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Melanoma Induces Immunosuppression by Upregulating FOXP3+ Regulatory T Cells[1]

Joel Baumgartner, M.D.[*], **Cara Wilson, M.D.**[†], **Brent Palmer, Ph.D.**[†], **Don Richter, M.S.**[*], **Anirban Banerjee, Ph.D.**[*], and **Martin McCarter, M.D.**[*]

[*] *Department of Surgery, University of Colorado at Denver and Health Sciences Center, Denver, Colorado*

[†] *Department of Medicine, Division of Immunology, University of Colorado at Denver and Health Sciences Center, Denver, Colorado*

Abstract

Background—The immune response to melanoma is rarely curative suggesting the emergence of immunosuppression. FOXP3-expressing regulatory T cells (T_{reg} cells) function to suppress immune responses. The objective of this study was to determine if melanoma evades immune surveillance, in part, by inducing T_{reg} cells.

Material and methods—Peripheral blood mononuclear cells (PBMCs) were isolated and exposed to melanoma-conditioned media (MCM) or control media for one week. The induction of T_{reg} cells in these PBMCs was determined by measuring the proportion of $CD25^+FOXP3^+$ T cells in all $CD4^+$ T cells by flow cytometry. FOXP3 expression was determined by mean fluorescence intensity (MFI) and Western blot. Supernatant cytokines were determined by ELISA.

Results—Normal PBMCs exposed to MCM revealed higher proportions of T_{reg} cells than those exposed to control media after six days (3.4% v. 1.3%, respectively, $P < 0.02$). The expression of FOXP3 in T_{reg} cells from PBMCs exposed to MCM increased over time by MFI and Western blot but was not significantly different than those exposed to control media. The level of IL-10 and TGF-β in supernatants after six days growth was higher in MCM than control media but this did not reach statistical significance.

Conclusion—Exposure of PBMCs to melanoma results in induction of $FOXP3^+$ T_{reg} cells.

Keywords

melanoma; regulatory T cells; FOXP3; cytokines

Introduction

Melanoma possesses the ability to elicit a profound immune response, as demonstrated by spontaneous regression of primary and metastatic disease [1–3]. The critical components of this immune response have been extensively investigated and exploited for immunotherapy in the form if IL-2, INF-α, tumor vaccines and adoptive cell transfer [4–8]. While some patients experience tremendous regression of their disease with these treatments, most immune

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Correspondence: Martin McCarter, M.D., University of Colorado Health Sciences Center, 4200 E. 9th Ave C311, Denver, CO 80262, martin.mccarter@uchsc.edu, (303) 315-8671 (office), (303) 315-5527 (fax).

therapies ultimately fail suggesting the development of a counter-regulatory immune suppressive mechanism.

Regulatory T cells (T_{reg} cells) are an immunosuppressive population of T cells expressing high levels of CD25 (IL-2Rα), constituting 2–3% of the total $CD4^+$ T cell population in the CD25 high group [9]. T_{reg} cells provide peripheral immune tolerance in normal individuals and elimination of T_{reg} cells results in severe autoimmunity [10]. Although the precise mechanism of T_{reg} cell-induced immune suppression is not understood, T_{reg} cells act in part by secreting immunosuppressive cytokines and suppressing the activation and proliferation of T cells in both cell contact-dependant and contact-independent mechanisms [11]. Increased frequencies of peripheral T_{reg} cells have been reported in a variety of malignancies including lung, breast, pancreatic, gastric, esophageal, colorectal, gallbladder, ovarian and cervical cancers and they may partially suppress the antitumor immune response in these tumors [12–16].

FOXP3 is a transcription factor in the forkhead/winged-helix family of transcriptional regulators and is predominantly found in $CD4^+CD25^+$ T_{reg} cells. Mutations in FOXP3 in humans lead to severe autoimmunity in the form of IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked), a lethal multisystem autoimmune disease characterized by a variety of mutations along the full length of the FOXP3 gene resulting in elimination of T_{reg} cells [17]. FOXP3 expression is both necessary and sufficient for the development and function of T_{reg} cells, making it one of the most T_{reg} cell-specific markers [18,19].

