

Quantum sensing to monitor changes in free radical generation by intracellular vesicles of polarized macrophages

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ABSTRACT

Macrophages are immune cells crucial in clearing our tissues from bacteria, viruses, dying cells, cell debris and other waste products. They also regulate inflammation by differentiating from non-activated (M0) cells into macrophages that initiate inflammation (pro-inflammatory macrophages, M1), or resolve inflammation (anti-inflammatory macrophages, M2). One of their key functions is to ingest pathogens within vesicles where they are degraded. The production of free radical (FR) plays an important role in this degradation process but also in macrophage differentiation and signaling. Here we used diamond-based quantum sensing to track free radical changes in vesicles with nanoscale resolution. We further followed the oxidative stress status, through free radical measurement during the macrophage activation process. We found that the three macrophage subtypes differed significantly in free radical generation in their vesicles. Additionally, we showed that the FR generation evolves over time in the different subtypes. We observed a 50 % increase in radical production in M0 after 24 h compared to the T1 values measured after 4 h of cell culture, a decrease in M1 and constant radical levels in M2 macrophages.

Statement of significance: Here we use quantum sensing for the first time to investigate the role that free radicals play in immune cells when they differentiate to fulfill their functions in the immune system. We were able to measure free radical generation specifically in vesicles while the macrophages differentiated.

1. Introduction

The human immune system is fundamental to disease development, regeneration, and organ homeostasis. Macrophages play a key role in the immune response [1]. Circulating monocytes, originating from bone marrow precursor cells, were once considered the exclusive source of macrophages. Later, it was recognized that some resident macrophages in the brain, liver, or heart may be generated during embryological development [2]. Macrophages are characterized by a remarkable phenotypic plasticity, adapting to different microenvironmental signals. Based on these stimuli, they polarize into various subtypes, with M1 pro-inflammatory and M2 anti-inflammatory macrophages, which are extremes among many subtypes in a spectrum [3]. In vitro, macrophage stimulation with lipopolysaccharide (LPS) and interferon-gamma

(IFN- γ) drives their differentiation into the M1 phenotype, while interleukin 4 (IL-4) and interleukin 13 (IL-13) promote differentiation into the M2 phenotype.

The most important role of macrophages is to internalize and neutralize anything that does not express receptors characteristic of healthy cells. This process is called endocytosis for objects smaller than 0.5 μm and phagocytosis for larger objects, and it acts as a defense system for the host against infection, injury, etc. [4]. The internalization process is also important from the perspective of the development of personalized nanomedicine, where nanoparticles are used as drug carriers and immunomodulators [5]. The uptake and intracellular processing of objects depend not only on their size but also on geometry, rigidity, surface roughness, and receptor recognition [4]. The internalized content is processed and transported through the cells in vesicles to

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be delivered to specific cell compartments, recycled back to the membrane, or fused with lysosomes. Throughout this process, enzyme pools such as NADPH oxidases (NOX) and inducible nitric oxide synthases (iNOS) become activated at specific stages, generating bursts of superoxide ions ($O_2^{\cdot-}$) and nitric oxide (NO $^{\cdot}$). These oxides degrade pathogens or cellular debris down to molecules and affect the stability of therapeutic nanoparticles and their cargo [6].

Saffi et al. have presented that free radicals (FRs) can prevent lysosome coalescence depending on their type [7]. They suggested that specific FRs could be involved in regulating localized processes such as membrane fusion and motor activity. Moving forward, research should focus on clarifying how the type and positioning of FRs influence vesicle dynamics. Goldring et al. suggested that redoxosomes could be pharmacological targets for disease therapy (e.g., osteoarthritis) [8]. Mitchell et al. have also raised the crucial topic of cell population heterogeneity, both within a patient and across a patient population, which creates diverse trafficking pathways and therefore barriers to the success of a one-fit-all nanoparticle-based therapy [5]. Therefore, gaining deeper insights into the role of free radicals in the vesicle trafficking process could bring significant improvements in nanoparticle design and disease treatment. However, the field lacks the capability to detect FR generation with subcellular resolution in real time using standard methods.

This work relies on nitrogen-vacancy (NV) centers in nanodiamonds, which alter their optical properties in response to changes in their magnetic environment [9–12]. Thus, by using different measuring modalities they can be used for nanoscale sensing of temperature [13,14], strain [15], pH, as well as electric [16] or magnetic fields [17,18]. Relaxometry is sensitive to magnetic noise and allows for high spatial resolution (several nanometers). As a result, the method reveals the local concentration of the sum of all radicals. So far, this technique has been used to detect FRs in biological systems at various organizational levels such as single molecules [19], organelles [20,21], single cells [22–25], and tissues [26,27].

The relaxometry experiment begins by polarizing the spins of NV-centers in the diamond lattice into a bright $m_s = 0$ ground state using a strong laser pulse. Once the laser is turned off, the NV-centers stochastically decay into an equilibrium of different magnetic states ($m_s = 0$ and $m_s = \pm 1$) [28,29]. The spin-polarized configuration shows stronger fluorescence compared to the equilibrium state, enabling optical manipulation and monitoring of this transition, as well as determining its rate. The equilibration process occurs more rapidly at higher levels of spin noise, and the associated relaxation rate depends on concentration.

While Wu et al. used relaxometry to study free radical generation in macrophages that ingested bacteria [30], we used T1 relaxometry to explore changes in free radical concentration during the endosomal trafficking of nanodiamonds. Given their size (70 nm), nanodiamonds predominantly follow an endocytic pathway rather than phagocytosis [31–33]. We investigated how free radical generation in endocytic pathway, specifically in the early endosomes and late endosomes/lysosomes varies depending on the macrophage polarization status. We aimed to explore if relaxometry can be applied in the future as a tool to evaluate the role of FRs in vesicle dynamics and, in the long run, impact the efficiency of nanoparticle-based immunotherapies and drug delivery in personalized medicine.

2. Materials and methods

2.1. Fluorescent nanodiamonds (FNDs)

Fluorescent nanodiamonds (FNDs) with a hydrodynamic diameter of 70 nm and oxygen terminated surface (ND-NV-70nm-Hi, Adamas Nanotechnology, USA), were used in this study. Briefly, FNDs were produced by grinding high pressure high temperature diamonds. NV centers were implanted in the diamond material by irradiation with 3

MeV electrons at a fluence of 5×10^{19} e/cm ^{2} , followed by high-temperature annealing, resulting in an average of 500 NV centers per diamond [34]. FNDs undergo a final cleaning in oxidizing acids, which results in an oxygen-terminated surface. Previous studies have already characterized these particles extensively [34,35].

