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Effects of beta-tricalcium phosphate particles on primary cultured murine dendritic cells and macrophages

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ABSTRACT

Beta-tricalcium phosphate (β-TCP) is widely used for bone substitution in clinical practice. Particles of calcium phosphate ceramics including β-TCP act as an inflammation mediators, which is an unfavorable characteristic for a bone substituent or a prosthetic coating material. It is thought that the stimulatory effect of β-TCP on the immune system could be utilized as an immunomodulator.

Here, *in vitro* effects of β-TCP on primary cultured murine dendritic cells (DCs) and macrophages were investigated. β-TCP particles enhanced expression of costimulatory surface molecules, including CD86, CD80, and CD40 in DCs, CD86 in macrophages, and MHC class II and class I molecules in DCs. DEC205 and CCR7 were up-regulated in β-TCP-treated DCs. Production of cytokines and chemokines, including CCL2, CCL3, CXCL2, and M-CSF, significantly increased in DCs; CCL2, CCL3, CCL4, CCL5, CXCL2, and IL-11ra were up-regulated in macrophages. The results of the functional assays revealed that β-TCP caused a prominent reduction in antigen uptake by DCs, and that conditioned medium from DCs treated with β-TCP facilitated the migration of splenocytes in the transwell migration assay. Thus, β-TCP induced phenotypical and functional maturation/activation of DCs and macrophages; these stimulating effects may contribute to the observed *in vivo* effect where β-TCP induced extensive migration of immune cells. When compared to lipopolysaccharide (LPS), an authentic TLR ligand, the stimulatory effect of β-TCP on the immune systems is mild to moderate; however, it may have some advantages as a novel immunomodulator. This is the first report on the direct *in vitro* effects of β-TCP against bone marrow-derived DCs and macrophages.

1. Introduction

Calcium phosphate ceramics, including β-TCP and hydroxyapatite (HA), have been widely used for bone substitution in orthopedic, maxillofacial, and dental surgery, as well as tumor resections, because of their excellent biocompatibility and bioactivity. β-TCP has been described as a fully resorbable, biocompatible, osteointegrative, and osteoconductive material [1–6]. HA has also been used to coat the metal parts of prostheses in order to improve biocompatibility. On the other hand, it is well known that these calcium phosphate ceramics also have properties that induce inflammatory cellular responses (reviewed in [7]). Extensive research on these ceramics has shown that their biological effects, including inflammatory mediation, are influenced by physicochemical properties, such as stoichiometry [8], crystallinity [9], solubility [10], surface area [11], rugosity, porosity [12], and granulometry [13].

Materials with inflammatory properties are thought to be adverse for bone substituents or coating materials; however, some researchers, including our group, have shown that these materials could be suitable as immunomodulators [14–16]. In our former study, we demonstrated that β-TCP evoked extensive migration of immune cells in normal C57BL/6 mice, and phenotypically and functionally stimulated cells of a murine monocyte/macrophage cell line, J774A.1 [16]. Furthermore, it has been shown that β-TCP caused anti-tumor effects in a xenograft tumor model of athymic mice injected with human colon cancer cells [17], and that injection of β-TCP enhanced the anti-tumor effect of tumor antigen in a syngeneic mouse tumor model within a prophylactic

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experimental setting [16]. These findings suggest that β-TCP has potential as an immunomodulatory agent or a vaccine adjuvant.

In the present study, the *in vitro* effects of β-TCP on primary cultured murine DCs and macrophages were investigated as the next logical step to our former study using the murine cell line. Here, β-TCP particles up-regulated the expression of costimulatory surface molecules, including CD86, CD80, and CD40 in DCs, CD86 in macrophages, and MHC class II and class I molecules in DCs. In addition, DEC205, C-type lectin-like antigen receptor, and chemokine receptor CCR7 were also up-regulated by β-TCP in DCs. Furthermore, production of cytokines and chemokines, including CCL2, CCL3, and M-CSF, significantly increased in DCs, and CCL2, CCL3, CCL4, CCL5, CXCL2, and IL-11ra were up-regulated in macrophages. The results of the functional assays revealed that β-TCP caused a prominent reduction in antigen uptake by DCs, and that conditioned medium from DCs treated with β-TCP facilitated the migration of splenocytes in the transwell migration assay. These findings indicate that β-TCP induced maturation/activation of DCs and macrophages, and that stimulation of antigen-presenting cells may contribute to the *in vivo* effect observed in our former study, where β-TCP induced extensive migration of immune cells. When compared to lipopolysaccharide (LPS), an authentic TLR ligand, the stimulatory effect of β-TCP on the immune systems is mild to moderate; however, it may have some advantages as a novel immunomodulator. Furthermore, this manuscript is the first report on the direct *in vitro* effects of β-TCP against bone marrow (BM)-derived DCs and macrophages.

2. Materials and methods

2.1. β-TCP preparation

Particles of β-TCP were prepared by Olympus-Terumo Biomaterials (Tokyo, Japan), as previously described [16]. In brief, a fine powdered formulation of β-TCP with a 1–50 μm diameter was prepared by chemical synthesis followed by sintering at 1000 °C for 10 h. Granulometry of the β-TCP particles was analyzed with a laser diffraction analyzer (SALD-2000J, Shimadzu, Kyoto, Japan). Particles had a diameter of 5.373 ± 0.342 μm (mean ± standard deviation), and >90% of particles were 1–20 μm with d50 and d90 values of 4.994 μm and 16.323 μm, respectively. The β-TCP preparation was endotoxin free, and its purity was >99%. Fine powder of β-TCP was subjected to experiments as a suspension in phosphate buffered saline (PBS).

