*nsplant Immunology* 1996; 4: 186–191

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# TGF-β1 pretreatment impairs the allostimulatory function of human bone marrow-derived antigen-presenting cells for both naive and primed T cells

Andrew Bonham, Lina Lu, Richard A Banas, Paulo Fontes, Abdul S Rao, Thomas E Starzl, Adriana Zeevi and Angus W Thomson

*Pittsburgh Transplantation Institute and Departments of Surgery, Pathology, and Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania*

Received 6 November 1995; accepted 19 January 1996

**Abstract:** Transforming growth factor-beta (TGF-β) exhibits strong antiproliferative effects upon lymphocytes and inhibits many of the effector functions of activated immune cells. However, its influence on the inductive phase of immune responses, and in particular its effect on antigen-presenting cells (APC), has not been well studied. In this investigation, we examined the influence of human TGF-β1 on the antigen-presenting function of human bone marrow (BM)-derived APC propagated in liquid culture for 11–17 days in response to granulocyte/macrophage colony-stimulating factor (GM-CSF). These cells were predominantly macrophages, accompanied by a minor population of dendritic cells. TGF-β1 had no effect upon the allostimulatory function of vertebral body whole BM cells cultured for 3–5 days in GM-CSF. However, it markedly reduced the allostimulatory capacity of BM-derived APC exposed to the cytokine for the last 3 days of culture. This inhibitory action could not be ascribed to cytokine 'carry-over', or to any consistent changes in the expression of cell surface molecules implicated in antigen presentation (HLA-DR), intercellular adhesion (ICAM-1; CD54), or costimulatory activity (B7-1; CD80). Mechanisms that may underlie the inhibitory action of TGF-β on APC function and the immunologic and possible clinical implications of the findings are discussed.

## Introduction

Transforming growth factor-beta (TGF-β) is a growth inhibitory cytokine that exerts multiple actions on most cells. It plays an important role in the regulation of immune reactivity, wound healing, and tissue development.[1,2] Three isoforms of TGF-β have been identified, each encoded by its own gene, and with a sequence homology of 70–80%.[1] The nature of its effects depends on several parameters, including cell type, differentiation, local environment, and the presence of other growth factors and cytokines. The immunomodulatory activities of TGF-β have been extensively described.[1–6] It has strong antiproliferative effects on lymphocytes,[6–9] and downregulates interferon-gamma (IFN-γ)-induced cell surface major histocompatibility complex (MHC) class II antigen expression.[10] TGF-β decreases the expression of many cytokines including IFN-γ, tumor necrosis factor-alpha and interleukins IL-1, IL-2 and IL-3. In contrast, it increases production of IL-1 receptor antagonist,[2,4–6,11] an observation consistent with its anti-inflammatory properties. TGF-β inhibits IL-2 receptor expression[4,5] and the effector functions of activated cells, such

Address for correspondence: Angus W Thomson, Pittsburgh Transplantation Institute, University of Pittsburgh Medical Center, W1544 Biomedical Science Tower, 200 Lothrop Street, Pittsburgh, PA 15213-2582, USA.

the cytolytic activity of NK (natural killer) and LAK ymphokine-activated killer) cells.[7,9] It deactivates differen-ıted macrophages, suppressing the respiratory burst and $H_2O_2$ lease.[12] However, its effects on the inductive arm of the ımune response (i.e. antigen presentation and lymphocyte tivation) have not been well characterized.

The bone marrow (BM) contains a number of cells with the otential to develop into or serve as antigen-presenting cells \PC).[13,14] These cells are of interest both in the context of M transplantation and rejection, and with regard to the aug-entation of donor leukocyte chimerism by BM infusion at e time of solid organ transplantation.[15] Among the potential PC resident in the BM are B lymphocytes, monocytes and ndritic cells. It has been postulated that donor BM-derived PC could play a key role in the establishment of tolerance llowing organ transplantation.[16–18] Furthermore, recognition at the relative tolerogenicity of the transplanted liver could related to its rich endowment of BM-derived leukocytes[16] ıs led to clinical trials using BM to augment donor cell chim-ism in solid organ transplantation.[15] BM is a rich source om which large numbers of APC may be propagated. The aturation and function of these cells is markedly affected the cytokine milieu. Thus, it has recently been shown that anulocyte/macrophage colony-stimulating factor (GM-CSF), ther alone or in combination with other cytokines, promotes e maturation and development both of macrophages and den-itic cells.[19–22]

## Objective

he objective was to explore the influence of TGF-β1 on the owth and allostimulatory activity of cells present in unmodi-ed human vertebral body BM, and in APC-enriched popu-tions grown in response to recombinant (r) human (h) GM-SF. The cells were tested using both naive and primed T cells responders and characterized with a panel of monoclonal ıtibodies (mAbs) directed against key cell surface phenotypic ıd functional markers.

