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The Role of Dendritic Cells in Bone Loss and Repair

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Abstract

The cells of innate immunity, such as neutrophils, macrophages, and dendritic cells (DCs), stuck to the bone implant walls release reactive radicals, enzymes, and chemokines, which induced subsequent bone loss. DCs do not play a big role in bone homeostasis in steady-state conditions, but could act as osteoclasts precursors in inflammation foci of bone. The potent antigen-presenting cells responsible for activation of native T cells and modulation of T cell activity through RANK/RANKL pathway and other cytokines associated with osteoclastogenesis determine critically situated at the osteoimmune interface. The titanium (Ti) and magnesium (Mg), the metallic candidate in implant, including calcium-phosphate coating formation on them by method plasma electrolytic oxidation were used to evaluate the immune-modulatory effects of DCs. The calcium-phosphate coating on metals induced mature DC (mDC) phenotype, while Ti and Mg promoted a noninflammatory environment by supporting an immature DC (iDC) phenotype based on surface marker expression, cytokine production profiles, and cell morphology. These findings have numerous therapeutic implications in addition to DC's important role in regulating innate and adaptive immunity. A direct contribution of these cells to inflammation-induced bone loss establishes DC as a promising therapeutic target, not only for controlling inflammation but also for modulating bone destruction.

Keywords: osteoimmunology, dendritic cells, bone, titanium, magnesium, biocompatibility

1. Introduction

There are two forms of the immune response of organism: innate and adaptive ones and dendritic cells (DC) serve as a bridge between them. The role of innate immunity cells

in inflammation-induced osteoclastogenesis and subsequent bone loss is critical, thereby establishing a new paradigm of osteoimmunology [1–3]. According to the long-standing definition, septic and aseptic total joint and bone replacement loosening are two distinct conditions with little in common. Septic bone replacement loosening is driven by bacterial infection whereas aseptic loosening is caused by biomaterial wear debris released from the bearing surfaces. The initial injury to the tissue surrounding a bone implant induces an inflammatory response mediated by the cells of innate immunity, such as neutrophils, macrophages, and DCs. The cells, which are stuck to the implant walls, release reactive radicals, enzymes, and chemokines, which cause a cascade of inflammatory responses [4, 5]. The mobilization of functional activity of innate immunity cells is directed by chemokines, cytokines, and integrins. Recently, it has been recognized that the mechanisms that drive macrophage activation in septic and aseptic total joint replacement loosening resemble each other [6]. Accumulating evidence indicates that in addition to mediating bacterial recognition and the subsequent inflammatory reaction, toll-like receptors (TLRs) and their ligands, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPS), play a key role in wear debris-induced inflammation and cellular activation. Furthermore, metal ions released from some total joint replacements can activate TLR signaling similar to bacterial derived PAMPs [7]. Likewise, metal ions can function as haptens activating the adaptive immune system similar to bacterial derived antigens [8]. Thus, it appears that aseptic and septic joint and bone replacement loosening share similar underlying pathomechanisms. DCs derive from the mononuclear cell pool and are characterized by high expression levels of CD11c/major histocompatibility complex (MHC) II. Conventional DCs are capable of antigen presentation and reverse migration, i.e., are able to migrate to lymph nodes via afferent lymphatic vessels. Immature progenitor conventional DCs are unable to prime T cells, but are equipped with high phagocytic activity [9]. It was shown that, DCs can influence the type of immune response through the induction of regulatory mechanisms. Immature DCs express chemokine receptors (CCR-1, −2, −5, −6 and CXCR-1) and are able to migrate actively to the inflammatory focus in response to the appearance of chemokines [10]. The process of maturation of DC is initiated by various factors, including biomolecules of microorganisms, pro-inflammatory cytokines and products of necrosis of cells and tissues. Maturation of the DC is a continuous process, in which there is a decrease in the endocytic potential, the expression of antigen-recognizing receptors, while, on the opposite side, the expression of adhesion molecules CD54 and CD58 increases. This changes the structural organization of cellular organelles, increases the activity of lysosomal enzymes, there are immunoproteasomes which process the intracellular protein antigens. Fully differentiated DCs actively produce pro-inflammatory cytokines, such as IL-12alpha (IL-12p35), through which T-lymphocytes are activated, including regulatory lymphocytes [11].

Researchers recently began to investigate a possible direct role of DC in inflammation related bone damage. DCs are known for their role of antigen presenting cells (APCs) and do not appear to play a role in bone homeostasis in nonpathological conditions, but some data suggest that DC could act as OC precursors in an inflammatory milieu, transforming into

DC-derived-OC according to phenotypic and functional characterization studies. Moreover, DCs modulate T cell activity through RANK/RANKL and osteoclastogenesis-associated cytokines [12–14]. The role of DC, as the key components of the defensive response of the organism in the pathology of bone, was demonstrated in the field of osteoimmunology research. It is indicated, that normally the localization of the DC in the stroma proper or adjacent to the bone tissue, is rare and DC do not take part in the restoration of its defects [15, 16]. On the other hand, the presence of DC in patient's synovial periodontal fluid during periodontitis and in joints of patients with rheumatoid arthritis has been documented [17, 18]. With these diseases, localized in the bone stroma the DC can form aggregates with T- cells, forming inflammatory foci, where migrate through chemotaxis and adhesion molecules RANK-RANKL. It is shown that, during the inflammation of bone tissues, the expression of these receptors on the surface of the DC induces indirectly through regulation of T-cell activity and through the process of differentiation and survival of osteoclasts bone degradation [15, 16, 19]. Rivollier et al. have shown that myeloid DCs of human peripheral blood can be transformed into osteoclasts in the presence of macrophage colony-stimulating factor M-CSF and the soluble form of receptor RANKL, suggesting direct participation of DC in osteoclastogenesis [16, 20, 21]. Further, co-cultivation of $CD11c^+$, $CD11b^-$ DC, similar to the classical precursors of osteoclasts, under the influence of granulocyte-macrophage colony-stimulating factor GM-CSF and interleukin 4 IL-4, their transformation into osteoclasts was demonstrated. This suggests that transformed into functional osteoclasts $CD11c^+$ DC, under the condition of their immune interaction with $CD4^+$ T cells and other factors in the surrounding bone tissue environment, can induce the bone resorption process in vivo. Also installed an important protein currently considered as a master regulator of osteoclastogenesis—dendritic cell-specific transmembrane protein (DC-STAMP). It is assumed that DC-STAMP plays an imperative role in bone homeostasis by regulating the differentiation of both osteoclasts and osteoblasts [22]. In general, these data point to a critical effect of DC on the process of osteoclastogenesis in inflammatory bone diseases, where they act not only as powerful antigen-presenting cells, that activate and regulate the cells of immune system, but also influence directly to the destruction of bone tissue. There is a lack of definitive evidence about the physiological relevance of this phenomenon in vivo but DCs could act as an osteoimmune interface, contributing to bone loss in inflammatory diseases [12, 16, 21].

