Human monocyte-derived macrophages and dendritic cells as targets for biomaterial cytocompatibility studies using an improved *in vitro* culture system

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Since macrophage plays a key role in the biocompatibility process, neoplastic macrophage cell lines and human blood monocytes are commonly used as target cells for *in vitro* biomaterial tolerance evaluation. However, tumor cells profoundly differ from normal tissue cells and monocytes are only precursors of macrophages. It has become possible to generate recently, under adherent-free conditions, fully mature macrophages and dendritic cells from human blood monocytes in the presence of GM-CSF and GM-CSF + IL4 respectively. In the present work, we examined the effects of titanium-alloy on morphology, adhesion, cell phenotype and TNF-α release activity of such differentiated cells grown in hydrophobic teflon bags. Scanning electron microscopy showed that macrophages substantially adhered and spread on titanium-alloy surface throughout the culture period, whereas only a few dendritic cells were adherent. The phenotype of both cell types remained unchanged in the presence of the tested material. However, titanium-alloy stimulated the secretion of TNF-α by the macrophages of some donors. This model of culture may offer new insights into the biomaterial evaluation and may be useful for studying individual responses induced by biomaterials.

1. Introduction

The interactions of host cells with prosthetic implants play a key role in determining their biocompatibility. The wide number of studies [1–23] focused on macrophage responses upon biomaterial stimulation confirms the important role of this cell type in the biocompatibility process. In response to biomaterial stimulation, macrophages have been found to release several mediators as cytokines that modulate the proliferation and the secretory functions of other cell types like fibroblasts and osteoblasts that are also involved in the biological integration of implants [21–23]. However, the use of macrophage cell lines [1–5] or fresh adherent human mononuclear cells [6–20] has made the existing literature difficult to interpret because the relevance of both cell types has not been clearly established. First, tumor cells have a profound altered cellular machinery and consequently, they differ from normal tissue macrophages. Second, adherent mononuclear cells constitute a very heterogeneous cell population containing various numbers of plastic-activated monocytes, the blood precursors of macrophages.

In the last few years, methods have been developed to differentiate adherent mononuclear cells into either functional macrophages [24, 25] or highly specialized antigen presenting cells (APC)-dendritic cells-[26–28] in culture with granulocyte macrophage-colony stimulating factor (GM-CSF) with or without interleukin-4 (IL-4). In a recent study, we have adapted this technique to generate substantial numbers of adherent-free macrophages and dendritic cells from highly purified blood monocytes for experimental and clinical use [29]. In this study, we have used this approach to investigate the effects of titanium-alloy on differentiating macrophages and dendritic cells with respect to morphology, attachment, phenotype and we have demonstrated that macrophages and dendritic cells exhibit different adhesion patterns while maintaining their proper

phenotype in the presence of titanium-alloy. Since the release of inflammatory cytokines is a clinically relevant biocompatibility parameter [21, 30–33], the effects of titanium-alloy on TNF-α release from cultured macrophages have also been analyzed. We have found that this material stimulates TNF-α secretion by macrophages of some donors. Finally, we have reported on the suitability of the model to evaluate biomaterial cytocompatibility *in vitro*.

2. Materials and methods

2.1. Biomaterial preparation

Beads of titanium-alloy containing 6% aluminum and 4% vanadium ($TiAl_6V_4$) ranged from 100 to 200 µm in diameter were supplied by the center Régional d'Innovation et de Transfert de Technologie (CRITT, Charleville-Mézières, France). They are generally used to bond cementless implant stem in view to reduce stem fatigue strength. The material was heat-sterilized by autoclaving (30 min, 121 °C) before use in culture.

2.2. Cell separation

The blood collected from healthy adult donors was submitted to cytapheresis for platelet collection using a Cobe Spectra continuous flow cell separator. The concentrated leukocyte residue was resuspended in phosphate buffer saline (PBS) (Gibco-BRL) without calcium and magnesium. Mononuclear cells were isolated by a 20 min centrifugation at 1000 g in a Cobe 2991 cell processor using a Ficoll Paque of density 1.077 (Eurobio, Les Ulis, France). Monocytes were purified using TS 745 solution (Braun Laboratories, Boulogne-Billancourt, France) in a counter current centrifuge (Beckman JE 6) as described by Lopez *et al*. [34].