## Materials and methods

### Preparation, culture and treatment of BM cells

ermission to use BM from human cadaveric organ donors for search purposes was obtained from the donor family as per niversity of Pittsburgh Medical Center Internal Review oard protocol. Thoracolumbar vertebral bodies were pro-ssed as described elsewhere.[15] BM cell suspensions were epared, and erythrocytes lysed with sterile water for 3 s. ysis was quenched with an appropriate volume of )× Hanks' Balanced Salt Solution (HBSS; Gibco BRL, rand Island, NY) to render the final suspension isotonic. The lls were then washed twice with HBSS. Viability was con-rmed by trypan blue dye exclusion. The cells were resus-ended in RPMI-1640 (Gibco BRL) supplemented with 5% /v) human AB serum (Nalgene, Miami, FL), nonessential mino acids, sodium pyruvate, L-glutamine, penicillin–strepto-ıycin, 2-mercaptoethanol (all from Gibco BRL), and .4 ng/ml rhGM-CSF (R&D Systems, Minneapolis, MN). Two $10^6$ cells were placed in each well of a 24 well plate in volume of 2 ml. The cultures were maintained at 37°C in umidified 5% $CO_2$ in air, and fed every other day by aspirating half of the supernatant and then replenishing the wells with an equivalent volume of fresh, GM-CSF-supplemented medium. To enrich for APC, the plate were swirled gently prior to aspiration to remove nonadherent granulocytes, without dislodging clusters of presumptive dendritic cell progenitors attached loosely to monolayers of plastic-adherent macrophages.[19,22] For cultured whole BM, no attempt was made to remove nonadherent cells over the 3–5 day culture period. The cultures were inspected daily and, when growing clusters of cells were abundant (at 8–14 days), subcultures were performed.

The mature 25 kDa dimer of rhTGF-β1 (R&D Systems; 0.6 ng/ml) was added to selected plates. The cells were cultured for an additional 3 days and then harvested by pipetting. They were washed three times in a large excess of HBSS to remove any free contaminating cytokines. Decanted supernatants were saved for later analysis. Harvested cells, which consisted of those easily dislodged by pipetting, were used in functional assays and immunophenotypic analysis. Cytospin preparations were stained with Giemsa to demonstrate cell morphology.

### mAbs

Mouse mAbs to the following human cell surface antigens were used: Leu-4 (T cell; CD3 epsilon chain), Leu-3a (T helper/inducer; CD4), Leu-2a (T suppressor/cytotoxic; CD8), Leu-19 (NK cell; CD56), Leu-12 (B cell; CD19), Leu-M3 (macrophage; CD14), Hle-1 (leukocyte common antigen, LCA; CD45), HLA-DR (MHC class II), CD11a (LFA-1α), Leu-15 ($CR_3$; CD11b), Leu-M5 ($CR_4$; CD11c), CD18 (LFA-1β), Leu-5b (LFA-2; CD2), CD80 (BB1/B7) (all Becton Dickinson, San Jose, CA); T6-RD1 (CD1a) (Coulter Immunology, Hialeah, FL); CD54 (ICAM-1) (PharMingen, San Diego, CA).

### Staining of cell surface antigens

Immunophenotypic analysis was performed by cytofluorography, using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Cells harvested from human BM cultures were stained with the appropriate mouse IgG isotypic subclass controls to detect the presence of nonspecific antibody staining. Two × $10^5$ cells per sample were first blocked with 10% (v/v) normal goat serum (Vector Labs, Burlingame, CA) in HBSS containing 0.1% bovine serum albumin (BSA; Sigma, St Louis, MO) for 30 min at 4°C. Cells were then washed once with 0.1% BSA/HBSS and centrifuged at 400 × *g*, 4°C for 6 min. Supernatants were decanted and the cells resuspended in 100 μl. They were then stained with FITC- or phycoerythrin-conjugated mouse mAbs for 45 min at 4°C. Excess antibody was removed by washing twice in 0.1% BSA/HBSS. Samples were fixed in 1% paraformaldehyde (Sigma) prior to analysis. Typically, 5000 events were analyzed from each sample. Cells were analyzed by gating on the large, granular population.