2.2. Reagents

All the reagents for cell culturinge, media, supplements, and fetal bovine serum (FBS) were purchased from Life Technologies (Carlsbad, CA, USA). Recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) were purchased from R&D systems (Minneapolis, MN, USA). Llipopolysaccharide (LPS; ultrapure grade from *E. coli* K12 strain) was purchased from InvivoGen (San Diego, *CA*, USA).

2.3. Mice

Male C57BL/6 (B6) mice of 5–6 weeks old were purchased from CLEA Japan (Tokyo, Japan), and subjected to experiments after a 1–2 week acclimatization period. All mice were housed and used for experiments in accordance with standard ethical guidelines for the care and use of laboratory animals [18], and the study was approved by the Animal Experiment Ethics Committee of the Shizuoka Cancer Center.

2.4. Cell preparation

BM was harvested from femurs and tibiae of B6 mice and filtered through a Falcon 40-μm nylon cell strainer (BD Biosciences, San Jose, CA, USA). Red blood cells present in the BM preparation were lysed with ACK Lysing Buffer (Life Technologies) and the resulting BM cells were used to prepare DCs and macrophages.

BM-derived DCs were generated as previously described [19]. In brief, BM cells were seeded into a 75 cm^2 flask at a density of 3×10^6 cells/mL in RPMI 1640 medium supplemented with 5% FBS and 50 μM 2-mercaptoethanol, and incubated at 37 °C for 1.5 h. After the incubation, non-adherent cells were collected and re-seeded at a density of 1×10^6 cells/mL for 5 days in the presence of 1000 U/mL GM-CSF. $CD11c^+$ cells were enriched from the culture with CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with a resultant purity of >90% $CD11c^+$ cells.

In the preparation of BM-derived macrophages [20], mononuclear cells were fractioned from BM cells by density gradient centrifugation using Lympholyte-M (Cedarlane, Ontario, Canada) and seeded into 25-cm^2 flasks at a density of 3×10^6 cells/mL in the presence of 10 ng/mL M-CSF in EMEM medium supplemented with 10% FBS and 15 mM HEPES. After 24 h, non-adherent cells were harvested, and re-seeded into 75-cm^2 flasks with 10 mL of the same fresh medium with M-CSF. The cells were incubated for 6 days, and then harvested and used as BM-derived macrophages; purity for $CD11b^+$ cells exceeded 90%.

2.5. Stimulation with β-TCP

BM-derived DCs and macrophages were cultured in 12-well or 24-well plates at 1×10^6 cells/mL and stimulated with medium alone, 100 μg/mL β-TCP, or LPS (2 μg/mL in DCs, 1 μg/mL in macrophages, respectively) for 24 h. A dose of 100 μg/mL β-TCP was determined by preliminary experiments (Supplementary Fig. 1), and the same dosage was used in macrophage experiments to facilitate comparison with DC experiments. After stimulation, cells were harvested for flow cytometric analysis and culture supernatants were collected for enzyme-linked immunosorbent assay (ELISA) or *in vitro* migration assay of splenocytes.

2.6. Flow cytometry

Cell surface marker expression, including that of major histocompatibility complex (MHC) molecules and costimulators, was analyzed by flow cytometry. Cells were pre-incubated with Mouse BD Fc Block (BD Biosciences, San Jose, CA, USA) for 5 min to reduce Fc receptor-mediated binding by antibodies of interest. They were then incubated with fluorescent monoclonal antibodies (mAbs) or isotype control antibodies for 15 min at 4 °C and subsequently washed with PBS containing 0.5% bovine serum albumin and 0.01% sodium azide, and analyzed using a BD Accuri C6 flow cytometer (BD Biosciences). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-mouse CD11c (clone HL3), CD11b (M1/70), MHC class I molecule H-2Kb (AF6-88.5) and H-2Db (KH95), MHC class II molecule I-A/I-E (M5/114.15.2), CD86 (GL1), CD80 (16-10A1), and CD40 (3/23) were purchased from BD Biosciences. PE-conjugated anti DEC205 (NLDC-145) -mouse was purchased from Miltenyi Biotec. PE-conjugated anti-mouse F4/80 (CI:A3-1) was purchased from AbD Serotec (Oxford, UK). Isotype control antibodies including fluorescent-labeled hamster IgG1 (G235-2356) and IgG2 (B81-3), rat IgG2a (R35-95) and IgG2b (A95-1), and mouse IgG2a (G155-178) and IgG2b (MPC-11), were purchased from BD Biosciences.

2.7. Analysis of cytokine and chemokine production

The production of cytokines and chemokines in culture supernatants was screened using the Mouse Cytokine Array Panel A kit (R&D systems) according to the manufacturer's instructions. The analytes that showed more than a two-fold increase in signal intensity in β-TCP-treated DCs or macrophages were selected (Supplementary Fig. 2), and their concentration was determined by commercially available ELISA kits (R&D systems). The analytes selected included tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-1ra, M-CSF, chemokine (C-C