2.3. Cell cultures

As previously described [29], human purified blood monocytes were cultured in teflon bags (Tech gen, Les Ulis, France) for the generation of macrophages and dendritic cells. Briefly, macrophages were differentiated in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco-BRL, Cergy Pontoise, France) supplemented with human pooled AB serum (5%) (Institut Jacques Boy, Reims, France), β-mercaptoethanol (3×10^{-5} M) (Gibco-BRL), non-essential amino acids (1%) (Gibco-BRL), sodium pyruvate (100 mM) (Gibco-BRL), penicillin (100 UI/ml) (Gibco-BRL), streptomycin (100 µg/ml) (Gibco-BRL), indomethacin (5×10^{-6} M) (Sigma-Aldrich, Saint-Quentin Fallavier, France) and granulocyte macrophage-colony stimulating factor (GM-CSF) (250 UI/ml) (Pepro Tech Inc., London, UK). To induce dendritic cells differentiation, monocytes were seeded at a cell density of 2.5×10^6 cells/ml in RPMI-1640 supplemented with l-glutamine (2 mM) (Gibco-BRL), heat-inactivated fetal calf serum (FCS) (5%) (Corning Costar, Brumath, France), penicillin (100 UI/ml), streptomycin (100 µg/ml), GM-CSF (800 UI/ml) and IL-4 (500 UI/ml) (R&D systems, Abingdon, UK). All cultures were maintained at 37 °C in humidified atmosphere of 5% CO_2.

2.4. Cell immunophenotyping

Flow cytometry analysis was used for phenotypic characterization. Monocytes were cultured at a cell density of 2.5×10^6 cells/ml in the presence of GM-CSF or GM-CSF + IL-4 with or without titanium-alloy particles (15 mg/ml) for seven days. For membrane immunolabeling, murine monoclonal antibodies (mAbs) directed against leukocyte differentiation antigens CD-1a (Coulter, Margency, France), CD-14 (Dako, Trappes, France), major histocompatibility complex (MCH) class II products HLA-DR (Becton Dickinson, San Jose, CA, USA) and CD-1c (Immunotech SA, Marseille, France) were used according to the manufacturer's instructions. Isotype-matched control antibodies were purchased from Coulter. 100 µl of each cell suspension, used at a density of 1×10^6 cells/ml, were incubated for 20 min at 4 °C with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated-mAbs raised against the antigens CD-1a, CD-14 and HLA-DR. For CD-1c labeling, an unconjugated mAb was used and PE-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG (Immunotech, Margency, France) were used as a second step reagent. Cells were then washed and analyzed using an EPICS XL flow cytometer (Coulter).

2.5. Scanning electron microscopy (SEM)

For the analysis of cell adhesion to titanium-alloy by SEM, monocytes were cultured for 4, 7, 11 and 15 days. The culture medium was replaced with fresh medium every three days when cells were grown more than 7 days. At the end of each culture time, a drop (80 µl) of control macrophage and dendritic cell suspensions and titanium-alloy particles with the adherent cells was placed on a glass slide in a humidified chamber at 37 °C in a 5% CO_2 atmosphere for 15 min. Samples were rinsed in PBS and fixed for 2 h in a 2.5% glutaraldehyde-PBS solution at 4 °C. They were then rinsed in PBS, dehydrated in graded alcohol series before being transferred into acetone and critical point dried. After coating with a layer of palladium gold (Jeol, Ion Sputter, I.F.C. 1110), specimens were examined under a Jeol 5400 LV SEM.

2.6. TNF-α assay

For cytokine measurement, 6 day-cultured macrophages were washed with fresh medium and incubated at a density of 1×10^6 cells/ml for 48 h in the presence of titanium-alloy particles. Cells cultured in medium alone served as negative control (non-stimulated cells). All supernatants were centrifuged to remove cell debris and material particles, and were aliquoted and stored at − 80 °C until the analysis.