Given our understanding of the immunosuppression associated with melanoma and the growing understanding of the activity of T_{reg} cells, we hypothesized that melanoma directly induces T_{reg} cells, as defined by the $CD4^+CD25^+FOXP3^+$ phenotype. We sought to determine if melanoma could induce T_{reg} formation in normal peripheral blood mononuclear cells (PBMCs) in an in vitro system. To do this, we exposed normal PBMCs to melanoma-conditioned media. We also investigated possible mechanisms of T_{reg} induction and activity, including FOXP3 expression and immunosuppressive cytokine secretion.

Materials & Methods

Cells and Culture Conditions

Peripheral blood was collected from healthy human volunteers without a known history of melanoma, and the PBMC fraction was isolated using a Ficoll-Paque density gradient (Amersham Biosciences AB, Uppsala, Sweden) following an institutional review board-approved protocol. After three washes with Dulbecco phosphate-buffered saline (DPBS) the cells were grown in various media. Control media consisted of Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 10% human serum. Melanoma-conditioned media (MCM) was generated by bringing a metastatic melanoma cell line (A375; American Type Culture Collection, Manassas, VA) to 80–90% confluence in control media, removing the media and washing the cells with DPBS, adding fresh control media, and collecting the conditioned media 24 hours later. This media was then centrifuged at 350*g*, passed through a 0.22μm filter and stored at −80°C until used. Additional melanoma-conditioned media was created using other melanoma cell lines. For use in experiments, the MCM was diluted to 20% in control media to ensure the presence of adequate levels of growth factors and micronutrients. Isolated PBMCs were incubated in a 24 well plate (Costar, Corning, NY) at 2×10^6 cells per well in control media or MCM at 37° C in 5% CO_2.

Cell Staining and Flow Cytometry

After incubation in media for up to one week, cells were harvested, washed with DBPS and stained with murine allophycocyanin (APC)-labeled anti-human CD3, tricolor (TC)-labeled anti-human CD4 (Invitrogen, Carlsbad, CA) and fluorescein isothiocyanate (FITC)-labeled anti-human CD25 (Beckman Coulter, Inc., Fullerton, CA) antibodies by incubating them for 15 minutes at room temperature. The cells were then washed and fixed using Fixation/Permeabilization solution purchased from eBioscience (San Diego, CA) according to the manufacturer's instructions. The cells were then incubated for 15 minutes at 4°C with normal rat serum and permeabilization buffer (eBioscience) to prevent nonspecific binding with Fc receptors before incubation with rat phycoerythrin (PE)-labeled anti-human FOXP3 antibody (eBioscience) for 30 minutes at 4°C. Cells were then washed and resuspended in 1% paraformaldeyde (PFA) in DPBS and stored at 4°C in the dark until flow cytometric analysis was performed. Two hundred thousand events were collected by flow cytometry on a FACSCaliber flow cytometer (Beckton, Dickinson and Company, Franklin Lakes, NJ) with gating based on forward versus side scatter for live lymphocytes, then on CD3+/CD4+ for $CD4^+$ T cells, then on CD25+/FOXP3+ for T_{reg} cells in which the mean fluorescent intensity (MFI) of FL2 (FOXP3-PE channel) was determined. The gating and calculation of the proportion of T_{reg} cells and MFI of FOXP3 was performed using FlowJo software (Tree Star, Inc., Ashland, OR).

Western Blot Analysis

Total cell lysates were extracted from cultures of PBMCs grown for various days in control media versus MCM. Lysates were solubilized in tris-buffered saline Tween (TBST) and boiled at 100°C for 5 minutes before undergoing SDS-polyacrylamide gel electrophoresis (PAGE) with equal volumes of cell lysate per lane. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) which was blocked with 5% nonfat dry milk in TBST overnight at 4°C. Immunostaining was performed with 1μg/ml rabbit anti-human FOXP3 antibody for 1 hour at room temperature and with 1μg/ml goat anti-rabbit horseradish peroxidase conjugated secondary antibody before development with the appropriate detection kit according to the manufacturer's instructions (Abcam, Inc., Cambridge, MA). Membranes were also probed for GAPDH as additional loading control.

In Vitro Cytokine Analysis

After six days in culture, levels of the supernatant cytokines TGF-β, IL-2, IL-4 and IL-10 were assayed by ELISA (ELISATech, Denver, CO).