2.2. FND uptake and cell polarization for confocal microscopy

Murine macrophages (J774A.1, LGC Standards, Germany) were seeded in a 4-quarter Petri dish with a glass bottom (CellView, Greiner Bio-One, The Netherlands) with a concentration of 30,000 cells per quarter in 500 μ L Dulbecco's Modified Eagle's Medium-Complete (DMEM-HG, Sigma Aldrich, The Netherlands) with 10 % FBS (Life Technologies, The Netherlands) and 1 % penicillin/streptomycin (Life Technologies, The Netherlands) for 24 h at 37 °C and 5 % CO $_2$. Hereafter, 70 nm FNDs were sonicated for 10 min in an ultrasonic bath. The medium was removed from the Petri dish and cells were incubated with 1 μ g/mL 70 nm FNDs for 30 min at 37 °C and 5 % CO $_2$ in DMEM-HG-Complete followed by rinsing with 500 μ L phosphate buffer saline (PBS, Gibco, The Netherlands). Finally, 500 μ L of fresh DMEM-HG-Complete was added. Polarization of M1 was induced by 1 μ g/mL LPS (InvivoGen, The Netherlands) for 4 h or 24 h. M2 was induced by 20 ng/mL IL-13 (Sigma Aldrich, The Netherlands) and 20 ng/mL IL-4 (Sigma Aldrich, The Netherlands) for 4 h or 24 h in 500 μ L DMEM-HG-Complete medium. The experimental design is presented in Fig. 1.

2.3. Flow cytometry for polarization markers

The polarization state of macrophages was assessed using fluorescent markers and flow cytometry. Macrophages were seeded in a 6-well plate with a density of 500,000 cells per mL and cultured overnight. Then, cells were incubated with FNDs as described above, 1 μ g/mL 70 nm FNDs for 30 min at 37 °C and 5 % CO $_2$ in DMEM-HG-Complete, followed by rinsing with PBS to remove excess FNDs and incubation in DMEM-HG-Complete. Finally, cells were activated using polarization factors for 4 h, 24 h and 48 h. Polarization into M1 macrophages was induced by 1 μ g/mL LPS, and polarization into M2 macrophages was induced by 20 ng/mL IL-13 and 20 ng/mL IL-4 in DMEM-HG-Complete medium. After activation, cells were rinsed twice with PBS, harvested by scraping and collected in 1.5 mL Eppendorf tubes for centrifugation. After centrifugation at 400 g for 5 min at 4 °C, the pellet was collected and resuspended in 200 μ L of PBS containing 10 % FBS. Staining was performed in a low-adherence U-bottom 96-well plate, using a primary antibody for 30 min at room temperature, followed by incubation with a secondary antibody for 20 min at room temperature on a microplate shaker. We used the following a) primary antibodies: anti-mouse CD40 monoclonal antibody hosted in rat (1:50, 1C10, eBioscience), FITC anti-mouse CD206 (MMR) antibody hosted in rat (1:100, C068C2, Biolegend), CD163/M130 polyclonal antibody (1:50, BS-2527R, Bioss), CD64/IGFR1 polyclonal antibody (1:100, BS-3511R, Bioss); and b) secondary antibodies: goat anti-rat IgG (H + L) cross-adsorbed secondary antibody conjugated with AlexaFluor $^{\text{TM}}$ 488 (1:100, Invitrogen), goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody conjugated with Texas Red (1:100, Invitrogen). Finally, cells were washed twice in PBS, centrifuged at 250 g for 10 min, and resuspended in PBS. Marker expressions were analysed with the NovoCyte Quanteon Flow Cytometer (Agilent Technologies, Santa Clara, California, US). The data was processed using NovoExpress (Agilent Technologies, Santa Clara, California, US). The median fluorescence intensity (MFI) normalized by the number of cells was plotted in a bar graph.

2.4. FNDs location in intracellular vesicles

An important parameter to investigate before relaxometry measurements is the amount of nanodiamonds that are in cells. Here we conduct relatively long experiments and thus it is especially relevant to

