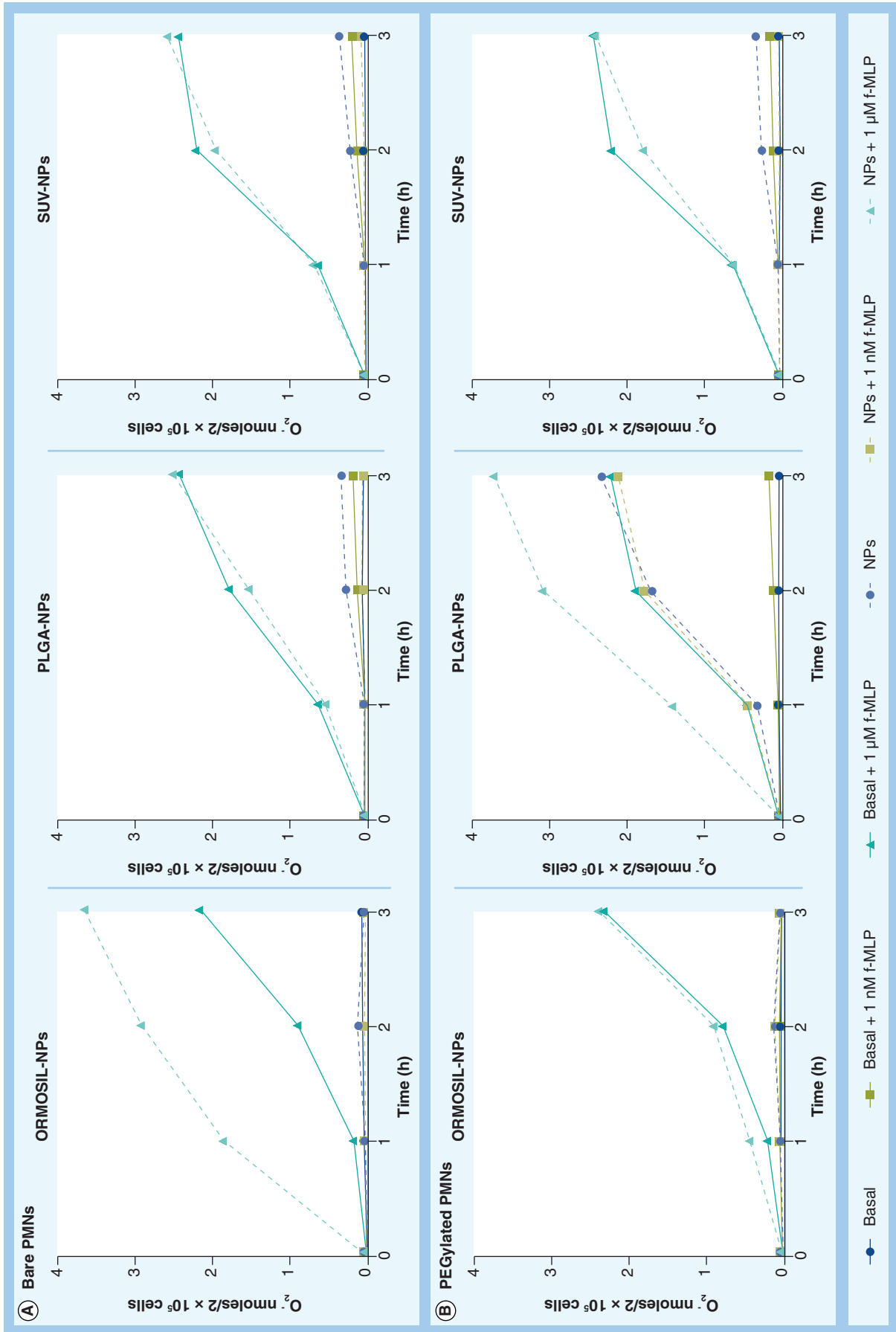
Figure 7. Effect of the nanoparticles on superoxide anion production in monocytes and polymorphonuclear leukocytes. PMNs were incubated at 37°C for 1 h with 50 μg/ml of different NPs (**A**) bare or (**B**) PEGylated) and then stimulated with 0–1–1000 nM f-MLP. The O_2^- release was estimated by cytochrome C reduction at 550 nm at different time points of f-MLP treatment (1–5 h).
f-MLP: Formyl-methionine-leucil phenylalanine; NP: Nanoparticle; PEG: Polyethylene glycol; PLGA: Poly(lactic-co-glycolic acid); ORMOSIL: Organic modified silica; PEG: Polyethylene glycol; PLGA: Poly(lactic-co-glycolic acid); PMN: Polymorphonuclear leukocyte; SUV: Small unilamellar vesicle.