Levels of TNF-α were determined using quantitative colorimetric sandwich ELISA kits (R&D Systems Europe Ltd, Abington, UK). The sensitivity of this cytokine assay was about 4.4 pg/ml and the detection limits were ranged from 15.6 to 1000 pg/ml. Stutent *t*-test ($p = 0.05$) was used to compare differences between non-stimulated cells and titanium-alloy treated macrophages TNF-α production.

3. Results

3.1. Titanium-alloy particles do not affect macrophage and dendritic cell differentiation

Freshly purified human blood monocytes were cultured in the presence of GM-CSF or GM-CSF + IL-4 for macrophage and dendritic cell generation respectively. After 7 days of culture with or without titanium-alloy, we analyzed the cell phenotype using flow cytometry. Results are represented as overlay histograms in Fig. 1. In comparison with freshly purified monocytes, control macrophages up-regulated CD-14 and HLA-DR whereas they remained negative for CD-1a and CD-1c. No significant alteration of this phenotype was observed in the presence of $TiAl_6V_4$. Control dendritic cells expressed CD-1a, CD-1c, HLA-DR, down-regulated CD-14 and maintained this phenotype in the presence of titanium-alloy.

3.2. Unlike dendritic cell, macrophage strongly adhere to titanium-alloy surface

SEM observations of the interface between monocyte-derived cells and $TiAl_6V_4$ were performed at the 7th day of culture. Control macrophages showed prominent cytoplasmic ruffles (Fig. 2A) whereas in presence of titanium-alloy particles macrophages flattened, overlapped with maximal cytoplasmic spreading, and subsequently formed a confluent cell layer on the material surface (Fig. 2B and C). Control dendritic cells exhibited many thin filopodia (Fig. 2D) and were much less adherent and spread on the material surface than macrophages and were connected together with their cytoplasmic extensions (Fig. 2E and F).

Kinetics of cell density on $TiAl_6V_4$ were analyzed by SEM after 4, 7, 11 and 15 days of culture (Fig. 3). On the 4th day of culture, we observed sparsely adherent macrophages on $TiAl_6V_4$, among which some were flattened (Fig. 3A). The density of adherent macrophages and the extent of cell spreading sharply increased on day 7 (Fig. 3B) and then showed a marked decrease on days 11 and 15 (Fig. 3C and D). At this stage, cells were characterized by an altered cytoplasmic membrane. The kinetic study of dendritic cell cultures confirmed their weak adhesion capacity; only a small number of dendritic cells adhered on the material on day 7 (Fig. 2E and F) while virtually no adherent dendritic cells were found on titanium-alloy surface on days 4, 11 and 15.

3.3. Titanium-alloy particles stimulate TNF-α production by cultured macrophages of some donors

We further investigated whether macrophage-$TiAl_6V_4$ interaction modulates the secretion of TNF-α, a pro-inflammatory cytokine. As shown in Fig. 4, spontaneous release of TNF-α by control cells considerably varied (43 ± 16 to 1272 ± 240 pg/ml) among the six healthy donors tested. For 2 out of 6 donors, the material induced