At the moment, in the field of endoprosthetics, there is a tendency in studies aimed at creating biomaterials that can replace damaged tissue sites of the human organism. Most successfully, these studies are made while treatment of the pathology of the musculoskeletal system, including in the endoprosthetics of large joints. At the moment, stainless steel and titanium alloys are the main materials used for the manufacture of immersion implants. Nevertheless, the use of fixatives from bioinert metals in osteosynthesis requires repeated surgical interventions aimed at removing the metal implants that have performed their role, and this is often no less traumatic, than osteosynthesis itself. Therefore, it remains relevant to search for bioresorbable materials that are suitable for creating implants used in osteosynthesis, that could be completely metabolized by the organism without exerting a pathological effect on surrounding tissues and the organism as a whole [23, 24]. Such materials include magnesium

alloys, which, due to the strength properties, are suitable for the production of various types of implants. This material has good biocompatibility, sufficient corrosion resistance and shows a positive effect of magnesium biodegradation products on osteogenesis, but the mechanism of their action is not fully studied [25]. Both bioinert and bioresorbable materials, when introduced into the organism, are contacted with antigen-presenting cells and their properties, such as topography of the surface, chemical composition, play an important role in initiating a pro- or anti-inflammatory immune response.

Thus, DCs are suitable cells for evaluating of their response to biomaterials because they can transform into osteoclasts under bone inflammation and also initiate and modulate the immune response to the implants materials. This way, the determination of the ability of biomaterial to influence on the phenotype of DC is quite applicable for determining their compatibility properties with the organism. Only several metal-based nanoparticles were reported to activate T cell responses or homeostasis. For example, TiO_2 nanoparticles provoke inflammatory cytokines and increase DC maturation, expression of co-stimulatory molecules, and prime native T cell activation and proliferation [26]. Most importantly, pattern recognition receptors signaling activations also can enhance antigen presentation via upregulating the expression of MHC and co-stimulatory molecules (CD80 and CD86) on DC leading to adaptive immunity activations [1, 7]. Thus, the study of the phenotype and functional activity of the DC after exposure to biomaterials corrected for properties suggests a direction in the development of the immune response induced by their introduction into the organism and makes it possible to compose an immunomodulating design of such biomaterial.

From all the listed above, the aim of the work is to reveal the immunomodulating properties of bioinert (titanium) and bioresorbable (magnesium) metal implants according to the degree of their influence on DC markers.

2. Materials and methods

2.1. Materials

Bacterial lipopolysaccharide (LPS, Abcam, USA) and the disks of implants were prepared from 1-mm thick sheets of commercially pure Ti (wt. %: Fe 0.25; Si 0.12; C 0.07; O 0.12; N 0.04; H 0.01, Ti—the remaining part balance) and Mg alloy MA8 (1.5–2.5 wt. % Mn; 0.15–0.35 wt. % Ce; Mg—balance) were used. The samples of a size of 15 mm × 20 mm × 2 mm have been undergone preliminary mechanical treatment until the roughness parameter of Ra = 0.12 μm. After mechanical treatment, samples were thoroughly washed with deionized water and ethanol and dried in the airflow. The samples appearance after volumetric tests was observed using a Stemi 2000CS stereo-microscope (Zeiss, Germany).

The electrolyte was prepared in 2 liters of deionized water by adding the following components: 30 g/l of calcium glycerophosphate dihydrate ($C_3H_7O_6P$), Ca·2H$_2$O and 40 g/l of calcium acetate monohydrate (Ca(CH$_3$COOO)$_2$·H$_2$O). The electrolyte pH was adjusted to 10.9–11.3 by adding 20% NaOH solution [27]. Plasma electrolytic oxidation was carried out using a reversible thyristor rectifier, as power supply, equipped with an automated control system with appropriate software. All the samples were treated in the unipolar PEO-mode at

a current density of 0.67 A/cm^2. The treatment time was 300 s and the final voltage equaled to 540 V. Experimental series were carried out with the sample coatings, which included calcium and phosphorus (Ca and P) on Ti and Mg alloy substrate. The samples were denoted in the text as: uncoated titanium—Ti 1; titanium with calcium-phosphate coating—Ti 2, and uncoated Mg alloy—Mg 1; with coating—Mg 1. The samples were punched to be 15 mm in diameter for snug fit in the wells of 6-well tissue culture polystyrene (TCPS) plates (Thermo Scientific, Germany). The samples were sterilized in a laboratory oven (Thermo Scientific, Denmark) at 180°C for 15 min (with controlling of surface properties), in accordance with the rules for sterilization of medical devices.

2.2. Animals

Study approval from the local Ethical Committee of the Pacific State Medical University (Vladivostok, Russia) was received under No. 2015–0102. For the experiments, adult, three-month old, 250 g of weight male rat was used. Animals were euthanized using carbon dioxide asphyxiation as approved by the MIT committee on animal care (National Institute of Health Guide for the Care and Use of Laboratory Animals, NIH Publications No. 80023, 1996).

2.3. Dendritic cell (DC) culture

The two primary cell types were used in this study are human peripheral blood mononuclear cell (PBMC) and rat bone marrow derived DCs (RMDC). Human PBMC were obtained from donor blood (Border station of blood transfusion, Primorye, Vladivostok, RU). All donors were in good health and were negative for blood-borne pathogens as detected by standard blood bank assays. The aphaeresis product was processed to enrich the PBMC fraction by using ficoll-hypaque (BioLegend, CA, USA) density gradient separation according to standard protocols as previously described [28]. RMDC were generated, as previously described, by Onai et al. [29]. Briefly, BM cells were removed from a male of rats and cultured in 24-well-culture plates, at a concentration of 5×10^6 cells per well, in 800 μl of RPMI-1640 (Lonza, Belgium) supplemented with heat-inactivated 10% fetal calf serum (FCS), 100 μg/ml of penicillin, 100 μg/ml of streptomycin, 5×10^{-5} m 2-mercaptoethanol (Lonza, Belgium) plus GM-CSF (50 ng/ml) and IL-4 (10 ng/ml). On days 3, 6, and 9 the supernatant was gently removed and replaced with the same volume of the supplemented medium. On day 9 of culture, ≈80% of the cells were CD11c$^+$ DC.

2.4. Cell viability assay

To determine toxicity levels of samples the cellular cultures RMDC were prepared at approximately 2000 and 20,000 cells per well, respectively, in 96-well flat bottom tissue culture plates. A mitochondrial colorimetric assay (MTT assay) by the percent of total succinate dehydrogenase (SDH) released was used [30]. In the each well with cellular monolayer, leaving 200 μL, to which 40 μL of MTT 1.2 mM solution (3-(4.5-dimethylthiazol 2-yl)-2.5-diphenyltetrazoliumbromide, Sigma-Aldrich, USA) was added. The cells were incubated at 37°C and 5% CO^2 for 4 h. The upper medium was removed carefully, and the intracellular formazan was solubilized by adding 200 μL of dimethyl sulfoxide to each well (Sigma-Aldrich, USA). Then, the contents of the wells were mixed thoroughly using a pipette. Two hundred microliters from

each well were transferred into a separate well on a 96-well ELISA plate (Corning Costar, Lowell, MA, USA). The absorbance was measured at 570 nm. The results expressed as optical density (OD) were obtained for three different experiments from each surface modification.

2.5. Dendritic cell phenotyping

Phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein (PerCP-Cy5.5)—conjugated monoclonal antibodies (mAbs) specific for human CD14 (M5E2), CD34 (MEC14.7), CD38 (90), CD86 (2331), CD83 (HB15e), and HLA-DR (L243) (all from BioLegend, CA, USA) were used to determine the cell surface receptors expressed on the reverse transmigratory human PBMC DCs. Isotype control antibodies included MIgG2a (G155–178) and MIgG1 (MOPC-21) were also purchased from BioLegend. DCs were collected and labeled with the abovementioned specific antibodies for 45 min at 4°C in PBS containing 2% bovine serum albumin (BSA) and 0.05% sodium azide. Flow cytometry was used to determine the abundance of each cell type (subpopulation) expressing markers using MACSQuant™ Analyzer 10 (Miltenyi Biotec GmbH, Germany) and Kaluza 1.5 analysis software (Beckman Coulter, USA).