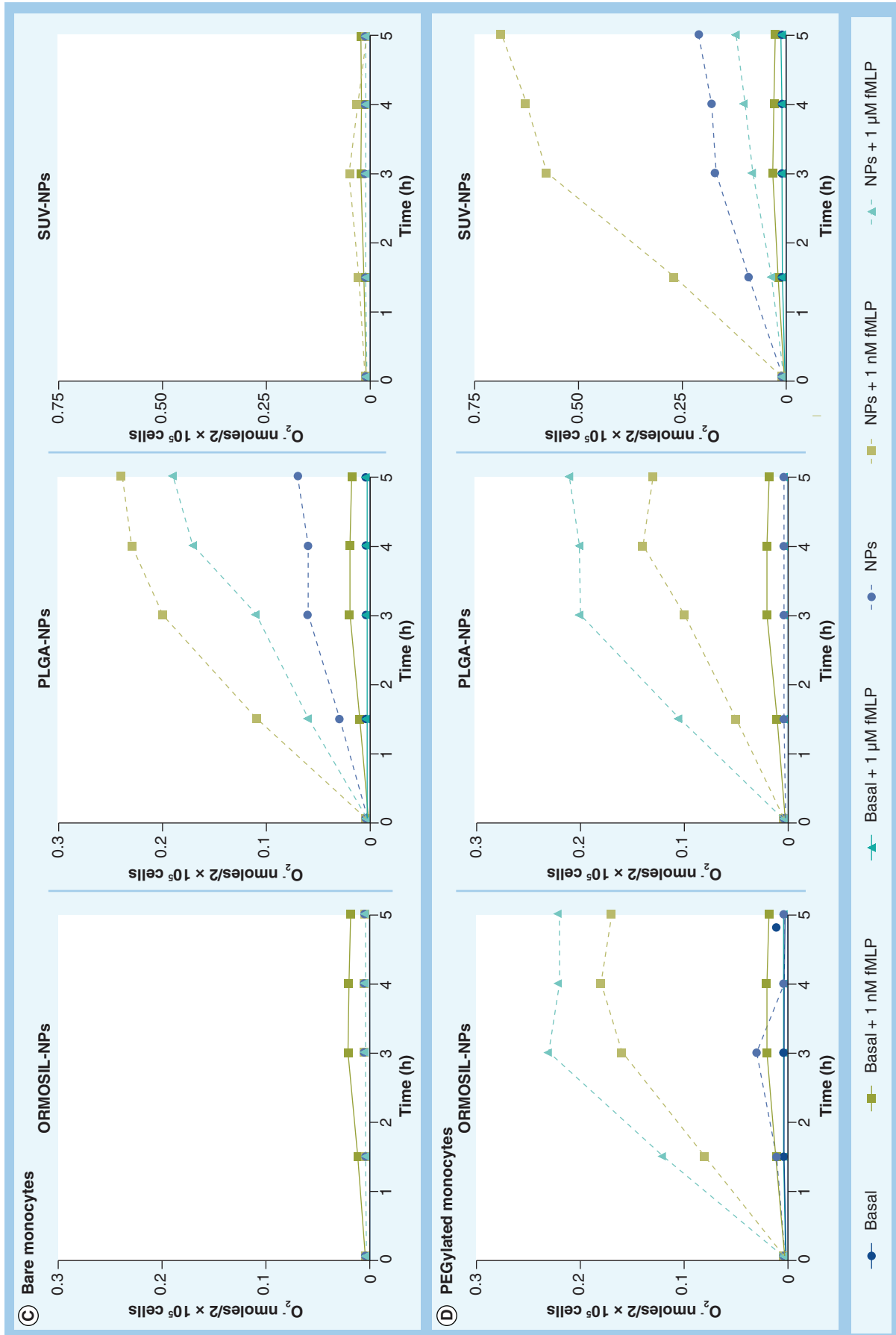
Figure 7 (cont.). Effect of the nanoparticles on superoxide anion production in monocytes and polymorphonuclear leukocytes. Monocytes were incubated at 37°C for 1 h with 50 µg/ml of different NPs ((C) bare or (D) PEGylated) and then stimulated with 0–1–1000 nM f-MLP. The O_2^- release was estimated by cytochrome C reduction at 550 nm at different time points of f-MLP treatment (1–5 h).
f-MLP: Formyl-methionine-leucil phenylalanine; NP: Nanoparticle; ORMOSIL: Organic modified silica; PEG: Polyethylene glycol; PLGA: Poly(lactic-co-glycolic acid); PMN: Polymorphonuclear leukocyte; SUV: Small unilamellar vesicle.