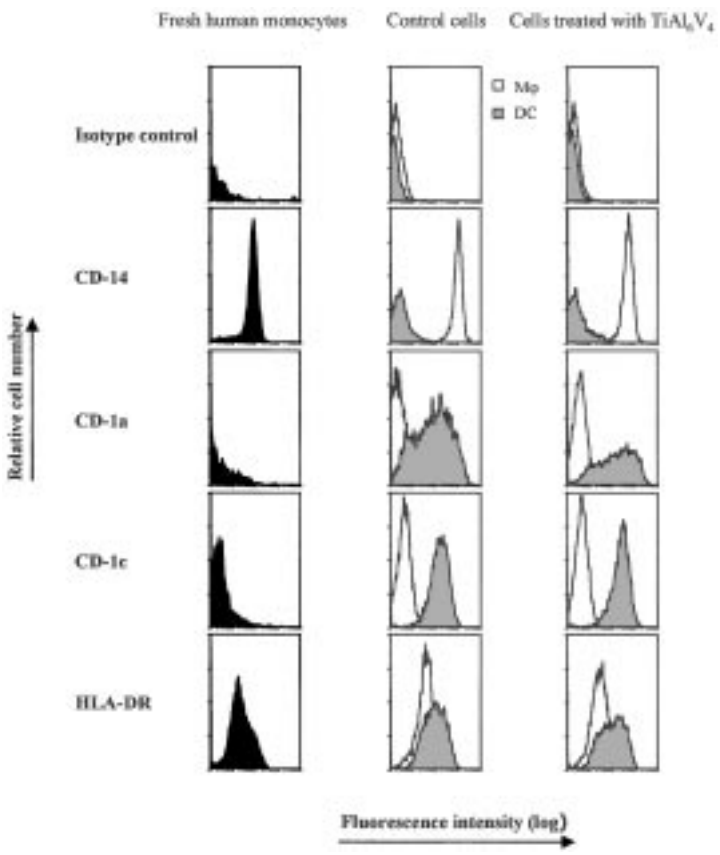

Figure 1 Surface antigen expression by macrophages and dendritic cells grown with and without $TiAl_6V_4$ particles. Representative overlay of a selected panel of surface markers are shown for freshly purified human blood monocytes (black areas), for cells cultured with GM-CSF + IL4 (gray areas) or with GM-CSF alone (white areas) in the presence and absence of titanium-alloy.