The mean fluorescence intensity (MFI) value for the expressing CD14, CD34, CD83 on RMDC (polyclonal antibody with species reactivity human, mouse, rat, 1:200, MyBioSource, Inc., USA) was analyzed using a confocal scanning laser microscope (Zeiss, Germany) connected to an Evolution MP Color Camera (Media Cybernetics Inc., Bethesda, MD, USA). The camera used Image-Pro Plus 7.0 software (Media Cybernetics Inc.), and the acquired digital images were processed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) for qualitative analysis.

2.6. Methods of determination of the cells functional activity

RMDC incubated with samples of implants at 37°C and separate supernatant were frozen and stored at −20°C. The disrupted cells were mixed with 100 μL of Griess reagent, which consisted of equal volumes of 0.1% N (1 naphthyl) ethylenediamine dihydrochloride and 1% n-aminobenzene sulfanilamide (ICN, USA) in 2.5% phosphoric acid solution. After incubation for 10 min, the absorbances were measured at 540 nm using a Multiskan Titertek Plus spectrophotometer (Flow lab, Finland). Determination of the ATPase was determined by adding to the cellular monolayer 20 μl of substrate for ATPase (8 mg ATP on 1 ml of Tris HCl buffer (pH 7.8), which contained 87 mg of NaCl, 28.7 mg of KCl, and 52 mg of $MgCl_2$ 6 H_2O, ICN, USA) and the samples were left for 30 and 60 min. The reaction was stopped by adding 100 μl mixture of ascorbic and molybdenum acids at a ratio of 1:1. After 20 min, the optical density of the substrates was measured on a spectrophotometer at a wavelength of 620 nm. For the determination of the activity of lactate dehydrogenases (LDH), the Lloyd method in his own modification was used. 100 μl of substrate was added into wells of plate with adherent cells (2 mg/ml iodine nitro tetrazolium on phosphate buffer pH 7.2 with 0.4% $MnCl_2$, Sigma-Aldrich, USA) and incubated at 37°C for 30 min. Diformazan pellets were dissolved by adding 100 μl of isopropyl alcohol and acidified with 0.04 M HCl for 20 min. The optical density of the substrates was determined on a spectrophotometer at a wavelength of 650 nm. The activity of cytochrome oxidase was determined by adding to the cellular monolayer 100 μl of 0.1 M acetate buffer, pH 5.5, containing 10 mg/ml of $MnCl_2$, 0.33% hydrogen peroxide, and 2 mg/ml of diaminobenzidine. After 10 min incubation at room temperature, the reaction was stopped

by the addition of 10% sulfuric acid (100 µl per well). The quantity of formed product was determined by measuring the absorption at 492 nm. Samples containing the substrate solutions and 10% sulfuric acid were used as the control.

The results were obtained for three different experiments from each surface modification. The spectrophotometric data of optical density were evaluated as the simulation index (T), which was calculated as the ratio of the difference between the mean values of the optical density of the solutions containing reaction products of control and experimental cells, versus the mean value of optical density of intact cells (and expressed as per cents).

2.7. Cytokine assays

Measurements of RMDC cytokines RANTES, TNF α, IL-1, IL-6, IL-10 and IL-12 in the supernatants were performed by using specific solid-phase sandwich enzyme-linked immunosorbent assay (ELISA). Capture and detection cytokines used were purchased from Mouse ELISA Kit (Abcam, USA), using the procedure recommended by the manufacturer. The absorbances were measured at 450 nm by use of a microplate ELISA reader.

2.8. Scanning electron microscopy (SEM) of adherent RMDC

The qualitative analysis of cell adhesion was determined at 1, 3, 6, and 9 days. The disks were washed three times with warm D-PBS to remove the non- or loosely adherent cells. The cells samples were fixed with 1 ml 0.2 M cacodylate buffer (pH = 7.4) included 2% glutaraldehyde, 3% paraformaldehyde and 0.02% (w/v) picric acid (Sigma) overnight. The cells were washed three times with 0.2 M cacodylate buffer and were post-fixed with 0.5 ml 1% osmium tetroxide (OsO_4, Sigma) for 1 h. The cells were then dehydrated in a sequential series of increasing concentrations of acetone: 15, 30, 45, 75, 90, and 100% acetone for 30 min at each concentration. Subsequently, the samples were dried in an E3000 Critical Point Dryer (Quorum Technologies, Canada) and sputter coated with a thin layer (~5 nm) of carbon (JEE-420, JEOL, Japan). The micrographs were collected using scanning electron microscopy ULTRA PLUS-40-50 (Zeiss, Germany) in accelerating voltage 5 kV.

2.9. Statistical analysis

Data for the differentiation assay were analyzed by analysis of variance (ANOVA) and the Mann–Whitney method for comparisons between groups. The levels of cytokines and cellular proliferation, as well as the fluorescence intensity of the mature DC, were also analyzed by "ANOVA" followed by the Newman-Keuls test to determine multiple comparisons. Values were considered significant when different at $P < 0.05$.

3. Results

3.1. The morphology and activity of dendritic cells

Innate immunity is nonspecific and the first line of organism defense is carried out with the help of pattern-recognition receptors, which plays a significant role in the early reaction and the