In our study we decided to characterize the interaction of our ORMOSIL-NPs with blood leukocytes to test the possibility that they are captured by circulating inflammatory cells well before being captured by the reticuloendothelial system (RES). Leukocytes, monocytes and PMNs can initiate inflammation, releasing a plethora of mediators like cytokines and chemokines. In addition they are endowed with a powerful antimicrobial system, the NADPH oxidase, responsible for the production of anion superoxide and derived ROS.

We initially showed that monocytes have the ability to capture ORMOSIL-NPs more efficiently than other blood leukocytes and that this cell-selectivity was kept over a large size-range (from 20 to 180 nm diameter), while PLGA- and SUV-NPs could also be significantly associated to other leukocytes, such as lymphocytes, NK cells and PMNs. Confirming our previous data [18,19], we proved that coating of ORMOSIL-NPs with PEG reduced uptake by monocytes and other leukocytes, even after a prolonged incubation time. Interestingly, we found that such ability to escape cell capture was less pronounced for 20 nm diameter PEGylated ORMOSIL-NPs and increased for larger NPs. This confirmed that the high density of PEGylation, which can be achieved with our synthesis procedure, results in NPs with good stealth capacity. This is consistent with the observation that the stealth feature of PLGA-NPs and SUV-NPs, in which PEGylation could not exceed 7.5 and 4%, respectively [19], was less pronounced or negligible. Since the same PEG polymer was used (PEG 2000) in all NP formulations, the increased NP stealth feature seems to correlate with PEG surface density. Moreover, we showed that, in absolute terms, approximately 50-nm diameter PEGylated ORMOSIL-NPs displayed the maximal stealth feature. Size-dependence experiments and measurements of the zeta potential of our NPs (see method section) showed no simple correlation of both cell-selectivity and stealth properties of our NP panels. In fact, although the absolute amount of NPs can vary, cell selectivity remains substantially the same for ORMOSIL-NPs with very different sizes. All used NPs are moderately negatively charged, especially in the presence of FCS, and so their difference in cell selectivity does not appear to be related to such parameter. This suggests that, while cell capture appears more efficient for larger ORMOSIL-NPs, cell selectivity depends more on the chemical composition of the different particles used.

Functional experiments showed that bare ORMOSIL-NPs (50 nm) stimulate IL-1β, IL-6,

TNF-α and IL-8 production in monocytes, to which they effectively bind. It is nevertheless important to note that, in spite of the much reduced cell-association, ORMOSIL-NPs also induced IL-8 secretion by PMNs. Contrarily to what might be expected, such effects were reduced but not totally eliminated, by surface PEGylation of ORMOSIL-NPs, in spite of an acquired marked stealth effect. Moreover, f-MLP costimulation allowed disclosing a high activity of NPs in both forms. In monocytes, IL-1β and IL-6 secretion induced by bare ORMOSIL-NPs was synergized by f-MLP concentrations occupying only high-affinity FRPs (1 nM), while doses engaging also low-affinity FRPs (1 μM) counteracted this enhancing effect. TNF-α release in monocytes and IL-8 secretion in both monocytes and PMNs, were on the contrary, not improved by 1-nM f-MLP, but increased by high f-MLP doses (1 μM). These data suggest that high-affinity and low-affinity FRPs can differently modulate ORMOSIL-NPs induced cytokine/chemokine secretion balance. High-affinity receptors induce a high IL-1β, IL-6/ TNF-α, IL-8 ratio, while low-affinity receptors revert this pattern favoring TNF-α and IL-8 and partially silencing IL-1β and IL-6 release. Again, it is important to note that PEGylated ORMOSIL-NPs, although captured by monocytes much less efficiently than their bare counterparts (>90% decrease), were still able to sensitize monocytes to nanomolar f-MLP. Consequently, the gulf between cytokine/chemokine production by bare and PEGylated ORMOSIL-NPs effects was further reduced. It then appeared that, in spite of a PEG-dependent acquisition of stealth properties, PEGylated ORMOSIL-NPs can mediate a significant proinflammatory mediator secretion in suitable conditions (i.e., f-MLP costimulation).