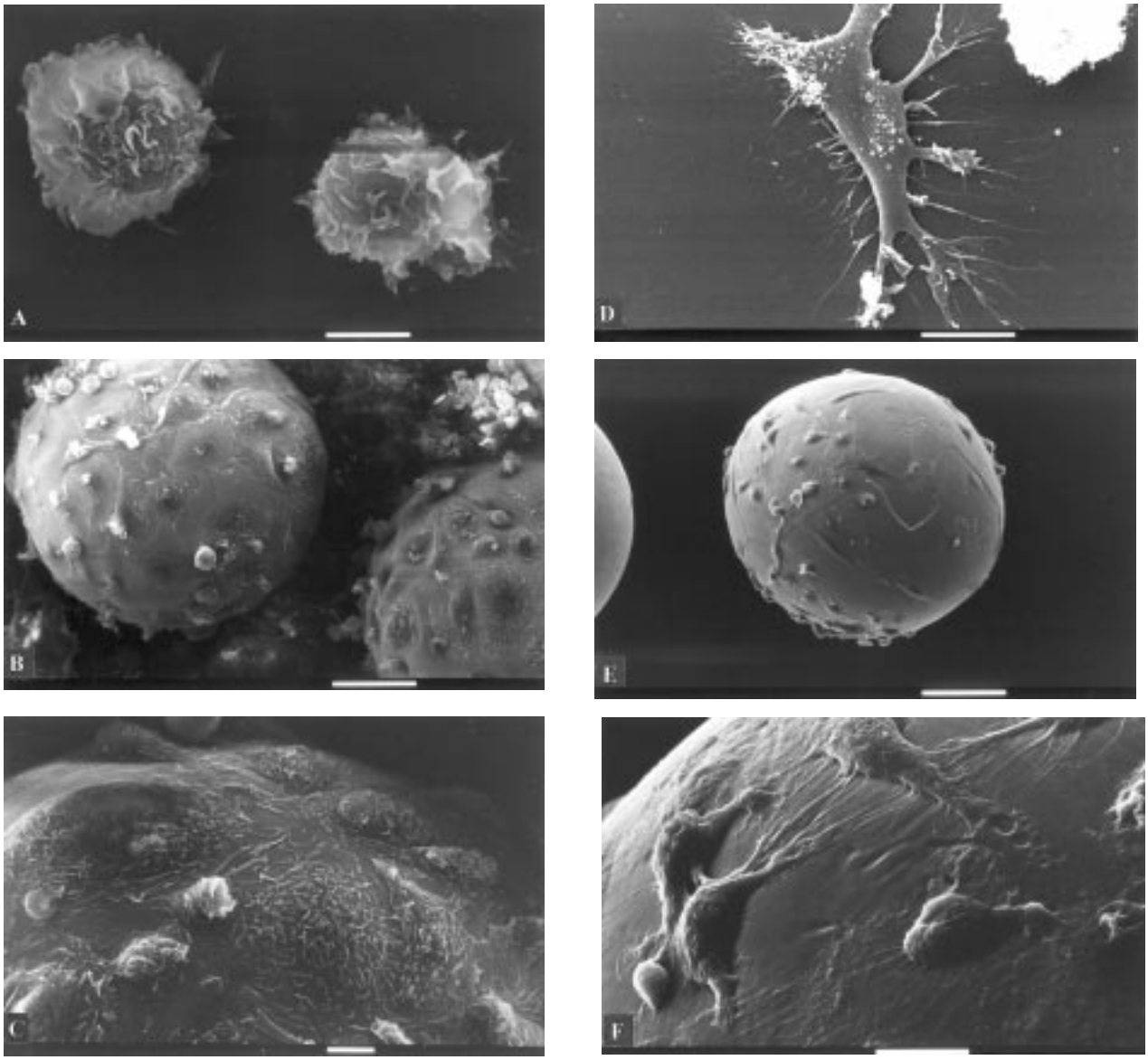

Figure 2 Scanning electron micrographs of human monocyte-derived cells cultured for 7 days with or without $TiAl_6V_4$ particles. Macrophages (A) displayed prominent ruffles and strongly adhered on titanium-alloy (B, C). Dendritic cells (D) processed filopodial extensions and were much less adherent on the substratum (E, F). Bars represent 10 μm A, C, D and F, 50 μm in B and E.

a 3–7 fold increase of TNF-α production that was not consistently altered by the presence of titanium-alloy in the other cases.

4. Discussion

Established cell lines with macrophage properties have been used for biocompatibility studies [1–5], because they provide a continuously available source of cells with uniform biological properties. However, these cells have a profound altered cellular machinery that subsequently may produce altered cellular responses. For example, unlike normal macrophages, the *in vitro* cloned cell line P388D1 from murine lymphoid neoplasm tumor does not secrete T cell activating factor [35] and does not respond to either the complement chimiotactic product C5a nor to lymphocyte-derived chemotactic factor [36]. Thus, cell lines do not appear as the best targets for *in vitro* studies, and data obtained using these cells should be completed.

In regard to this problem, non-tumor human blood monocytes are used as alternatives [6–20], but monocytes commonly obtained by plastic adherence constitute an heterogeneous population of mononuclear cells that contain about 35–45% monocytes and varying numbers of lymphocytes and NK cells [16, 37]. Besides, freshly isolated or short-term cultured monocytes have not yet matured and lack the morphological, phenotypic and antigen capture ability properties of fully differentiated macrophages [29]. Furthermore, the need of a plastic adherence step for the isolation of monocytes may alter the cell physiology [38–40] and this can subsequently modify their responses to other stimuli. Finally, cell detachment involved in some culture procedures may represent a cell stress, since it may result in decreased cell viability [41]. These results taken together made difficult the interpretation of data obtained from adherent monocytes.

The need for a more realistic cellular model to study the biocompatibility of biomedical materials prompted us to use improved methods of culture that allow *in vitro* macrophage and dendritic cell generation [24–29] including: (1) highly purified human blood monocytes