subsequent pro-inflammatory response. The physical and chemical properties of the implants initiate various cellular reactions, such as absorption and intracellular biodistribution, which lead to a certain form of immune response [10, 31]. SEM micrographs showed that RMDC after contact with Ti 1 and Mg 1 had a spherical shape with an estimated size of 20 μm in diameter (**Figure 1B**, **E**). DCs treated with calcium phosphate coated exhibited more dendritic processes associated with mature DCs, the cells were larger with a folded surface; the formation of numerous dendritic appendages was also observed (**Figure 1D**, **F**). While in contact with Mg 1, the diameter of the cells was within the limits, the architectonics of the surface had folding, which indicated their activation (**Figure 1E**). In contact with coating Mg 2 the morphology of DC changes were similar with the morphology of cells contacted with Ti 2 (**Figure 1F**).
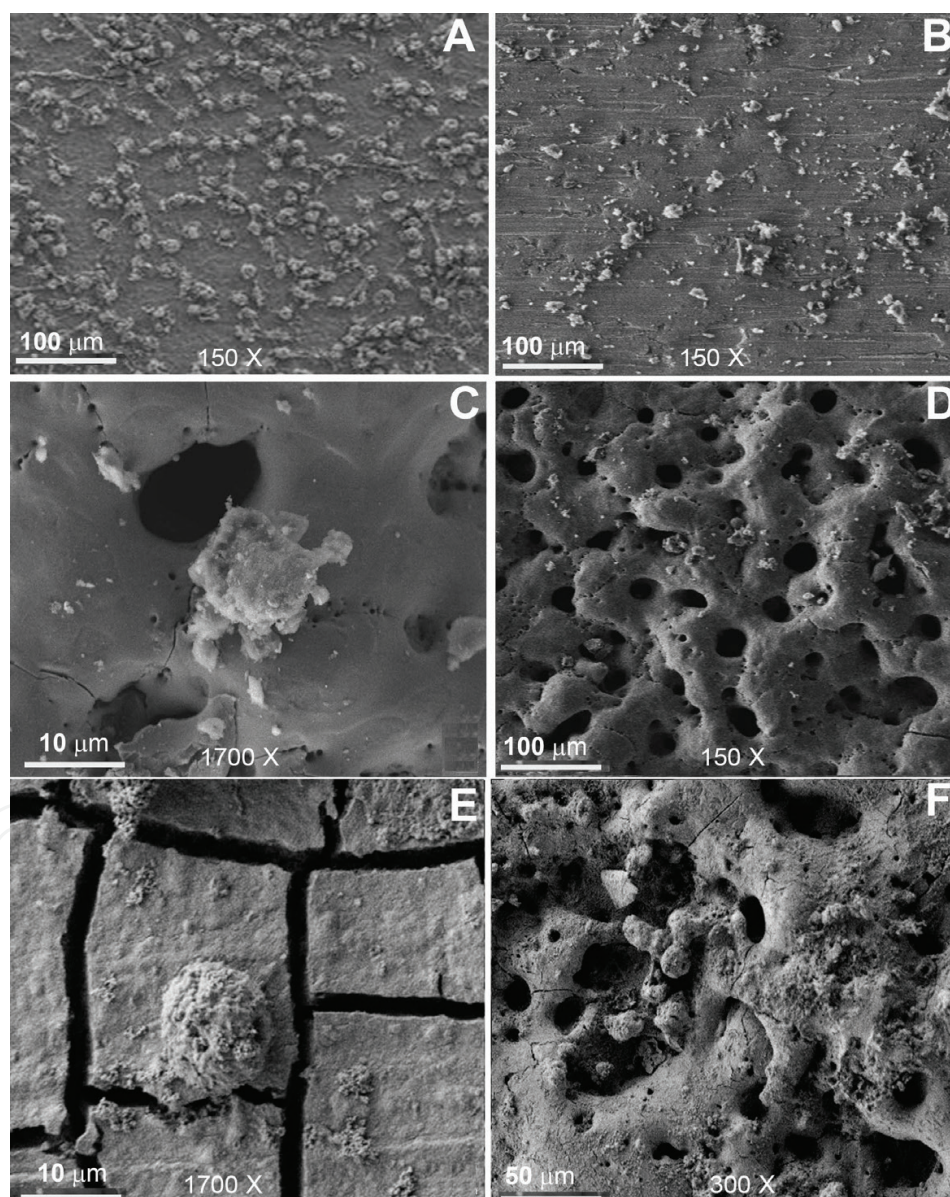

Figure 1. SEM of RMDC on the surface of collagen-coated glass (A) and samples. A typical architectonica of surface cell adhered to titanium Ti 1 (B) and Mg 1 (E); DC has a flattened shape without the presence of dendrites which is associated with immature DCs. In contact with calcium phosphate PEO-coated Ti 2 (C, D) and Mg 2 (F), the cells exhibited dendritic morphology which is associated with mature DCs; there have been numerous folds, and this surface was round and detected numerous pseudopodia. Data are from one of the two separate experiments, both with comparable results.

3.2. Cell viability

It is known that the MTT assay provides an estimate of total enzyme succinate dehydrogenase (SDH) released. SDH is a flavoprotein dehydrogenase and belongs to the succinate oxidase enzyme complex that forms the membrane respiratory chain of mitochondrion. The flavin group of this enzyme contains four iron atoms and is bound covalently to the protein [32]. We found no decrease of SDH activity of RMDC in the initial observation period (2 days) in DC contacting with Ti 1, Ti 2 and Mg 1, which indicates to a lack of cytotoxic effect of the studied samples (**Figure 2A**). A significant decrease of intracellular content after 3 days in cells in contact with Mg 1 and Mg 2 indicated a stimulated effect of these samples. It should also be noted that the dynamics of the cellular response on the samples with coatings was similar despite the different materials.

3.3. Enzymes activity of cells

Nitrite levels, an indirect measure of nitric oxide production, were assessed by the Griess Assay. Indicators for RMDC stimulated by Ti 1 was 14.5 uM, which was below than that stimulated by Ti 2, Mg 1 and Mg 2 (22.8; 21.9 and 19.03 uM), but the difference was not significant ($P = 0.07$). The level of cellular membrane released enzymes (ATPase) was significantly lower in the titanium Ti 1 and Mg 1 (T = −3.75 ± 0.5 and T = −6.25 ± 0.6%, respectively, 3 days contact) than in the coated samples ($P = 0.024$, **Figure 2B**). ATPase of membrane participates in
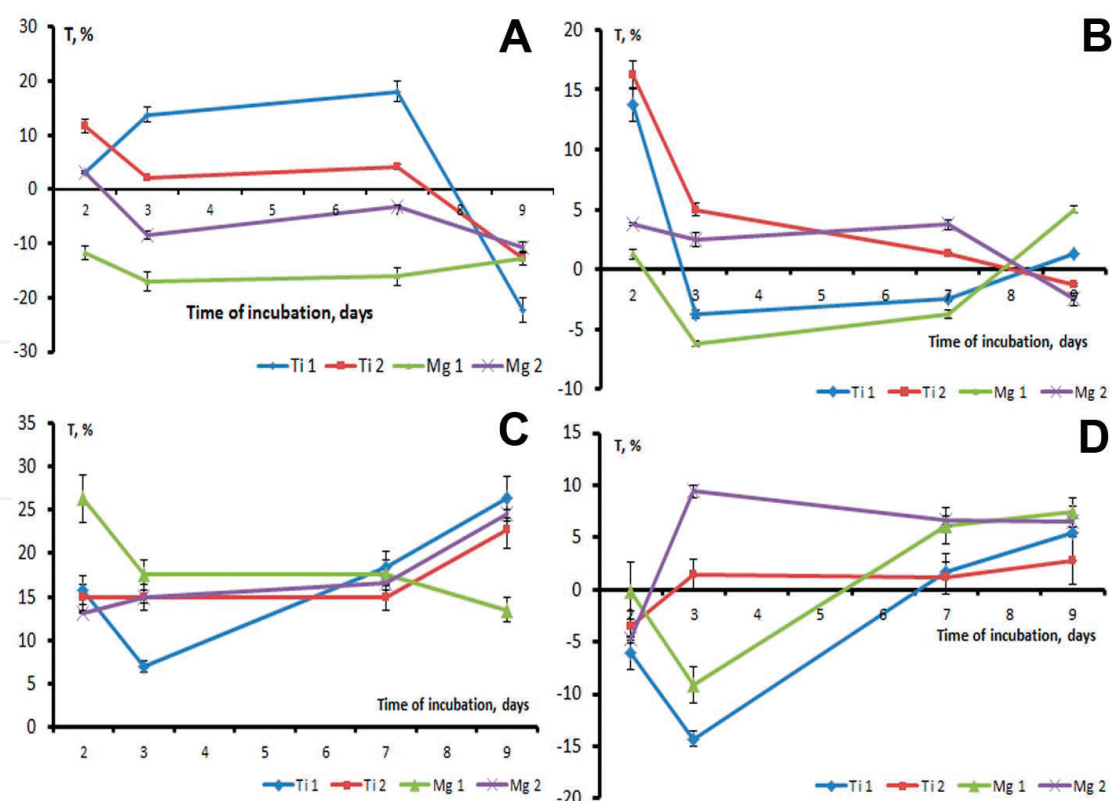

Figure 2. Enzyme activity of RMDC after contact with samples: A—succinate dehydrogenase; B—ATPase; C—lactate dehydrogenases; and D—cytochrome oxidase. Data presented as mean ± standard deviation of three independent experiments of time-course of cell contact. Ti 1—titanium without coating; Ti 2—calcium phosphate PEO-coating; Mg 1—Mg alloy without coating; Mg 2—calcium phosphate PEO-coating.

the hydrolysis of phosphate bonds and is indicators of stimulation of the cellular metabolism with a decrease in intracellular content. These data shows maximum stimulation of cells associated with the adhesion on samples surface within the first days and a difference ($p < 0.05$) of indices for coated on the titanium and magnesium (Ti 2, Mg 2) with less stimulating effects as compared with pure metals (Ti 1, Mg 1).