When analyzing the activation of the NADPH oxidase, we observed that bare ORMOSIL-NPs primed PMNs to f-MLP at concentrations involving low-affinity receptors (1 μM). In PMNs, PEGylated PLGA-NPs were paradoxically more active than their bare version and this activity summed up with the one induced by 1 μM f-MLP *per se*.

In monocytes, the pattern of NP activation of the O_2^- generating system, although much less intense compared with PMNs, was found to be more complex and unpredictable. f-MLP-dependent NADPH-oxidase activation was observed using both low and high tripeptide doses when cells were preincubated with bare PLGA-NPs, while not with bare ORMOSIL- and SUV-NPs. Surprisingly, PEGylated

ORMOSIL-NPs and PEGylated SUV-NPs did prime monocytes to f-MLP (low and high doses in the case of ORMOSIL, while only low doses in the case of SUV). The variable activity of NPs in terms of cytokine/chemokine and O_2^- production could not be ascribed to LPS contamination since the endotoxin level was always below 0.05 EU/µg of NPs (Limulus test). The result that the secretion of some cytokines is induced in NP-treated cells stimulated with f-MLP doses that do not evoke ROS production and *vice versa* is intriguing. Controversial data are present in the literature regarding the role of ROS in cytokine secretion: some authors demonstrated that ROS increases cytokine synthesis by activating transcriptional regulators such as AP-1 and NF-κB [29], whereas others found that oxidative stress reduces the cytokine production [30]. Whichever is the case, these findings could explain the lack of correlation between cytokine synthesis and ROS generation observed by us. Further experiments are required to elucidate the complex relationships between ROS and cytokine production in our experimental conditions.

At present, we have no easy explanation in molecular terms of the complex behaviours of heterogeneous NPs when engaged with monocytes and PMNs. Nonetheless some generalization, which brings novel perspectives in the field of nanomedicine can be drawn (see scheme in FIGURE 8). First of all, we document that ORMOSIL-NPs are more inflammogenic than PLGA- and SUV-NPs in both monocytes and PMNs. Another important observation is that cellular effects of NPs do not simply correlate with their accumulation. In fact, it clearly emerged that:

- NP capture does not necessarily imply cell activation, as exemplified by both bare and PEGylated PLGA- and SUV-NPs, which associate with monocytes and PMNs without inducing cytokines/chemokines. Clearly, we confirm that the constitutive material is important in determining the inflammogenic action of NPs and not the fact that they enter the cell;