Statistics

The student's *t*-test was used to compare the percentage of T_{reg} cells and the MFI of FOXP3 in control media versus MCM. Observations on cytokine data between the two groups was also compared by the student's *t*-test. JMP software (Cary, NC) was used for all calculations, with a $P < 0.05$ deemed statistically significant.

Results

Increased T_{reg} Cells in Melanoma-Conditioned Media versus Control Media

Normal PBMCs cultured in MCM demonstrated an increased proportion of T_{reg} cells over time compared to control media. Fig. 1 depicts the gating strategy used to identify $CD4^+CD25^+FOXP3^+$ T_{reg} cells. The percentage of $CD25^+FOXP3^+$ T_{reg} cells of all $CD4^+$ T cells increased in MCM and went down in control media over time (Fig. 2A, representative time-course experiment), while the MFI of FOXP3 in T_{reg} cells increased after day two in both conditions over time. After six days, the proportion of T_{reg} cells was significantly higher in

PBMCs cultured in MCM versus control (3.4% v. 1.3%, respectively, *$P < 0.02$; Fig. 2B). The expression of FOXP3 in T_{reg} cells, as measured by the MFI in the T_{reg} gate, was not significantly different in PBMCs grown in MCM versus control media after six days (468 units v. 344 units, respectively, $P = 0.29$). The induction of FOXP3 by MCM on normal PBMCs over time was confirmed by Western blot (Fig. 3).

Cytokine Production

Supernatant cytokine production from normal PBMCs grown in MCM or control media is shown in Fig. 4. Although there appears to be more TGF-β and IL-10 in the MCM group compared to control media, this did not reach statistical significance (3410 pg/ml v. 2862 pg/ml, respectively for TGF-β, $P = 0.70$; 114 pg/ml v. 62 pg/ml, respectively for IL-10, $P = 0.37$; Fig. 4). Levels of IL-2 and IL-4 were undetectable in all supernatants tested.

Discussion

In this study, we have demonstrated that melanoma induces immunosuppressive $FOXP3^+$ regulatory T cells. Specifically, this study shows the ability of melanoma-conditioned media to result in a higher proportion of T_{reg} cells compared to control media. The method of induction remains unclear and may involve stimulation or activation of T_{reg} cells already present, induction of de novo T_{reg} cells or expansion of the existing population of T_{reg} cells. FOXP3 expression as determined by Western blot and MFI also seemed to increase within T_{reg} cells over time in MCM, though the difference in MFI from PBMCs grown in control media did not reach statistical significance.

The method by which MCM induces T_{reg} cells remains to be elucidated. This induction may involve interactions with antigen-presenting cells or with other T cells. In addition, other investigators have identified soluble factors in a variety of malignancies implicated in inducing T_{reg} cells, including TGF-β, CCL22 and H-ferritin [20–22]. Furthermore, the induction mechanism within T_{reg} cells is poorly understood, but may involve a FOXP3-dependant pathway. One proposed activity of FOXP3 in T_{reg} induction is as a transcriptional repressor of the IL-2 gene through interactions with nuclear factor of activated T cells (NFAT) [23]. Additional questions regarding the development and upregulation of T_{reg} cells remain to be answered. Specifically, does exposure to melanoma pre-condition existing T_{reg} cells for later induction or influence naïve lymphocytes to become T_{reg} cells after additional tumor exposure? We surmise that further exposure of PBMCs from melanoma patients to melanoma either in vitro with conditioned media or in vivo with advanced disease would result in enhanced induction of T_{reg} cells. Further investigation of MCM may reveal induction mechanisms.

We have also shown a trend of immunosuppressive cytokines secreted by PBMCs in response to melanoma exposure. The precise origin of these cytokines in culture is unclear, but T_{reg} cells are a potential source as they have been shown to secrete IL-10 and TGF-β in tumor models [24]. Thus, either directly from T_{reg} cells or from other PBMCs affected by T_{reg} cells, these results reveal a potential mechanism of T_{reg}-induced immunosuppression. Other possible mechanisms include cell-contact dependant interactions or antigen specific responses as found in other model systems [25]. Future inquiries will investigate more precisely the cytokine expression profile of melanoma-induced T_{reg} cells and their suppressive activity on effector cells.