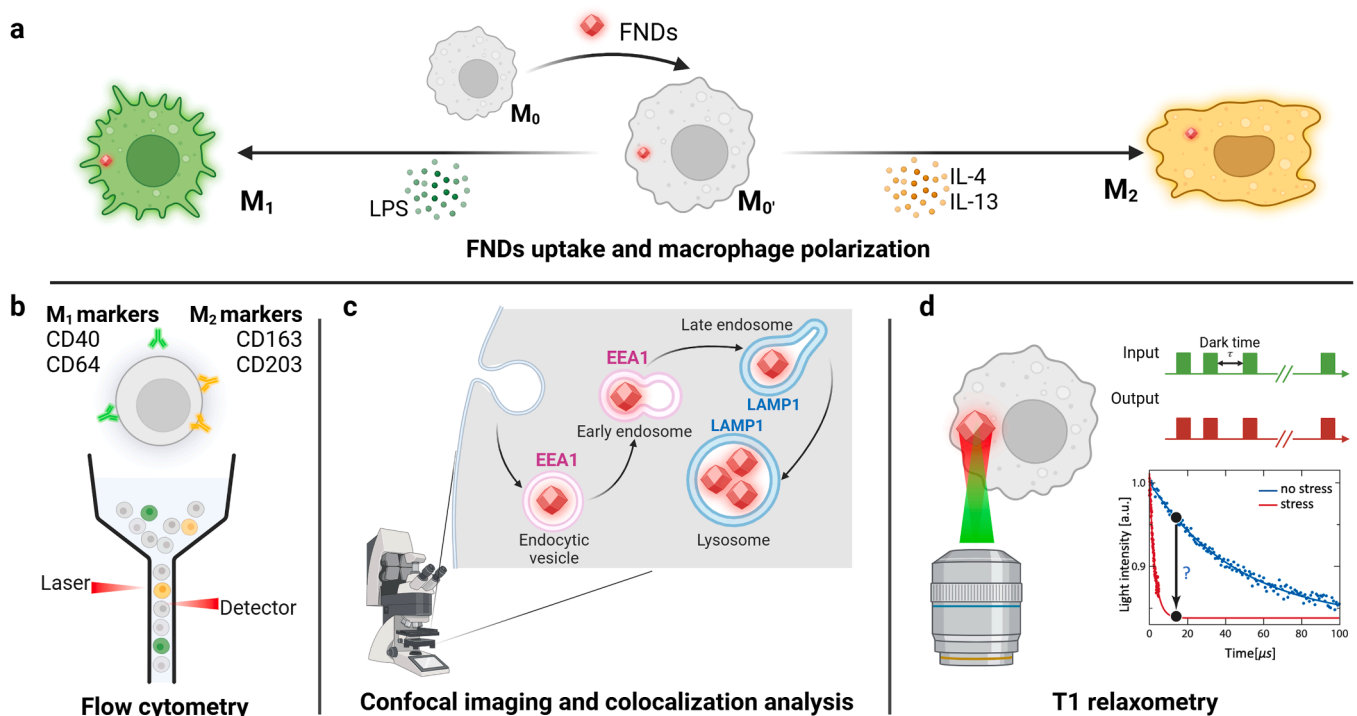


Fig. 1. Schematic of the experimental design for macrophage polarization and FND (Fluorescent Nanodiamond) treatment. (a) Overview of the process for FND uptake by macrophages and their subsequent polarization into M1 and M2 phenotypes. (b) Flow cytometry analysis is used to quantify and assess the polarization of macrophages using markers. (c) Confocal imaging and colocalization analysis are used to visualize the distribution and interaction of FNDs within macrophages in endocytic vesicles and early endosomes (EEA1, in light red) and late endosomes/lysosomes (LAMP1, in light blue). (d) T1 relaxometry measurements are used to evaluate the free radical concentration on polarized macrophages, providing insights into the local microenvironment.

determine if the number of particles is still sufficient for measurements at the later time point. The intracellular location of FNDs was investigated after 4 h and 24 h post-treatment. After FND uptake and macrophage polarization, cells were fixed with 4 % PFA for 10 min, rinsed with PBS, and 0.1 % Triton X-100 in PBS was added for 15 min at room temperature. Hereafter, cells were washed 3 times with PBS and incubated with 2 % bovine serum albumin (BSA) in PBS for 60 min at room temperature. Cells were washed another 3 times with PBS and incubated with the primary early endosome antibody EEA1 (10 μ g/mL, Rabbit- α -LAMP1 EEA1, Thermofisher, The Netherlands) or late endosome LAMP1 (10 μ g/mL, Rabbit- α -LAMP1, Thermofisher, The Netherlands) in 0.1 % BSA for 3 h at room temperature [33]. After washing 3 times with PBS, the secondary antibody Alexa 488-Conjugated Goat Anti-Rabbit (10 μ g/mL, Abcam, The Netherlands) was incubated for 45 min at room temperature in the dark. Finally, cells were washed 3 times with PBS and imaged with a Nikon spinning-disk (SD) confocal microscope, which is a Yokogawa CSU-W1 SD system with 50 μ m pinholes. It is an inverted Nikon Ti2 motorized body with XYZ stage, connected to lasers at 405 nm, 488 nm, 561 nm, and 638 nm. Images were acquired with a scientific complementary metal-oxide-semiconductor (sCMOS) camera through a 100-fold oil immersion objective (chrome-free infinity (CFI) Plan Apochromat Lambda, numerical aperture (NA) 1.45, WD 0.13 mm). Image stacks were obtained from three independent experiments. In each experiment, 6 images were recorded and from each image, 6 cells were analyzed. Colocalization analysis was calculated with the Fiji (ImageJ) plugin DiAna [36]. Briefly, the confocal images set was deconvolved using Iterative Deconvolve 3D and a theoretical point spread function (PSF) Gaussian PSF 3D plugin in Fiji [37]. The regions of interest (ROIs) defining the cell borders were manually selected. Vesicles and FNDs within the ROIs were automatically segmented based on an appropriate threshold for each channel and Gaussian filters. Distance analysis was performed on the segmented objects, and the FNDs over vesicles colocalized fraction is reported.

2.5. Free radical detection with T1 relaxometry

Free radical levels were evaluated using data from T1 relaxometry measurements. These relaxometry experiments were conducted with a custom-built magnetometer, a standard tool in the field, previously described in [35]. The laser (532 nm, CNI, Changchun, China) was adjusted to 50 μ W (2×10^{-5} W cm $^{-2}$) at the sample location. Fluorescence was collected using an Olympus UPLanSapo40 $\times$ NA = 0.95 air objective. A 550 nm long-pass filter and a confocal pinhole were employed to separate scattered laser light from the fluorescence. Detection was carried out with an avalanche photodiode (SPCM-AQR15-FC) in single-photon counting mode. NV-centers were polarized with 5 μ s laser pulses, interspersed with dark periods ranging from 0.2 μ s to 10 ms. The spin state of the NV-centers was determined by observing the fluorescence within the first 0.6 μ s of each laser pulse. To avoid large aggregates, we chose particles with a count rate of around 1 million. Due to the movement of particles within cells (and for drift correction in stationary particles), particle tracking was necessary. Measurements were automatically paused every 5 s for a quick scan to locate the particle and adjust the laser position accordingly. Each measurement lasted several microseconds, and the sequence was typically repeated about 10,000 times to achieve a high signal-to-noise ratio. Six individual experiments were conducted, recording T1 values from six cells per sample variant in each experiment. In each cell, T1 values were recorded from three fluorescent nanodiamonds.

2.6. Statistical analysis

For flow cytometry data, statistical analysis was performed with 2-way ANOVA. Statistical difference ($p < 0.05$) and post hoc Fisher's LSD test were evaluated in GraphPad Prism 10. Colocalization data was plotted in a bar graph and statistical analysis was performed with a 2-way ANOVA (GraphPad Prism 10), a statistical difference ($p < 0.05$).

and post hoc Tukey's test.

Data from T1 measurements were plotted in a bar graph, and statistical analysis was performed using a 2-way ANOVA, with statistical significance ($p < 0.05$) and post hoc Tukey's test in GraphPad Prism 10.