- Cell activation is not necessarily correlated to endocytosis, as evident from the observation that stealth NPs like PEGylated ORMOSIL-NPs, can induce, at appropriate doses or in the presence of an adequate costimulus (f-MLP), a cytokine–chemokine production not dissimilar to the one induced by bare and more efficiently endocytosed ORMOSIL-NPs.
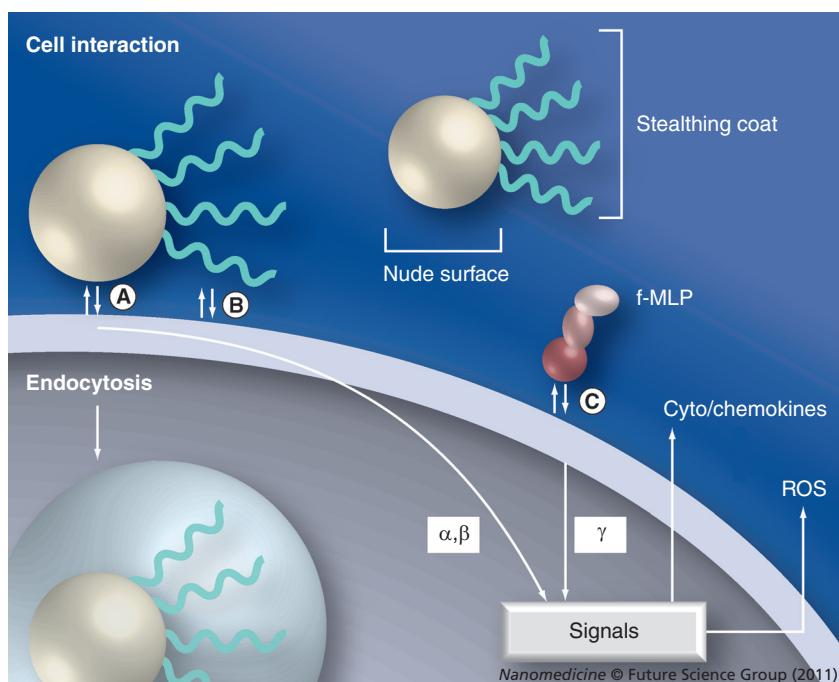
Figure 8. Model of the interaction between nanoparticles and monocytes/polymorphonuclear leukocytes and of the factors involved in determining the cell capture and biological actions of NPs. Both **(A)** the bare surface and **(B)** the stealthing layer (PEG in our study) are proposed to mediate the binding to components on the cell surface, which can result in either NP endocytosis or the transduction of intercellular signals (α and β). Opsonines from host tissue fluids (plasma primarily) likely contribute to such interaction (not depicted for simplicity). **(C)** Other ligands may bind to specific receptors (f-MLP receptor in our study) and generate additional signals (γ). The interplay between the three generated intracellular signals produces the final proinflammatory outcome. The biological response is not strictly related to endocytosis.
f-MLP: Formyl-methionine-leucil phenylalanine; NP: Nanoparticle; PEG: Polyethylene glycol; PMN: Polymorphonuclear leukocyte; ROS: Reactive oxygen species.

This suggests that NP–cell interactions with plasma membrane determinants, without endocytosis, may be sufficient to trigger cell biological effects. This implies that PEG coating, although eliminating, if sufficiently thick as in the case of ORMOSIL-NPs, some important drawbacks such as RES clearance or major procoagulant effects, and even a significant part of proinflammatory effects [18,19], still keep a residual bioactivity. It appears that PEG chains are bioactive, but only in the context of dense crowding on the NP surface, since purified PEG derivatives used to coat NPs did not stimulate monocytes and PMNs *per se* or in conjunction with f-MLP at any concentration up to 0.5% (w/w) [PAPINI ET AL., UNPUBLISHED DATA]. Our data are in line with other observations that PEG coating does not completely abolish the capability of NPs to interact with host factors [9]. Complement activation occurs effectively on stealth PEGylated liposomes, contributing to the described hypersensitivity to antitumor stealth PEG-covered doxorubicin loaded liposomes [31].

Moreover, monocytes bound to PEG hydrogel coated polystyrene surfaces secreted IL-1β, TNF-α and granulocyte–macrophage colony-stimulating factor much more efficiently than when bound to bare polystyrene [32]. Consistently, our highly PEGylated ORMOSIL-NPs, although confirmed to have excellent stealth properties, keep a significant residual capacity to stimulate blood phagocytes. This suggests that PEG polymer coating is probably not the best strategy to fully eliminate phagocytosis-independent proinflammatory effects. An important implication of our data is that NP endocytosis by phagocytic cells *in vitro*, although testing the propensity to escape clearance by RES *in vivo* does not fully evaluate NPs biocompatibility.

Another major contribution of our study is the novel concept that nanostructures full proinflammatory potential is scantly estimated if they are tested as single agonists. As better evidenced when analyzing the induction of a respiratory burst, various NPs can have no apparent intrinsic activity, but nevertheless predispose cells to be more responsive to other stimuli. Our data, although limited to a single kind of microbial mediator like the model peptide f-MLP, clearly indicates that strong proinflammatory effects may take place when NPs interact with immune-inflammatory cells in the biological fluids bathing tissue, including blood. In fact, the receptors recognizing f-MLP are part of a innate system sensing not only nonself pathogen-associated molecular patterns, such as bacterial leader peptides, but also peptides that may be released in the presence of tissue and cell damage, such as mitochondrial derived peptides [23].