Although we found that media conditioned by melanoma resulted in an increased percentage of T_{reg} cells in normal PBMCs over control media, T_{reg} cells are induced by other conditions as well. In addition to induction by cancer cells, other in vitro studies with benign cells have shown the capacity to induce T_{reg} cells [26]. In addition, they have been found to be induced by inflammation resulting from a large variety of disease states [27]. Thus, given the role of

T_{reg} cells to confer natural tolerance which occurs to prevent autoimmunity, T_{reg} cells mitigate the immune response in many disease states, including malignancy.

Other investigators have demonstrated an increased proportion of T_{reg} cells in lymph nodes with metastatic melanoma [28]. Furthermore, the failure of an objective clinical response in patients undergoing immune therapies has been mechanistically linked with elevated percentages of T_{reg} cells in these patients [29,30]. Although the percentage of T_{reg} cells was relatively small in our study (ranging from 1.3% to 3.7% of $CD4^+$ T cells), T_{reg} cells are quite active in small concentrations and have been shown to inhibit cellular proliferation and cytokine secretion in effector cells in in vitro studies at concentrations as low at 3% of $CD4^+$ T cells [31]. In addition, the immunosuppressive activity of T_{reg} cells follows a dose response curve and small increases in their concentration greatly enhance their immune suppression [31]. These findings, taken together with the results of our study, point toward a mechanism of immunosuppression in melanoma involving T_{reg} cells.

We have shown that melanoma has the potential to mitigate the antitumor immune response by the induction of T_{reg} cells. As much of tumor immunity is generated from the expression of self-antigens, T_{reg} cells may act in melanoma to suppress tumor-induced immunity. A better understanding of the mechanisms by which melanoma evades the immune response is crucial to developing more effective immunotherapies, as most have not resulted in durable clinical responses. Currently, clinical trials are underway or proposed to investigate the control of the T_{reg} response by their elimination or blockade to subvert tumor-induced immunosuppression by using anti-CD25 antibodies, anti-CTLA-4 antibodies, IL-2-toxin chimeric proteins, glucocorticoid-induced TNF-like receptor (GITR) ligands and CD123/OX-40 ligands [32]. In addition to the therapeutic role of T_{reg} cell elimination, they may serve as a diagnostic or prognostic marker for melanoma patients, as has been shown with other tumors [33].

In summary, our results support the hypothesis that melanoma evades the immune response, in part, by induction of immunosuppressive $FOXP3^+$ regulatory T cells from normal PBMCs. This may be through induction of FOXP3 and the T_{reg} cells may subsequently act partly through secretion of immunosuppressive cytokines. Further study is warranted to understand the mechanistic details of the induction and activity of T_{reg} cells in melanoma.