3. Results and discussion

3.1. FNDs promote increase of M2 polarization markers in M0 and M1-stimulated cultures

We have tested if FND treatment influences the polarization of macrophages. Macrophages were activated into M1 or M2 polarization, for pro-inflammatory or anti-inflammatory profile, respectively, using a standard protocol that relies on cell stimulation with an immunogenic stimulator, such as LPS or a mix of interleukins (IL-4 and IL-13) up to 48 h. This polarization time is similar to what has been reported earlier for the J774A.1 cell line [38,39] while longer polarization times have been reported in THP-1 cells [40]. The expression level of key markers for each phenotype without FND treatment is shown in Fig. 2. We have found increased levels of M1 activation markers i.e. CD40 and CD64 (Fig. 2a,b) compared to their expression in the untreated M0 and M2 groups. The CD40 level increased above the control threshold after 24 h of polarization, whereas significantly higher expression of CD64 was observed after 48 h exposition to cytokines, which shows a time-dependent expression of these markers. We have found that the level of the M2 phenotype markers i.e. CD206 and CD163, increased significantly after 48 h of exposure to the interleukins (Fig. 2c,d). Other authors have shown that human monocyte-derived macrophages require between 24 and 72 h of stimulation to show expression of CD64 (M1 marker) and CD206 and CD163 (M2 markers) [41]. The effect of FNDs on the activation status is presented in Fig. 3. No significant difference was found in the expression profile of pro-inflammatory markers (Fig. 3a,b) compared to the phenotypes which were not treated with FNDs (further details in Supplementary Fig. 1). That FNDs themselves do not induce ROS production in immune cells has been shown elsewhere

[30]. In groups M0 and M1 exposed to FNDs, we observed a slight increase in the presence of polarization markers towards the M2 profile, especially pronounced after 48 h of culture. Surprisingly, we found a lower presence of the CD206 M2-polarization marker in the M2-stimulated group exposed to FNDs (Fig. 3c,d). Further details are in supplementary Fig. S2.

Our results add to insights in the literature. Although numerous studies have explored nanoparticle (NP)-cell interactions, the mechanisms through which NPs mediate macrophage polarization remain poorly understood and conflicting results can be found in the literature. Recently it became clear that the physicochemical parameters of NPs and incubation time are crucial factors for determining the type of macrophage polarization. Xiao et al. showed that NP protein corona governs NP uptake and subsequent macrophage response [42].

Fuchs et al. demonstrated that polystyrene nanoparticles (NPs) with carboxyl and amino surface groups can significantly influence M2 macrophage polarization without impacting M1 markers. Additionally, both types of surface terminations helped reduce the expression of M2 surface receptors like CD200R and CD163 [43]. Among metal particles, gold nanoparticles (Au NPs) were found to be more effective in inducing M1 macrophage polarization compared to silver nanoparticles (Ag NPs) of the same size. The researchers suggested that the different cellular uptake mechanisms of the two NPs might explain their varying abilities to induce macrophage polarization. They showed that Ag NPs enter macrophages via pinocytosis, while Au NPs use both receptor-mediated endocytosis and pinocytosis [44].

Other studies have indicated that the pro-inflammatory effects of Au NPs in macrophages depend on exposure time. As exposure time increased, the pro-inflammatory response triggered by Au NPs decreased. Research showed that the inflammatory response was activated by Au NPs after 3 and 6 h of treatment, but the expression levels of pro-inflammatory markers decreased after 24 h [44,45]. In some studies, Au NPs did not induce the expression of M1-related markers [46].

Zanganeh et al. found that iron oxide NPs could significantly inhibit tumor growth by inducing M1 macrophage polarization [47].

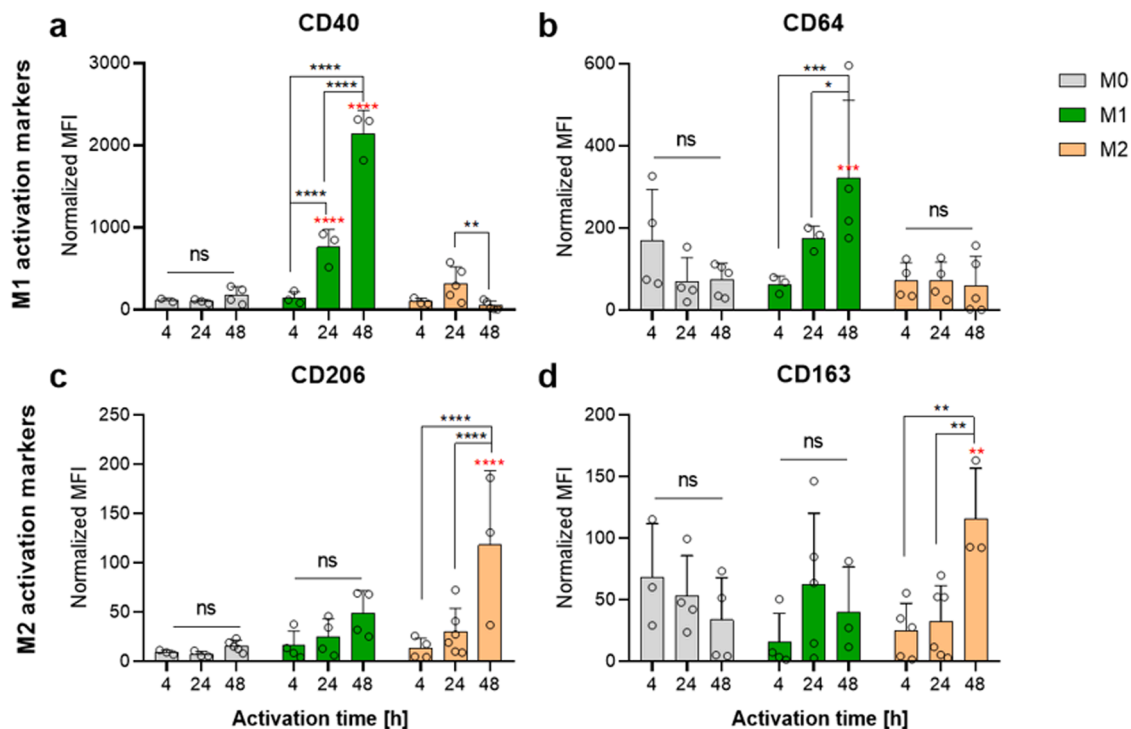


Fig. 2. Flow cytometry analysis of M1 (panels a, b) and M2 (panels c, d) activation markers following cell (without FNDs) exposure to stimulants for 4, 24, and 48 h. We show the median fluorescence intensity (MFI) collected for each group. Red stars indicate statistically significant differences compared to the M0 group at the corresponding activation time, only statistically significant differences compared to M0 are shown.