Conclusion

This study confirms that highly PEGylated ORMOSIL-NPs are promising drug-delivery systems. In fact, we provide evidence that a high PEG coating is critical to diminish the NPs interaction with circulating monocytes primarily, but also with other leukocyte types. However, we also documented that PEG chains, although useful to confer stealth properties to NPs, are not totally devoid of bioactivities and in particular may contribute to proinflammatory effects such as cytokine/chemokine secretion and ROS production. The other new observation of our study is that bare ORMOSIL-NPs are endowed with an intrinsic ability to stimulate inflammation, but may also sensitize immune cells to other relevant proinflammatory agonists. In particular we found that high- and low-affinity FRPs strongly modulate ORMOSIL-NPs cytokine/chemokine release and ROS production. Induction of inflammation reactions by the combination of nanostructures and other proinflammatory signals generated by pathogens or by the same host tissue in the complex *in vivo* environment may be a general condition for nanomedical tools. This prompts to caution when evaluating NPs proinflammatory potentials since an apparently biologically inert NP may turn out to be active in the presence of appropriate synergizing stimuli.

Future perspective

Full characterization of proinflammatory effects of nanostructures *in vitro* is a complex and difficult task: the molecular mechanisms involved are not only material-dependent but also cell-dependent and moreover, may be critically influenced by the molecular environment sensed by the NP-interacting cells. A combination of high-throughput analysis of the effect of NPs on cells and of mechanistic molecular investigations are needed to overcome the empirical approach to bio-hemocompatibility of proposed nanotheragnostics in the future. Our study points to the need to:

- Find innovative inert stealthing layers for NPs

- Identify any other independent signal that may amplify the biological effects NPs

In vivo evaluation of ORMOSIL-NPs proinflammatory effects are planned in our laboratory.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Aim
- Capturing efficacy and proinflammatory effects of bare and highly polyethylene glycol (PEG)ylated vinyltriethoxysilane (VTES)-based organic modified silica (ORMOSIL)-nanoparticles (NPs) were studied in human blood leukocytes and compared with poly(lactic-co-glycolic acid) (PLGA)- and small unilamellar vesicles (SUV)-NPs.

Leukocyte selectivity & stealth properties of NPs
- ORMOSIL-NPs selectively associated to monocytes over 20–180-nm diameter in size, but 40–50-nm NPs were captured by these cells to a lesser extent compared with smaller and particularly to bigger NPs.
- PEGylated ORMOSIL-NPs was the best stealth NP formulation compared with other NPs: capture by all blood leukocytes was maximally reduced in absolute terms using PEGylated 50-nm diameter NPs.

Cytokine/chemokine production induced by NPs in monocytes & polymorphonuclear leukocytes & their modulation by f-MLP
- ORMOSIL-NPs induced IL-1β, IL-6, TNF-α and IL-8 in monocytes and IL-8 in polymorphonuclear leukocytes (PMNs).
- PEGylated ORMOSIL-NPs only partially reduced pro-cytokine/chemokine activity in monocytes.
- Monocytes and PMNs treated with bare ORMOSIL-NPs for 1 h and subsequently stimulated with formyl-methionine-leucil phenylalanine (f-MLP), which improved their cytokine/chemokine production.
- High-affinity f-MLP receptor engagement enhanced IL-1β and IL-6 secretion in monocytes but had no effect on TNF-α secretion by monocytes and on IL-8 secretion by both monocytes and PMNs.
- Low-affinity f-MLP receptor engagement counteracted high-affinity receptor effects, inducing a decrease of IL-1β and IL-6 release from monocytes but enhanced TNF-α secretion by monocytes and IL-8 release by both monocytes and PMNs.

Activation of monocytes & PMN NADPH-oxidase by NPs & its modulation by f-MLP
- The reactive oxygen species (ROS) generating system, NADPH oxidase, was not activated by NPs *per se* (with the exception of PEGylated PLGA-NPs in PMNs).
- Low-affinity f-MLP receptors synergized ROS production by PMNs preincubated with bare ORMOSIL-NPs.
- f-MLP also synergizes with PEGylated ORMOSIL-, PLGA- and SUV-NPs, inducing a significant ROS production by the NADPH oxidase of monocytes.

Conclusion
- Proinflammatory effects of ORMOSIL-NPs are not totally eliminated by high-PEGylation in spite of the acquisition of strong stealth properties.
- f-MLP synergizes with both bare and PEGylated ORMOSIL-NPs to produce cytokines and chemokines in monocytes and PMNs.
- ROS production is also induced in cells treated with NPs by f-MLP costimulation.

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