The LDH is a coenzyme dependent dehydrogenase and catalyzes the transfer of a reduced equivalent (hydrogen) from lactate to NAD$^+$ or from NADPH to pyruvate. LDH acts on the last step of hydrolysis that occurs under anaerobic conditions and results in the reduction of pyruvate yielding lactate and NAD$^+$. Most of the enzyme in the cell is weakly bound to the cell structure and localized in the cytoplasm, a smaller part being attached firmly to mitochondrial membranes [33]. We found no decrease LDH activity in the observation period, which indicates to a lack of cytotoxic effect of the studied samples (**Figure 4C**). A significant increase of intracellular content of this period reflected the increase of metabolic activity cells after contact with the samples at higher index stimulation for cells with Mg 1 ($p = 0.035$).

Cytochrome oxidase and SDH are the main components of the normal aerobic oxidative system of the tissue cells that are also known as the succinate dehydrogenase complex, where SDH is the first component and cytochrome oxidase is the second. Cytochromes are subdivided into three groups according to their chemical structure and spectrum: cytochromes a, b, and c. Oxidized cytochrome oxidase is reduced by cytochrome c catalyzing the transfer of four electrons to the oxygen molecule. Thus, cytochrome oxidase is a representative of the third group of oxidases. The difference was observed between indicators of enzymes depending on the sample type: the highest one was detected in cells contacted with Ti 2 and Mg 2 (2 and 3 days, **Figure 2D**). Thereafter, these parameters de-creased, thus showing the cells stabilization. Such a change in cell metabolism was associated with the components contained in coatings on the samples.

3.4. Effect of samples on DC maturation

Influenced of various stimulus DCs are undergoing a process of maturation that allows them to become more potent inducers of the adaptive arm of the immune response. In the absence of stimuli, the vast majority of DCs are immature. It is unclear whether or not treatment with metals implants can trigger DC maturation, but the ability of calcium phosphate PEO-coating to induce ROS in phagocytes suggests the possibility that these materials might activate this potent antigen-presenting cell (APC) population. DC maturation can be connected to the increased expression of the activation markers, CD1a and CD83, similar to what is observed in vivo [10, 11]. The RMDC treated with RPMI 1640 showed the typical expression of cell surface molecules as immature DC. In contrast the RMDC treated with PEO coated showed a clear change of the expression levels of CD14, CD34, and CD83 (**Figure 3**).

The CD34$^+$ cells in bone marrow are precursors of both the DC and granulocytes, and such cells are of the "intermediate" type on the 6th day of culture under the influence of an inducer are able to differentiate in DC or in leukocytes. In order to study the role of implants as maturation inducers, the receptor phenotype of human PBMC was analyzed. The primary culture of human PBMC was placed in vials with samples and cultured in the presence of GMCS and IL-4. As a control, cells adhered to the surface of specialized plastic coated with lectin
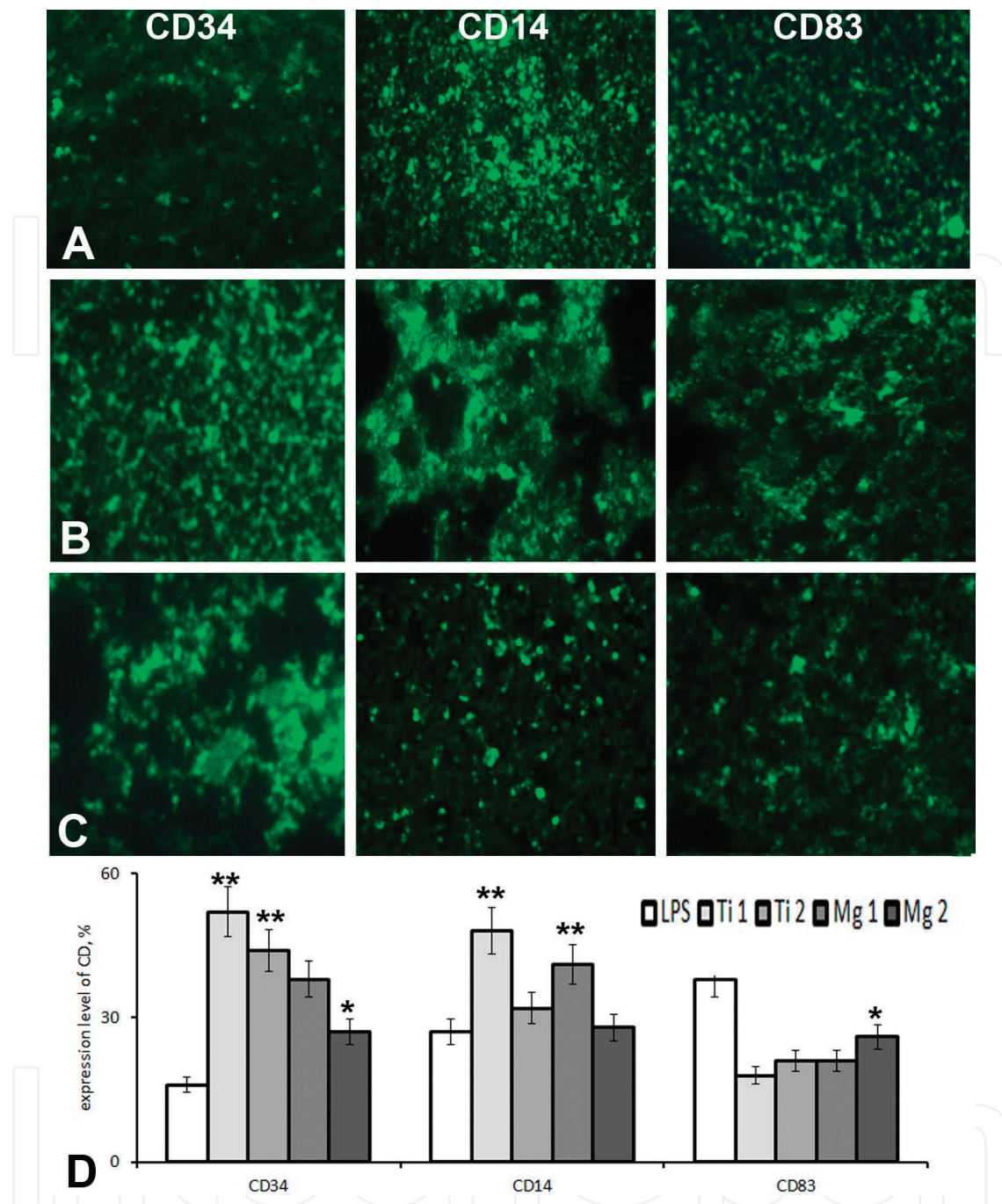
Figure 3. RMDC were cultured 9 days with LPS (A), magnesium Mg 1 (B), and calcium phosphate PEO-coating Mg 2 (C) and isolated. Representative photographs of immunofluorescent staining. DCs for FITC-conjugated anti-rat CD34, CD14, and CD 83 were analyzed by confocal scanning laser microscope, magnification 400×. Data are representative of three independent experiments (D); error bars represent mean ± SD (N = 20) with *p ≤ 0.05 and **p < 0.01 as compared to LPS-stimulated DC.