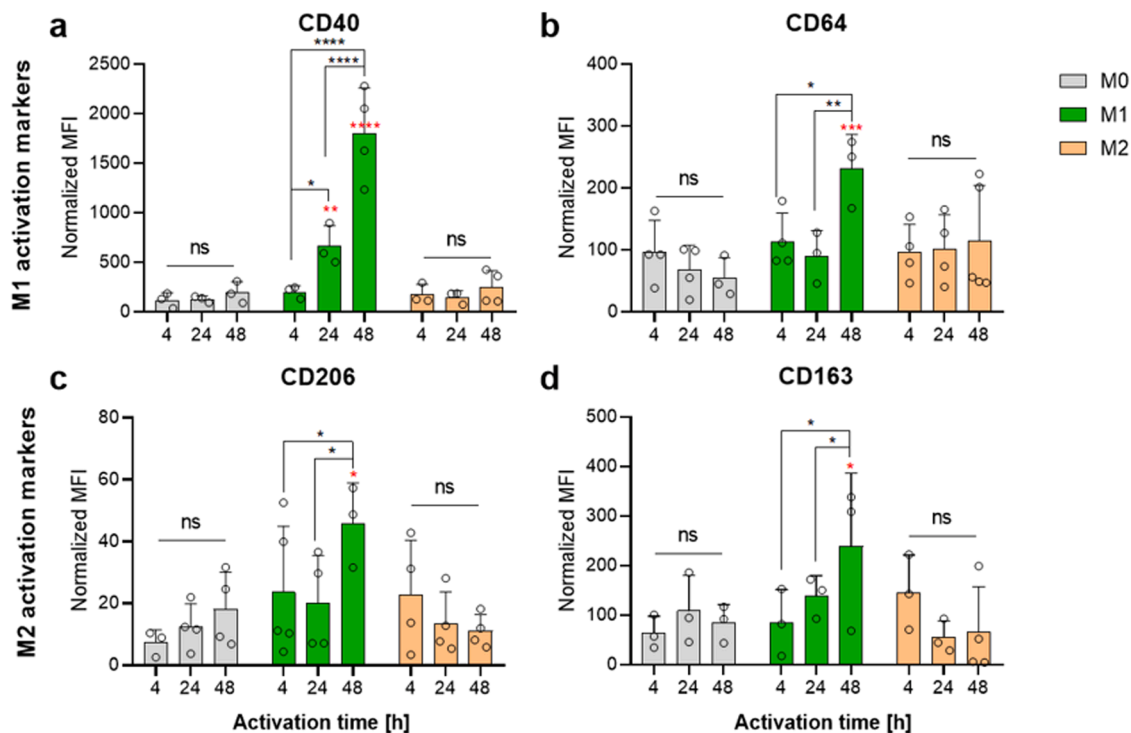


Fig. 3. Flow cytometry analysis of M1 (panels a, b) and M2 (panels c, d) activation markers following treatment with 70 nm FNDs. Cells were exposed to stimulants for 4, 24, and 48 h to assess the effect on polarization and marker expression. We show the median fluorescence intensity (MFI) collected for each group. Red stars indicate statistically significant differences compared to the M0 group at the corresponding activation time.

Superparamagnetic iron-oxide nanoparticles (SPIONs) were shown to shift macrophages from the M2 to the M1 phenotype, evidenced by increased levels of CD86 and TNF- α . Another study indicated that the iron in SPIONs contributed to the phenotypic shift in THP1-derived M2 macrophages toward the M1 phenotype [48].

Overall, ceramic NPs of different chemical compositions have distinct effects on macrophage polarization. For instance, titanium oxide (TiO₂) NPs (70 nm) and zirconium oxide (ZrO₂) NPs (5–30 nm) did not induce an inflammatory phenotype in primary macrophages [49]. Among carbon-based nanoparticles, graphene oxide (GO) was shown to induce macrophages to polarize to the M1 subtype in a size-dependent manner. Although the smallest GO had the highest cellular uptake, it did not activate the TLR4 signaling and NF- κ B pathways to induce macrophage polarization. In contrast, the largest GO had lower cellular uptake but tended to adhere to the plasma membrane, resulting in greater macrophage activation [50].

So far there are no other studies than presented here that would evaluate the effect of the fluorescent nanodiamonds on macrophage polarization. Niora et al. have explored the uptake of FNDs and how these NPs affect the viability of THP-1 monocytes and M0 macrophages derived from them [51]. They have not found any adverse effect of FNDs on the PMA-triggered differentiation of monocytes to the M0 state. However, these authors did not study if FNDs caused any further polarization of monocytes towards M1 or M2 phenotypes. The polarization effects observed in our studies could be due to any of the above-discussed physicochemical characteristics of FNDs. For future work requesting to use FNDs as inert biosensors, one would need to search for an appropriate surface modification strategy to avoid activation of the immune cells. Especially acid treatment which removes all residues of organic carbon has been effective for this purpose for other immune cells [52].

3.2. FNDs intravesicular location depends on polarization status

Before intravesicular quantum sensing measurements with FNDs, we

characterized their retention and location inside the cells. We performed immunostaining of early endosomes (Fig. 4) and late endosomes/lysosomes (Fig. 5) to analyze the colocalization of FNDs with those vesicles using confocal microscopy. Based on flow cytometry, we found that after 4 h post-treatment with FNDs, the M0 and M1 macrophages contain a similar number of nanoparticles (Fig. 6a), whereas only half of that number could be found in the M2 macrophages. Furthermore, the M0 phenotype showed higher particle retention over time, compared to other phenotypes. The number of FNDs in the M1 phenotype decreased by more than half after 24 h of culture. The M2-activated cells showed almost a 90 % decrease in the FNDs number after 24 h of culture. These observations indicate differences in the excretion profile and/or proliferation rate between phenotypes. Excretion of nanodiamonds as the primary mechanism for reducing the number of particles per cell has also been reported in yeast cells where nanodiamonds are excreted almost completely within 24 h [53]. On the other hand, Liu et al. reported no excretion in cancer and stem cells and attributed the reduction in particle numbers entirely to proliferation [54]. Also, a combination of both effects has been reported before with differences between cell types [55]. For relaxometry measurements, the reduced numbers of particles are not problematic since we measure single particles and macrophages ingest relatively high numbers of particles initially. Also, it has to be noted that low particle concentrations are beneficial to prevent artifacts from jumping between different particles during tracking.