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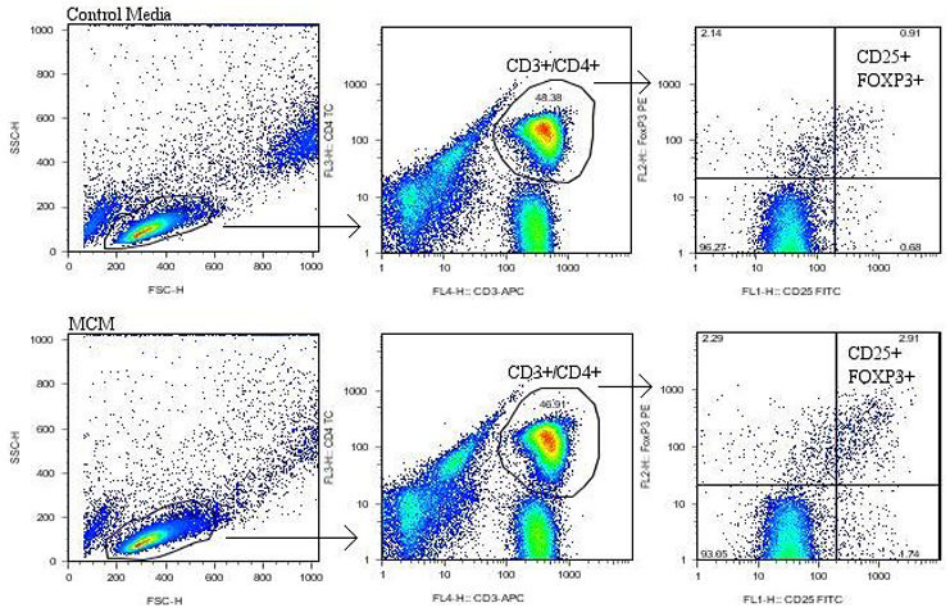
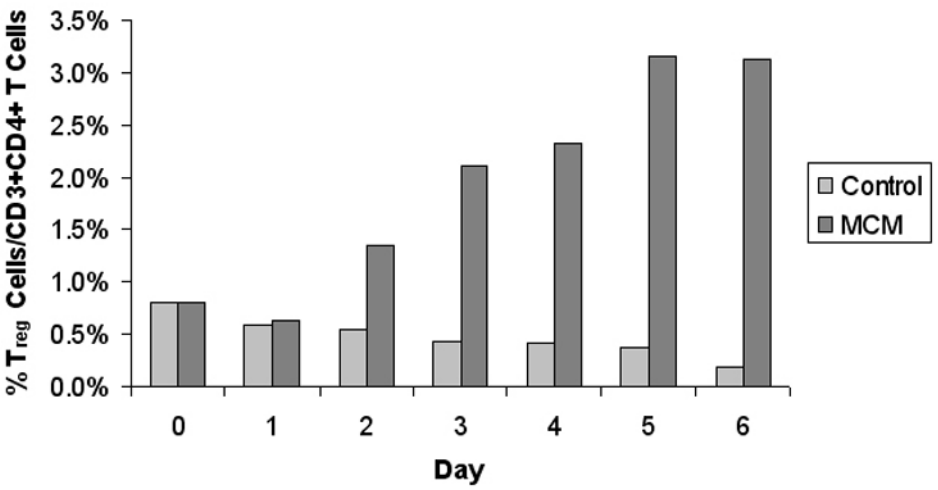


FIG. 1.

Gating strategy used for $CD4^{+}CD25^{+}FOXP3^{+}$ T_{reg} cells. Representative examples of flow cytometry plots and gating strategies of T_{reg} cells from PBMCs grown in control media and MCM. T_{reg} cells were those in the CD25+/FOXP3+ double-positive quadrant after gating for live lymphocytes and CD3+/CD4+ T cells, respectively. The quadrant labels showed the proportion of single or double-positive cells.

A



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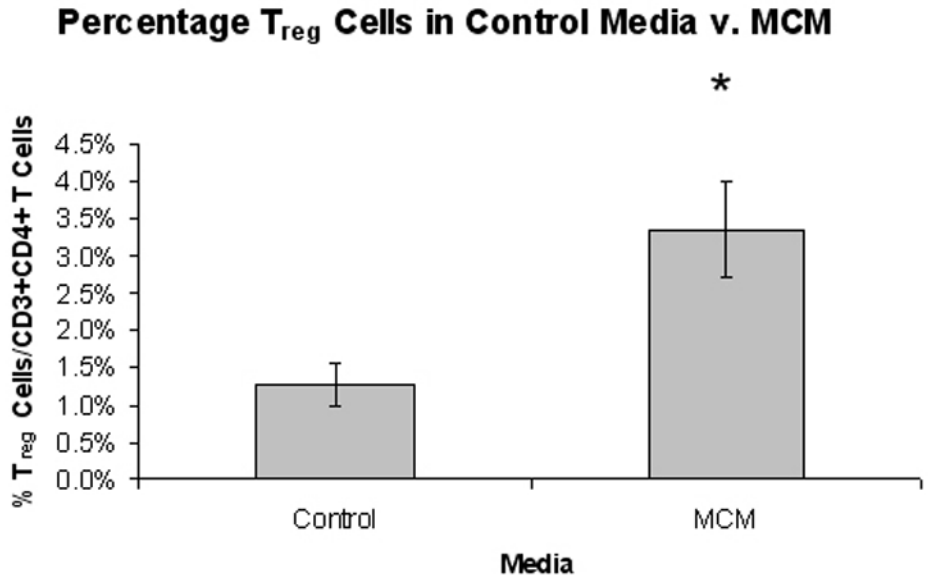

FIG. 2.