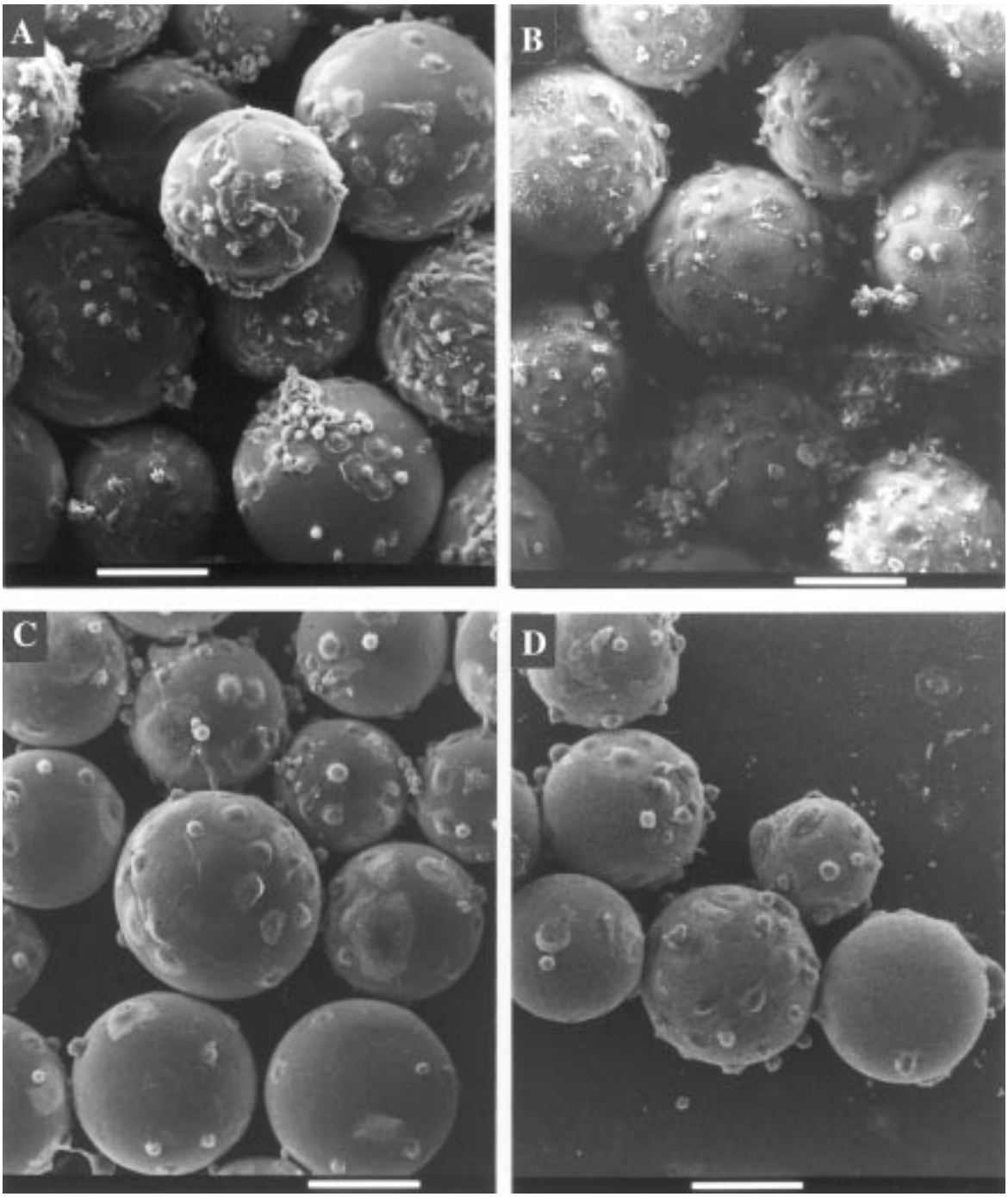

Figure 3 Behavior of human macrophage morphology on titanium-alloy surface after 4, 7, 11 and 15 days of culture. (A) Note rosettes formed by macrophages and $TiAl_6V_4$ beads after day 4 of culture. Seven-day-old cultures (B) had a higher density of adherent macrophages with maximal cytoplasmic spreading. After 11 days (C) and 15 days (D) of culture, SEM showed a low density of attached and spread macrophages with numerous necrotic cells. Bars represent 100 μm.

as cell source; the purification of monocytes from peripheral leukocytes collected by apheresis by counter current elutriation allowed the recovery of $90 \pm 2\%$ (2) hydrophobic teflon bags as culture support that prevented the adherence of cells, which were subsequently investigated without step of trypsinization; (3) cytokines as paracrine factors for long-term culture and full differentiation of macrophage and dendritic cell. The main point of this approach is the use of macrophages and dendritic cells that share functional and phenotypic characteristics of interfacial cells between the body and foreign substances [26, 28].