Besides the number of particles, the exact location within the cell is crucial. Here, we investigated two different intracellular locations: early endosomes and late endosomes/lysosomes. Due to the specificity of the immunostainings we have decided for these two locations: EEA+ vesicles correspond to endocytic vesicles and early endosomes, and LAMP1+ vesicles correspond to late endosomes and lysosomes. For further investigation within these two groups, a combination of functional markers such as pH labels can be used, however those are not within the scope of this study. The colocalization analysis has shown that FNDs are almost equally distributed between early endosomes and late endosomes/lysosomes in M0 macrophages after 4 h of culture. After

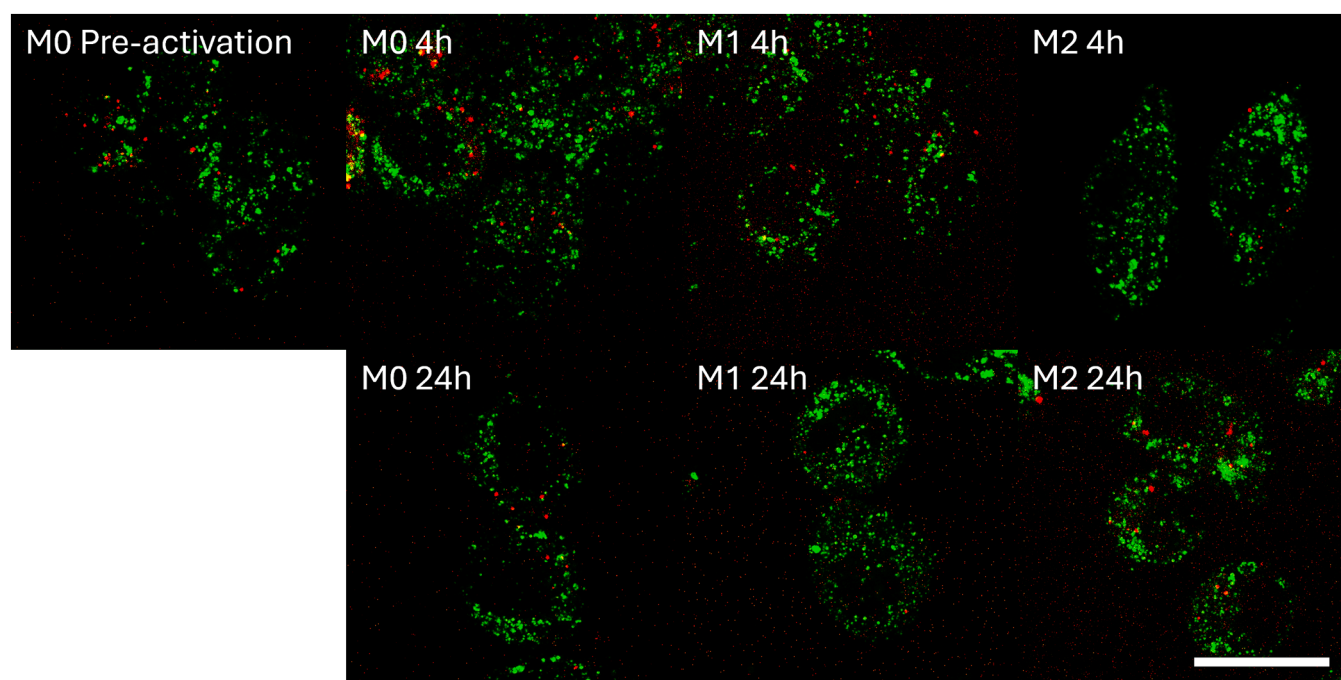


Fig. 4. Distribution of 70 nm FNDs (red) and early endosomes stained with EEA1 (green) in macrophages of M0, M1, and M2 phenotypes at different time points: 30 min (pre-activation), 4 h, and 24 h post-FND treatment and polarization. Scale bar: 20 μ m.