Proportion of T_{reg} cells from normal PBMCs in MCM versus control media (n = 5). (A) Representative graph of the percentage of CD25+/FOXP3+ T_{reg} cells in control media versus MCM over time. (B) There was a significantly higher percentage of T_{reg} cells in MCM versus control after six days (3.4% v. 1.3%, respectively, *$P < 0.02$). Graph represents mean values ± SEM of the percentage of $CD25^{+}FOXP3^{+}$ T_{reg} cells in $CD3^{+}CD4^{+}$ T cells from PBMCs grown in control media versus MCM after six days.

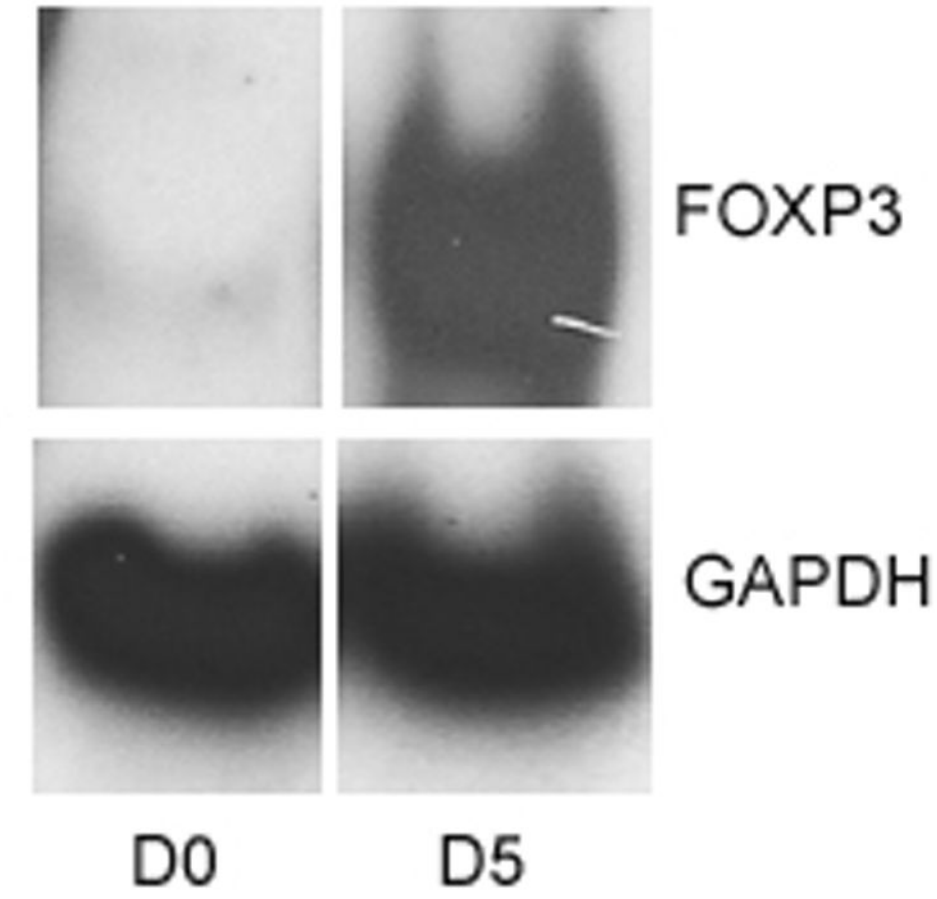

FIG. 3.
MCM increases expression of FOXP3 with time. Typical Western blot analysis showed increasing amounts of FOXP3 expression in PBMCs over time cultured in MCM. Lanes were loaded with equal volume of cell lysate and probed for GAPDH as loading controls.