### Functional assays

The allostimulatory activity of whole BM cells and APC-enriched BM-derived populations was tested in primary mixed lymphocyte reactions (MLR) and pooled primed lymphocyte tests (PLT). Cultured cells γ-irradiated with 20 Gy served as stimulators. Cryopreserved donor-derived splenocytes were irradiated to serve as positive controls. For use as responders in MLR, peripheral blood lymphocytes (PBL) were isolated

om heparinized blood of normal healthy donors by centrifugtion over Ficoll-Hypaque. Six-day unidirectional MLR cultres were established with a fixed number of PBL responders $1 \times 10^5$ cells per well) and varying numbers of irradiated imulators. Triplicate cultures were performed in 96 well lates, in 200 μl of tissue culture medium supplemented with % (v/v) human AB serum, at 37°C, in a humidified atmosphere of 5% $CO_2$ in air. During the final 18 h of incubation, ch culture was labeled with 1 μCi of [$^3$H]thymidine (ICN adiochemicals, Costa Mesa, CA). Cells were harvested onto lass fiber disks using a multiple cell harvester, and the degree f thymidine incorporation was determined in a liquid scintiltion counter. To test for TGF-β1 'carry over', supernatants om washed cells were added to lymphocytes stimulated by onor spleen cells at the initiation of culture. Thymidine ptake was measured during the last 18 h of culture.

For the PLT, PBL from healthy donors were primed by a rge, pooled panel of irradiated cells of different HLA types ooled PLT reagent). Alloreactive lymphocytes served as sponders in a secondary assay using irradiated BM-derived PC as stimulators. The number of responders was fixed at $5 \times 10^4$ cells per well, while the number of stimulators was ried. Cultures were performed in triplicate (200 μl per well), 96 well plates, for 3 days and [$^3$H]thymidine uptake was sessed as described above.

### tatistics

atistical analysis was performed using paired *t*-tests on Statew 4.0.1 software (Abacus Concepts, Berkeley, CA). A $p$ lue $< 0.05$ was considered significant.

## esults

### ffects of TGF-β1 on the growth and morphologic ppearance of GM-CSF stimulated BM-derived PC

total of $4.8 \times 10^7$ whole BM cells were placed initially in ch culture plate ($2 \times 10^6$ cells per well in a 24 well plate). ter extended culture in GM-CSF for 11–17 days, typically 75–1 $\times 10^6$ cells per plate were recovered from cultures riched for APC. When TGF-β1 was added for the last 3 ys of culture, similar number of cells were recovered, demstrating that TGF-β1 had no adverse effect on the viability the cultured cells. Geisma-stained cytocentrifuge preptions revealed that the APC-enriched population resembled lls reported previously in GM-CSF-stimulated liquid cules.[20,21] The morphologic appearance of the cells (approx. –70% macrophages and 20–30% dendritic cells) was not ected by 3 days of exposure to TGF-β.

### fects of TGF-β1 on the phenotype of cultured hole BM and GM-CSF-stimulated BM-derived PC

r purposes of comparison with cultured cells, flow cytome was performed on samples of freshly isolated BM cells m seven cadaveric donors. These cells expressed the leukote common antigen CD45. The incidence of the T cell mark CD3, CD4 and CD8 was low, as was that of the B cell rker, CD19, and HLA-DR (MHC class II). There was also low incidence of cells expressing the monocyte marker D14; NK cell (CD56) and dendritic cell (CD1a) markers re also identified. The adhesion molecules CD11a, CD11b, CD18 and CD54 (ICAM-1) were all expressed on up to 50% of the cells. CD2 and CD11c were not detected. B7-1 (CD80) expression was very low. After 3–5 days of culture in GM-CSF, there were no significant differences in the percentages of whole BM cells staining for each of these markers either between samples or in comparison to fresh BM. In addition, cells incubated in GM-CSF, and with TGF-β1 added for the last 3 days of culture, exhibited a phenotype similar to that of cells grown in GM-CSF alone (data not shown).