Titanium-alloy was selected as a model biomaterial for our study, because it represents a reference material widely used in implantology. We examined its interactions with macrophages and dendritic cells, and we showed that titanium-alloy offered a favorable surface for adherence of macrophages, that flattened on material with a pronounced cytoplasmic spreading, as phagocytes do when they undergo phagocytosis of too large particles. Furthermore, the increased macrophage density on titanium-alloy surface and the extent of cell spreading between 4–7 days suggest that the adherence of cells depend on the stage of cell differentiation. Interestingly, only a few dendritic cells adhered to the biomaterial in spite of the known high adhesiveness of titanium. Adherent dendritic cells did not spread but maintained their characteristic filopodia extensions. The low

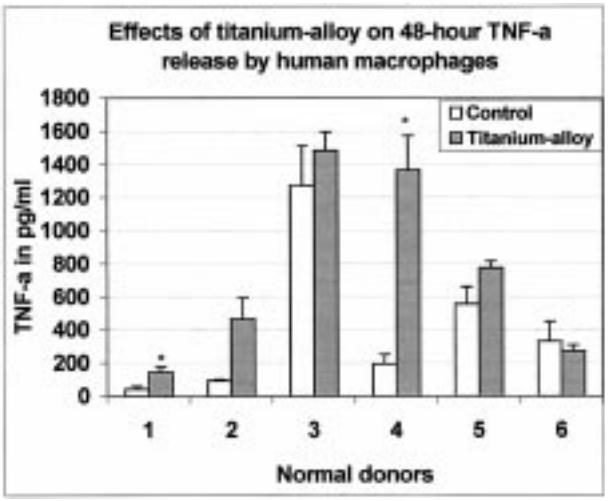

Figure 4 TNF-α release by age-matched healthy volunteers cultured macrophages. Tumor necrosis factor-α (TNF-α) was measured by ELISA in 48 h culture supernatants harvested from human macrophages cultured alone (control) or with titanium-alloy particles. Results represent the mean + SD of two independent duplicate experiments. *, significantly different from control cells cultured without titanium-alloy; $p = 0.05$ determined using Student *t*-test.

percentage of adherent dendritic cells suggests that monocytes presumably differentiate into various subsets of dendritic cells with different adhesion capacities.

Dendritic cells have been previously characterized by a very weak plastic adherence ability. Although most of them failed to attach on titanium substratum, dendritic cells may be involved in the complex process of biocompatibility, since they have been identified in sites of inflammation, such as rhumatoid arthritis synovial fluid and tissue where they play an important role in perpetuating T cell-mediated inflammation characteristics of rhumatoid arthritis [43–46]. Because only one study [47] to our knowledge has identified dendritic cells type in periprosthetic inflammed tissue, it should be of great interest to complete this unique work by elucidating the role of dendritic cells in the surrounding tissue of loosened implants. Therefore, in agreement with the other previous investigations [26–29], the possible approach to generate *in vitro* sizable numbers of dendritic cells described here offers new insights in a biomaterial cytocompatibility study. For example, the effects of various biomaterials in the induction of T cell responses, including mixed leukocyte reactions and immune cytokines-chemokines release, may contribute to a better understanding of some implant failures.

In this study, the release of TNF-α by cultured macrophages was consistently variable according to monocyte donors. We found a 3 to 7 fold stimulation of TNF-α release by large sized beads of $TiAl_6V_4$. In agreement with Rogers *et al*. [18], we attributed some of the variations of mediator concentrations to errors in culture conditions and cytokine measurement, but most of them were due to differences in response between donors, since our data were reproducible in two separate experiments. This suggests that the cellular responses to biomaterials vary with different donors. Therefore, the variability of data obtained by using primary cell cultures from different donors generally considered as a disadvantage may account for individual responses that perhaps can explain some chronic inflammatory reactions around loosened devices. The use of human normal cultured macrophages and dendritic cells for biocompatibility *in vitro* tests may be more appropriate than cell lines to mimic the responses found in periprothetic tissues.

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