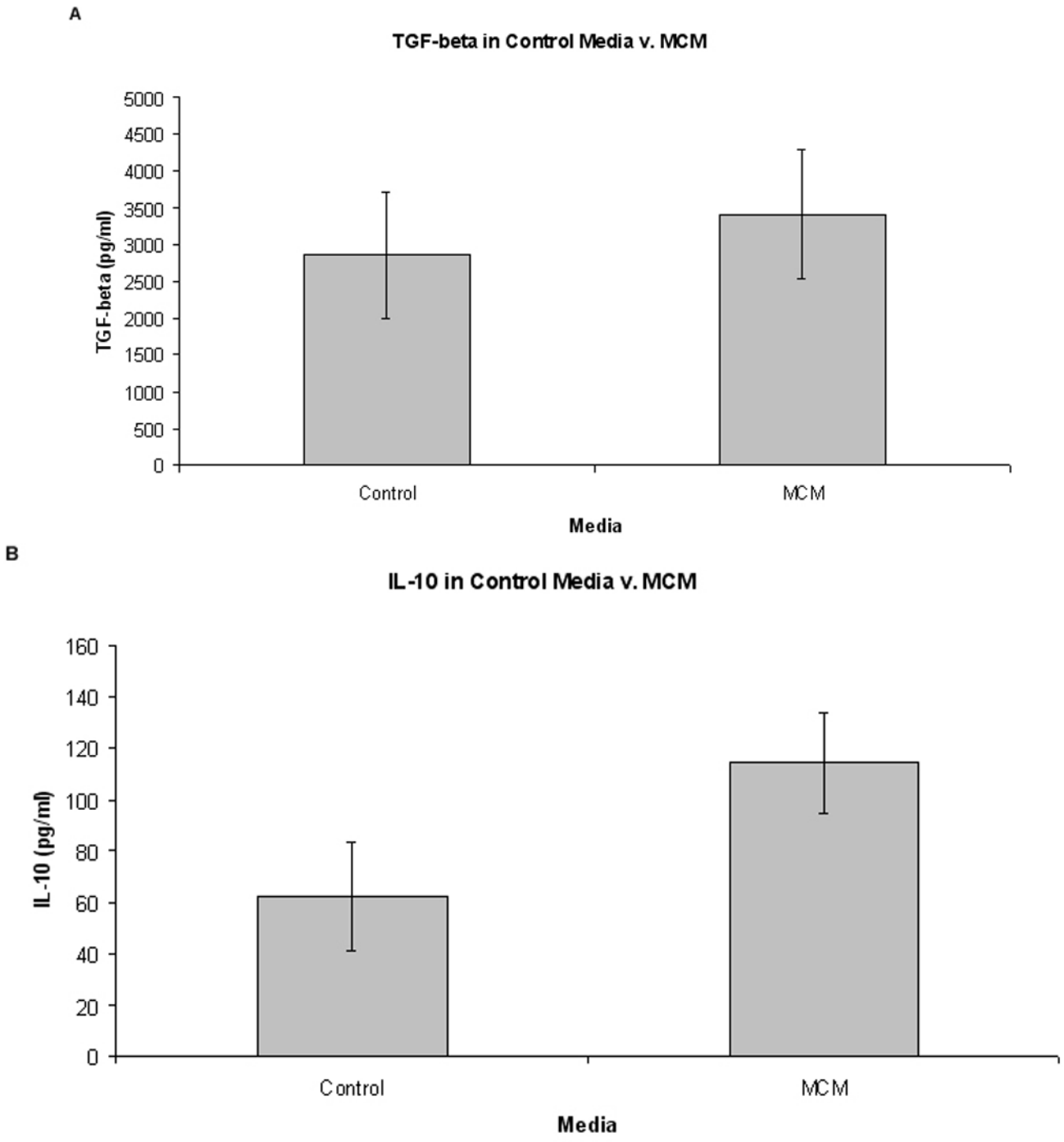

FIG. 4.

Cytokine levels in control media versus MCM after six days growth (n = 6). Graphs represent mean values ± SEM of levels of TGF-β and IL-10 as determined by ELISA. (A) There was an increased level of TGF-β in supernatants from PBMCs grown in MCM versus control media after six days (3410 pg/ml and 2862 pg/ml, respectively, $P = 0.70$), but this did not reach statistical significance. (B) Concurrently, there was no significant difference in the level of

IL-10 in MCM v. control media after six days growth (114 pg/ml and 49 pg/ml, respectively, $P = 0.37$).