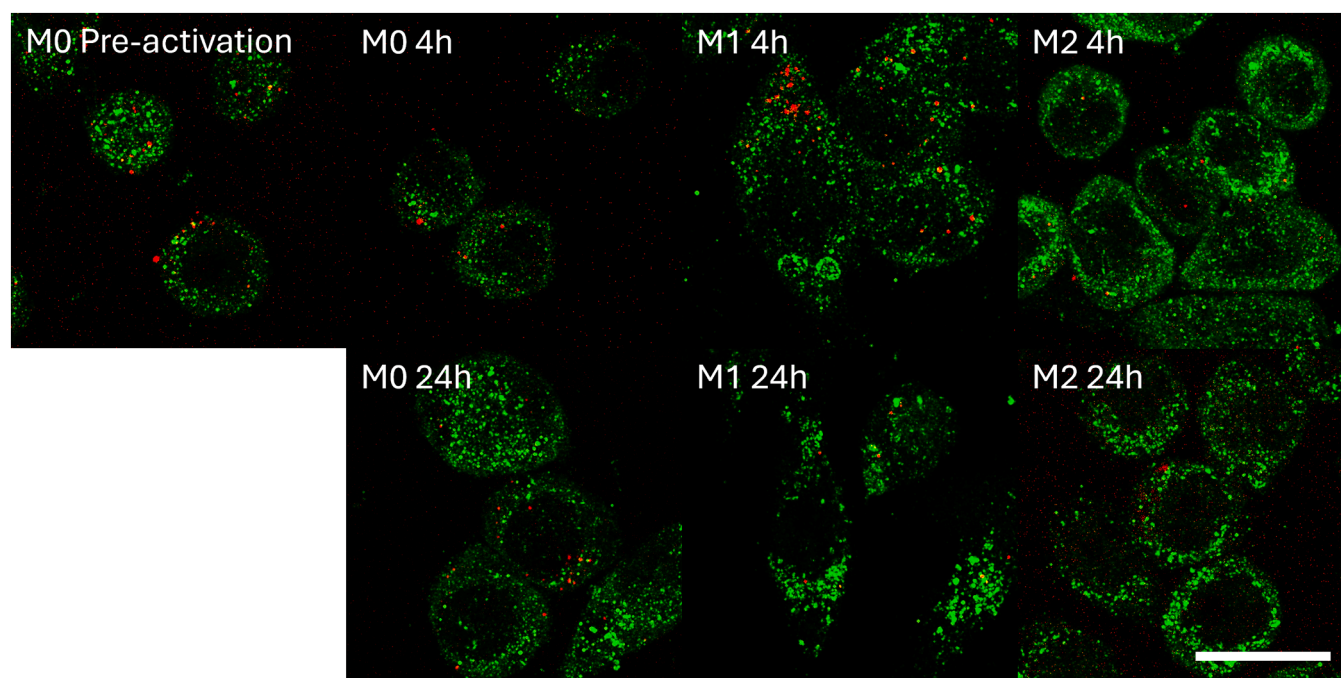


Fig. 5. Distribution of 70 nm FNDs (red) and late endosomes/lysosomes stained with LAMP1 (green) in macrophages of M0, M1, and M2 phenotypes at 30 min (pre-activation), 4 h, and 24 h post-FND treatment and polarization. Scale bar: 20 μ m.

24 h, however, they are located mostly in late endosomes/lysosomes (Fig. 6b,c). In the M1-activated cells FNDs were found mainly in the late endosomes/lysosomes after 4 h of culture. This status was maintained after 24 h. In the M2-activated cells, after 4 h around 20 % of FNDs were retained in early endosomes and around 10 % in late endosomes/lysosomes. After 24 h of culture almost no nanoparticles were colocalized with the early endosomes, while the number of FNDs in late endosomes/lysosomes only slightly increased compared to 4 h activation time, which may suggest that FNDs from early endosomes escaped vesicles. At

both time points, we have found that a significant fraction of FNDs per cell (around 80 %) was not co-localized with either early endosomes or late endosomes/lysosomes, which indicates they were released to the cytosol or located within other unstained vesicles. Other authors have also shown endosomal escape of nanodiamonds and attributed the effect to the sharp edges of the particles [55–58].

However, large differences have also been reported between cell types both concerning timing and the number of particles that escaped the endosomes [59]. In the studies where bigger vesicles as phagosomes

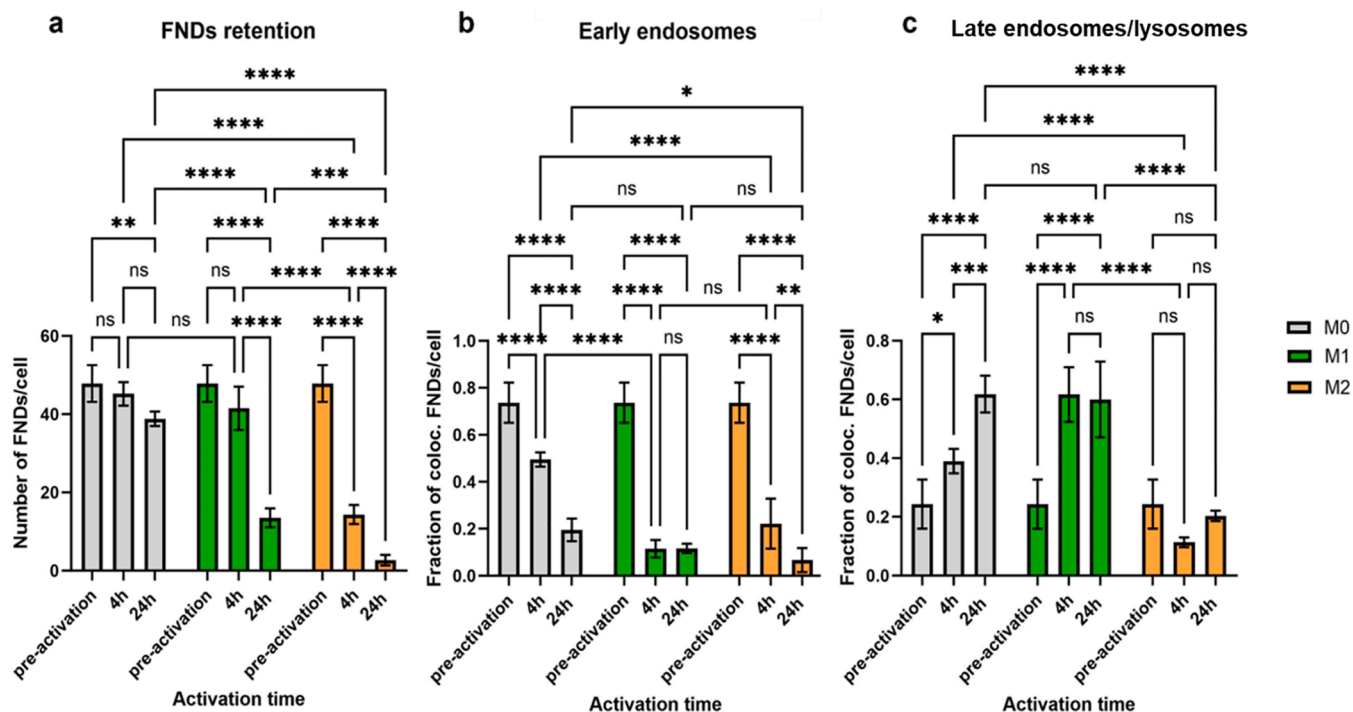


Fig. 6. FND retention (a), colocalization with early endosomes (b), and colocalization with late endosomes/lysosomes (c) in macrophages at different time points: pre-activation (30 min post-FND treatment), 4 h, and 24 h after macrophage activation.

and bacterial infections were investigated, the M1 phagosomes were associated with slower fusion rate with late endosomes/lysosomes. It has been proven that the delayed kinetics of vesicle fusion are caused by the inability of M1 phagosomes to acidify the environment due to increased activity of NADPH oxidase. In M1 cells, the elimination of pathogens is facilitated by the activation of reactive oxygen species at the expense of acidification and vesicle maturation delays. On the other hand, M2 vesicles promotes rapid acidification to efficiently clear apoptotic bodies, and therefore these phagosomes fuse and mature faster [60]. These studies focused on vesicles created during stimulation/infection and did not exceed 4 h, so could be referred to as short-term cell responses.