were used, and lipopolysaccharide *Escherichia coli* (LPS) was added to obtain a population of mature DCs. We already know that culturing of DC in the presence of GM-CSF and IL-4 supplemented with 2.5 ng/ml of LPS stimulates maturation of DC and reduces the number of macrophages in culture. It was determined that the maximum expression of CD34 on the DK surface was observed on the 1st day of joint incubation with LPS, and the cell content was 72 ± 5.8%. Later, their number decreased, reaching the minimum figures by the end of

the observation period (1.6 ± 0.08%). Under the influence of implants, the number of CD34⁺ cells compared to the control was lower. This way for samples with titanium after 1 day the index was 56 ± 4.8% and for samples with magnesium 48 ± 4.6%. The minimum number of these cells was noted at the end of the observation period (21 s) and amounted to 1.8 ± 0.2% and 2.4 ± 0.6%, respectively. Thus, the data obtained by us indicate an identical effect of the implants on the expression of the adhesion receptor, and the percentage of the content of these cells, reduced relative to the control, on their expressed effect as inducers of cell maturation.

As an indicator reflecting the direction of hematopoietic pool cell differentiation under the influence of implants, the degree of expression of membrane glycosylphosphatidylinositol-bound CD14 protein was determined. This component is an element of the CD14/TLR4/MD2 receptor complex, which recognizes the LPS, and is expressed on the surface of myeloid cells, especially on macrophages. We found that, in the control sample under the conditions indicated above, the minimum number of cells with a high degree of CD14 expression was determined on the ninth day, while the content of cells positive for detecting the DC CD83 terminal differentiation marker at these times the observation was maximum. The indicators were 14 ± 1.8 and 67 ± 5.8%, respectively. These data indicate that the introduction of LPS has a pronounced effect on the maturation of the DC population. During studying the degree of expression of these receptors in cell populations that contacted the implants, it was found that magnesium had the most activating effect on differentiation in the direction of the DC. Thus, on the 9th day of a joint incubation with magnesium, the CD14⁺ cell count was 26 ± 2.8% and CD83 + 58 ± 4.6%, while on contact with titanium 32 ± 3.1 and 48 ± 3.6%. In subsequent observation periods, the number of CD14⁺ cells was at the specified level, and when in contact with the implants, it decreased slightly. The data cited indicate a pronounced effect of the magnesium implant on the directionality of differentiation of hematopoietic pool cells mainly toward the DC.

The great interest is in the data obtained by us on the degree of expression of costimulatory molecules CD83 and CD86 on the surface of the DC during their interaction with implants, depending on the incubation time. These receptors of intercellular adhesion interact with the corresponding ligands with high avidity, under condition of their expression on cell membranes by clusters. Under effect of LPS on the degrees of expression receptors on day 9, the indicator of CD14⁺ DC was minimal against the maximum of CD83⁺ (2.4% ± 0.2 and 62.4 ± 0.6%, **Figure 4C**). Despite the fact that, at the initial time of observation, LPS activated maturation of DC more than implants, over time the level of expression of costimulatory molecules on the cell surface under the influence of titanium and magnesium increased (**Figure 4C**). Moreover, with relatively low values of CD83⁺ and CD86⁺ DC, the intensity of their luminescence increased (**Figure 4**) and expression of CD83 molecules on DC, incubated with implants remained elevated (23.8 ± 2.6 and 21.2 ± 3.4%, respectively) in comparison with DC treated with LPS (0.6 ± 0.2%) until the end of the observation period. In contact with titanium Ti 1, the number of CD14⁺ cells in this period was 32 ± 2.1% and CD83⁺ − 48 ± 2.6%, with coated titanium Ti 2–26.4 ± 2.1% and 52.6 ± 4.6% (**Figure 4**). These data indicate to the effect of coated on titanium as inductors of DC differentiation.

The leukocyte antigen CD38 is a bifunctional enzyme that combines ADP-ribosyl cyclase and cADP-ribosyl hydrolase activity, is expressed on hematopoietic cells, respectively, by their
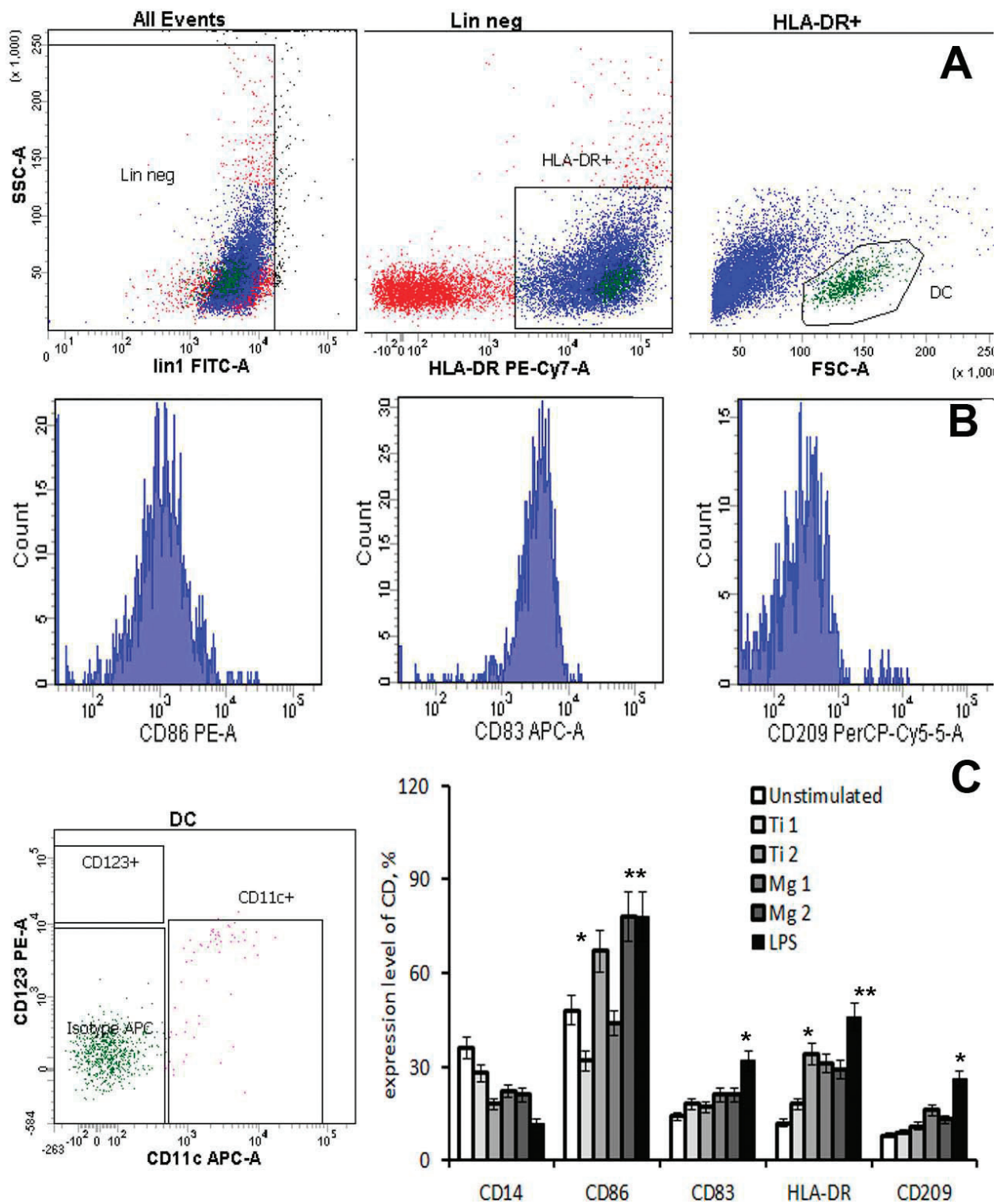
Figure 4. Phenotype profiles of DCs from human PBMC. The cells were cultured for 9 days in the presence of GM-CSF and IL-4 immature DCs (unstimulated), maturate LPS-stimulated DC (LPS) controls, and samples were isolated. (A) DCs were analyzed by flow cytometry for HLA-DR surface marker. (B) DC were stained with PE-conjugated CD86, APC-A-conjugated CD83, PerCP-Cys5–5-A conjugated CD209 and then examined by flow cytometry. (C) DCs were examined for CD11c, CD14, CD 83, CD 86, CD123 and CD 209 expression by flow cytometry. Data are representative of three independent experiments; error bars represent mean ± SD (N = 20) with *p ≤ 0.05 and **p < 0.01 as compared to unstimulated DC.