In contrast, in our research, M1 and M2 stimulation was performed after FNDs were internalized by M0 macrophages and at a minimum of 4 h post-stimulation, therefore in this work, we presented the long-term macrophage response.

3.3. FND-based quantum sensing enables the monitoring of vesicle-dependent changes in free radical generation

We have used FNDs as quantum sensors to monitor changes in T1 values, which inversely correspond to changes in the level of free radicals. In other words, lower T1 values indicate higher free radical concentrations. Here, we chose to use all optical T1 measurements instead of using T1 measurements with an additional microwave pulse which is used elsewhere [61,62]. The microwave step was omitted here (and in most articles published in the field) to avoid microwave heating of the biological samples. Further, charge transfer from NV- to NV0 are not problematic in large ensembles and at low laser powers which are used here [63]. Results of T1 relaxometry measurements are presented in Fig. 7. In the M0 cells we found a 50 % decrease in T1 values after 24 h compared to the T1 values measured after 4 h of cell culture. Considering the location of FNDs in M0 macrophages after 24 h we postulate those higher free radical concentrations after 24 h correlate with the higher redox activity of late endosomes/lysosomes. The level of FRs generated in the early endosomes of M0 cells is lower than that in their

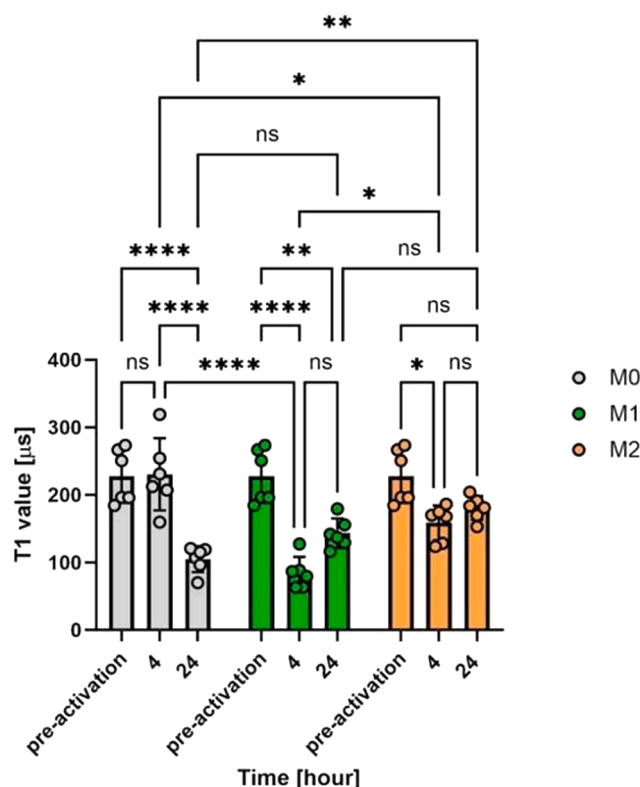


Fig. 7. T1 values in pre-activated (30 min. post treatment with FNDs) and in M0, M1, M2-activated macrophages after 4 and 24 h of polarization. We measured 3 FNDs per cell, in 6 cells per variant and we repeated the experiment 6 times.

late endosomes/lysosomes.

Among investigated phenotypes, the lowest T1 values were measured in M1-activated macrophages. Herein, the higher free radical generation could be associated with the location of FNDs in late endosomes/lysosomes and their high redox activity. We have also found that this lysosomal activity is higher within 4 h and slightly decreases after 24 h. The T1 values measured after 4 h stimulation in M2 macrophages were significantly lower than the corresponding ones recorded for M0 macrophages and higher than for M1 cells. We have not observed any significant change in the T1 value after 24 h culture. It has been reported that a key characteristic of fully differentiated M1 macrophages is a marked increase in the expression and assembly of NADPH oxidase, enabling the swift and strong production of FRs in response to inflammatory stimuli [60]. On the other hand, anti-inflammatory M2 macrophages exhibit lower expression levels of NADPH oxidase subunits and therefore lower FR levels. To this date, the state-of-the-art methods allowed researchers to investigate only short-term FRs generation in polarized macrophages. These studies have found that M1 phagosomes, containing zymosan particles up to 3 µm, generate FRs for at least 30 min in a continuous manner, whereas in M2 phagosomes, the oxidase activity is not detected after 5–10 min [60]. In contrast, in this work, due to the photostability, sensitivity and high spatial resolution of nanodiamond-based quantum sensing protocols, we were able to detect changes in FR generation as the consequence of the long-term effects of macrophage polarization.

4. Conclusions

Diamond-based quantum sensing offers a tool to investigate the FR generation within vesicles in macrophages. We further followed the polarization process and found differences in free radical levels in the different phenotypes. Compared to conventional optical free radical sensing methods, our technique offers greater spatial resolution which allows vesicle-specific measurements. Herein, we focused on the long-term response of macrophages to polarization as it has not been studied yet. In this study, we showed retention, time-dependent changes in the location of nanodiamond sensors and location-dependent FR generation. We found that FNDs have a slight impact on the direction of the polarization process, which may be useful in cases where a specific macrophage phenotype is desired. However, in the future, surface modification approaches should be developed to ensure that FNDs are inert for macrophage polarization to serve as sensors and drug nanocarriers.

CRediT authorship contribution statement

Aldona Mzyk: Writing – original draft, Methodology, Data curation, Supervision. **Claudia Reyes-San-Martin:** Writing – original draft, Visualization, Methodology, Data curation. **Yasemin Doğan:** Data curation. **Willem Woudstra:** Data curation. **Yue Zhang:** Data curation. **Ezgi Yilmaz:** Data curation. **Reinier Bron:** Data curation. **Willy de Haan-Visser:** Data curation. **Kirstine Berg-Sorensen:** Writing – review & editing, Supervision. **Romana Schirhagl:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

R.S. is the founder of QTsense, a spin-off company from the University Medical Center Groningen, that commercializes quantum sensing equipment.

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Data availability statement

The data is available from the authors on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2025.04.024.

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