degree of differentiation or proliferation. The product of the enzymatic activity of CD38-cyclic ADP-ribose is a universal catalyst of calcium from the internal depot. Moreover, the main function of the CD38 receptor is to regulate the activity of bone marrow cells, lymphoid tissue and peripheral blood, stimulating their production of cytokines, and also participates in the migration of the DC. Increased regulation of CD38 serves as a marker of cell activation, in particular, the process of differentiation of B-lymphocytes into plasmocytes. When studying the amount of CD38$^+$ phenotype in a pool of undifferentiated cells of the myeloid series before contact with samples and LPS, their content was determined to be $19.29 \pm 1.74\%$. In the dynamics of interaction of these cells with LPS, the amount of CD38$^+$ increased, with a maximum value of 3 seconds after incubation, the indicator was $98.2 \pm 9.7\%$. Then, in the samples incubated with magnesium, an increase in the number of CD38 $^+$ cells on day 2 ($91 \pm 8.92\%$) was revealed with a subsequent decrease to the end of the observation period ($28 \pm 2.4\%$). Upon contact with titanium, the maximum CD38$^+$ cell content was observed only for 9 s of incubation and their value was $82 \pm 7.8\%$. The above data indicate the presence of the inducing effect of implants on the maturation of DC, depending on the contact time, and more magnesium than titanium.

Treatment of pathogenic associated molecules generated a population shift from a precursor DC phenotype, traditionally CD14$^+$ and HLA-DR$^+$, to an increased number of immature and mature DC phenotype CD14$^-$/HLA-DR^{+low}, CD14$^-$/HLA-DR^{+high}, respectively. CD14$^+$ DCs express C-type lectin DC-specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN, CD209), which may also be found on monocyte-derived DCs, especially those generated under tolerogenic conditions such as IL-10. Analysis of the stimulated CD14$^-$/HLA-DR$^+$ population demonstrated significantly enhanced expression of mature DC-specific marker CD83, CD209 and the costimulatory molecule CD86 after contact with calcium phosphate PEO-coating implants compared to unstimulated controls and was similar to cultures stimulated with LPS (**Figure 4C**). There is an approximate 3-fold increase in DC-specific maturation marker CD83 expression on DCs treated with Ti 2 and Mg 2 over those given for cells on titanium ($p < 0.05$). This shift can be explained by stimulation of the resident cells population exposure properties of calcium-phosphate coating and the presence of molecular point-like effects on receptors. The increase in CD86 and CD123 indicate the DCs maturing ability for the costimulation of lymphocytes, triggering their subsequent activation and proliferation, further suggesting the potential to drive such an adaptive response.

3.5. Cytokine production of cells

DCs are a unique antigen-presenting cell that can both participate in inflammatory reactions, by producing a variety of inflammatory mediators, and directly respond to the product of these innate pathways. The study of titanium Ti and its coating effect on cellular production of cytokines showed that the level of pro-inflammatory cytokines, much difference between the indices for intact cells and after their contact with the samples, was found for the two cytokines TNF α and regulated on activation of cellular expressed and secreted RANTES (**Figure 5**). The greatest number of cytokine producing was in contact with titanium Ti 1 and a lower number in contact with calcium phosphate-coated Ti 2. This dependence was established in relation to anti-inflammatory cytokines production by cells—interleukin 6, 10, and 12. These data indicates that, in comparison with other studied samples, the smallest immune stimulatory effect applies to the calcium phosphate coated Ti 2.
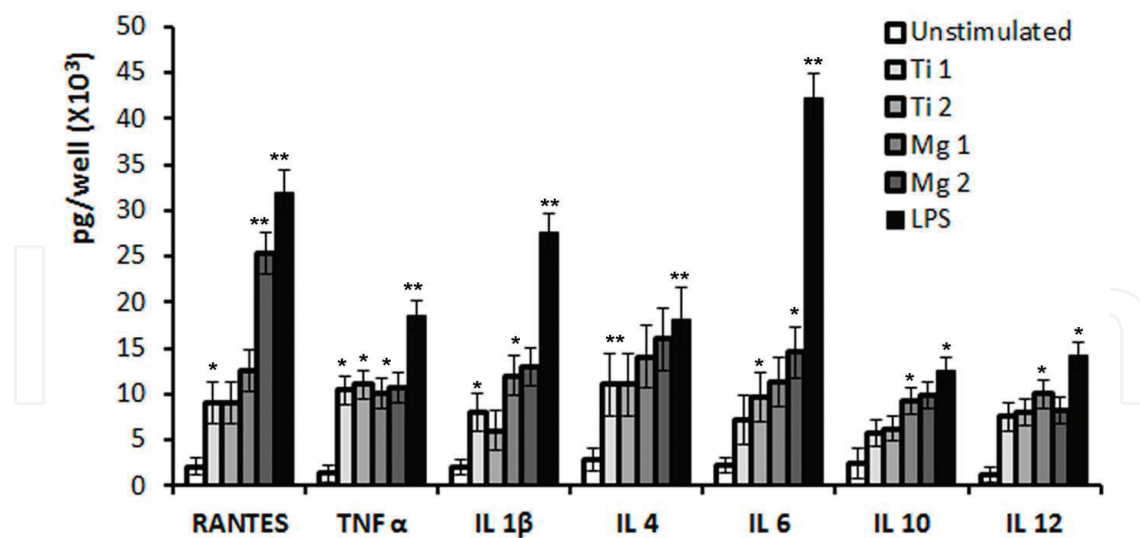
Figure 5. Cytokine release of RMDC treated with samples (Ti 1, Ti 2, Mg 1 or Mg 2) as compared to the immature DC (unstimulated) and maturate DC (LPS-stimulated DC) controls. Supernatant was harvested from each well and examined by cells ELISA. The cytokine amount was normalized to the total cell number in the well. Error bars represent mean ± SD (N = 120) with *p ≤ 0.05 and **p < 0.01 as compared to unstimulated DC.

4. Discussion

During the maturation of the DC, the endocytic potential, the degree of expression of antigen-recognizing receptors decrease, and, on the opposite, the expression of adhesion molecules, which are bound to the plasma membrane, increases. The tolerant properties of highly differentiated DCs in triggering an immune response are manifested not only by their ability to present antigens to lymphocytes, but also by unique migration properties, that allow them to attach antigens to various tissues of the organism and transport them to regional lymphoid organs. In addition to the changes in cell organelles morphology, the activity of lysosomal enzymes and immunoproteas, which process the intracellularly synthesized protein antigens, is increased, the production of pro-inflammatory cytokines and various growth factors, through which T-lymphocytes and connective-tissue cells are activated [1, 10, 34]. These unique properties allow DC to be important participants in the process of bone tissue regeneration as initiators of osteolysis, by activating the differentiation and maturation of osteoclasts. From this point of view, the property of bioresorbable magnesium implant, revealed in our study, has a more pronounced effect, in comparison with bioinert titanium, on the process of directed differentiation and maturation of the DC, which is of a particular interest to us.

The data obtained by us indicate the identical effect of the implants on the expression of the CD34 adhesion receptor of hemopoietic cells, and the percentage of these cells' content, reduced relative to control factor, to their expressed effect as inducers of cell maturation. The cited data indicate a pronounced effect of the magnesium implant and calcium phosphate coated on the directionality of hematopoietic pool cell differentiation, mainly toward the DC, in comparison with titanium. Despite the fact that at the initial time of observation, LPS activated DC maturation more than implants, over time the level of expression of co-stimulatory molecules on the cell surface under the influence of titanium and magnesium has increased. The CD38 receptor

appears on CD34$^+$ committed stem cells and specific progenitor cells of lymphoid, erythroid and myeloid cells. It is considered that CD38 expression persists only in lymphoid progenitor cells during the early stages. The above data indicate the presence of the inducing effect of implants on the maturation of DC, depending on the contact time, and more magnesium than titanium.

The phenotype and morphology of DCs was differentially modulated by metal and calcium phosphate coated surfaces. Specifically, although the expression levels of DC maturation marker, CD83 and HLA-DR were not altered significantly, calcium phosphate coated treatment of DCs induced higher co-stimulatory molecule, CD86, expression relative of iDCs (Unstimulated). DC treatment with Ti 1 did not affect CD86 expression as compared to iDCs, presumably promoting a noninflammatory environment. Was showed that CD86 is the most sensitive marker for DC response to biomaterial treatments and is a valid variable for determining DC maturation levels [31]. Furthermore, DCs contacted with calcium phosphate coated exhibited much more extensive dendritic processes, a morphology associated with maturated. Consistent with the CD86 expression results, DCs incubated with Ti 1 and Mg 1 possessed a rounded morphology that is associated with immatureted. Despite the non-stimulating nature of metal implants cells were able to fully mature upon LPS challenge (data not shown).

The bio-anodized surface contains Ca and P ions incorporated from the electrolytic solution improves biological properties of metal implants [35, 36]. The developed unique electrolyte composition and the formation method helped the creation of biologically active PEO coating on the surface of titanium and magnesium, which might affect the occurrence of aseptic inflammation. The results presented herein that calcium phosphate coated contacted DCs were non-stimulating indicated the importance of surface porosity as a material property that modulates DC phenotype and enzymes activity. Whereas maximum stimulation of cellular enzymes associated with the adhesion on surface within the first hour and a difference between indices for coated titanium with less stimulating effects as compared with titanium. Most expressed stimulation of the cytochrome oxidase in DCs in contact with a hydroxyapatite coated was established. DC in the inflammatory response by regulating cytokines such as nitric oxide and pro- and anti-inflammatory cytokines including the development of inflammation in the tissue surrounding the implant [37]. Cytochrome oxidase and SDH are the main components of the normal aerobic oxidative system of the tissue cells that are also known as the succinate dehydrogenase complex, where SDH is the first component and cytochrome oxidase is the second. Oxidized cytochrome oxidase is reduced by cytochrome c catalyzing the transfer of four electrons to the oxygen molecule. The cytochrome oxidase activity in the cells reflects the level of oxidative metabolism. This enzyme contains cytogemmin with which molecule of NO communicates. In this case, at interaction of super oxygen anion with NO is formed peroxynitrites—the powerful oxidizer capable inhibits activity of mitochondrial enzymes cell. Definition of activity this enzyme allows indirectly estimating ability of cells to production NO on nitrite reductase ways [38]. Reduced cell response upon contact with the coating indicates to better properties with respect to biocompatibility as compared to metal implants. In general, these data indicate to ambiguous reaction of cell in contact with coatings and property of metal.

In addition, DCs contacted with samples surfaces produced differential cytokine profiles. Contrary to the high expression level of CD86 and dendritic morphology, calcium phosphate coated and Mg 1 contacted DCs released higher amounts of anti-inflammatory cytokine,

RANTES, IL 1β, and IL 6, compared to immature DCs or Ti 1 treated DCs. Although some trends in the release of TNF-α and IL-12 were observed, the differences were not statistically significant. A wider array of cytokines and chemokines were subsequently analyzed in order to better delineate the cytokine responses upon DC treatment with Ti surfaces. Treatment of DCs with calcium phosphate coated on Mg promoted enhanced production of the chemokine RANTES of the mediator acute and chronic inflammation, compared to immature DCs, and to a level similar to LPS treated mature DCs.

Unlike titanium the magnesium after a certain time interval in the insertion into the injury site, is resorbed by osteoclasts and other professional phagocytes and the highly-differentiated DCs, already presented at the site of aseptic inflammation, are of a great importance. These cells, due to the fact that they have already acquired the properties of highly specialized antigen-presenting participants in the process of elimination of undesirable components of inflammation, can also have an indirect effect on the process of osteosynthesis by producing a variety of factors, including cytokines, into interstitial space to attract connective tissue cell elements. The relatively low degree of activating effect of the titanium implant on the DC confirms its property of bioinertness in relation to the immune system, which indicates its positive qualities as a material that continuously stays in the organism. DC participates in the inflammatory response by regulating cytokines such as nitric oxide and pro- and anti-inflammatory cytokines. The materials of coating exhibited better biological compatibility than metal implants. The immunomodulatory properties of currently available implant coatings need to be improved to develop personalized therapeutic solutions. DCs exposed to the implantable materials ex vivo can be used to predict the individual's reactions and allow selection of an optimal coating composition, that take prospects for use of this cells for diagnostic and therapeutic approaches to personalized implant therapy.

5. Conclusions

Calcium phosphate-coated surfaces have a very similar chemical composition, but differs a kind of metal substrate with different properties; titanium is bio inert and magnesium is bioresorbable. The comparable levels of CD86 expression for DCs contacted with Ti 2 or Mg 2 surfaces suggested that kind of metal substrate is not crucial in modulating DC phenotype. The calcium phosphate coated surfaces have the same roughness, were prepared to retain their high surface energy by plasma electrolytic oxidation and were treated in the unipolar PEO-mode.

In this study, different of surfaces metal implants and coated were shown to induce differential DC phenotype upon treatment. DCs treated with calcium phosphate surfaces exhibited a more mature phenotype, whereas DCs treated with Ti 1 and Mg 1 surfaces maintained an immature phenotype. These results indicate another benefit of metal surfaces for promoting bone formation and integration by providing a local noninflammatory environment. Furthermore, calcium phosphate surfaces indicated possible material property–DC phenotype relationships for implant design. There is mounting evidence to suggest the involvement of the immune system by means of activation by metal ions released via biocorrosion, in the pathophysiologic mechanisms of aseptic loosening of orthopedic implants.

However, the detailed mechanisms of how metal ions become antigenic and are presented to T-lymphocytes, in addition to how the local inflammatory response is driven, remain to be investigated.

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Conflict of interest

The authors state that there is no conflict of interest.

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