The results of FACScan analysis of the APC-enriched BM-derived cells are presented in Table 1. The putative APC showed a comparatively high incidence of CD45$^+$ and HLA-DR$^+$ cells. As anticipated from the morphologic appearance of the cells, B cell, T cell and NK cell markers were very low. The incidence of cells expressing the monocyte marker CD14, however, was relatively high, and staining was comparatively bright compared to cells from whole bone marrow. Both the incidence and staining intensity of cells expressing the dendritic cell marker CD1a was low. The adhesion and costimulatory molecule CD54, however, was present on most of the APC population. B7-1 was found on only a small proportion of the cells. Similar observations on GM-CSF-stimulated human blood-derived cells have recently been reported.[20,21] As with whole BM cells, the addition of TGF-β1 during the final 3 days of culture did not affect the cell surface phenotype of the APC-enriched population, although there was a trend towards lower percentages of cells expressing CD1a and B7-1.

### Allostimulatory activity of BM-derived APC

Cultures of whole BM cells and those enriched for APC were tested as stimulators in one-way primary MLR (Figure 1). Donor splenocytes from cryopreserved cell suspensions were used as control allogeneic stimulators. As shown in Figure 1, whole BM cells were poor stimulators of allogeneic T cells when compared to splenocytes. In contrast, GM-CSF-stimulated BM-derived APC were consistently three to five times more potent than spleen cells at inducing T cell proliferation. At low relative stimulator cell numbers, at which spleen cells could no longer induce T cell proliferation, the APC exhibited marked allostimulatory activity.

Table 1 Expression of cell surface antigens by TGF-β1-treated BM-derived APC

| Antigen | GM-CSF only | | GM-CSF + TGF-β1 | |
|---|---|---|---|---|
| | % positive[a] | MFI[b] | % positive[a] | MFI[b] |
| CD45 | 57.7 (46–70) | 33.8 | 63.6 (29–64) | 46.0 |
| HLA-DR | 48.9 (29–80) | 136.6 | 42.3 (38–64) | 135.3 |
| CD3 | 3.5 (0–7.1) | 9.1 | 2.2 (1.7–7.1) | 7.5 |
| CD19 | 0.6 (0.1–3.5) | 6.8 | 2.3 (0–2.4) | 12.0 |
| CD14 | 62.7 (53–79) | 140.5 | 61.4 (57–65) | 113.0 |
| CD1a | 16.9 (5.8–43) | 12.8 | 8.4 (7.2–22) | 11.6 |
| CD54 | 89.0 (84–92) | 168.8 | 83.8 (77–90) | 184.0 |
| B7-1 | 11.6 (0–16) | 19.1 | 2.5 (2.3–11) | 19.1 |

[a]Values shown are median values of the percentage of positively staining cells determined in cultures from 3–7 bone marrow donors. Ranges are shown in parentheses.
[b]Mean fluorescence intensity of cells.

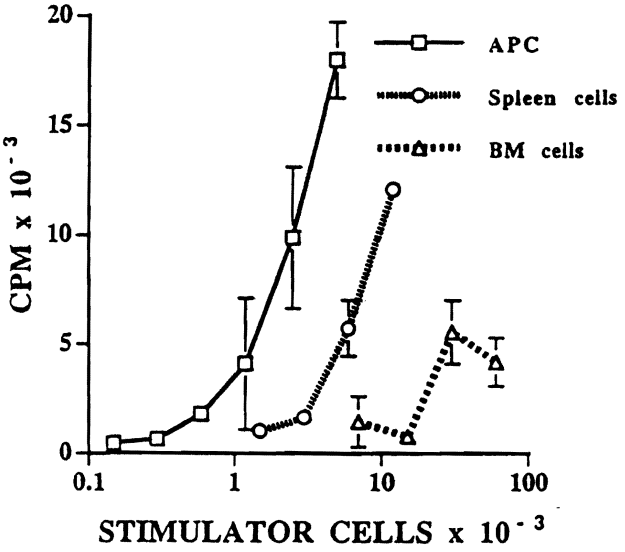


Figure 1 Six day primary MLR showing allogeneic T lymphocyte activation in response to three different stimulator cell populations. Eleven to 17 day GM-CSF-stimulated APC were better stimulators of naive allogeneic T cells than spleen cells from the same donor. In contrast, 3–5 day GM-CSF-stimulated whole BM cells were poor stimulators of the MLR. Vertical bars denote standard deviations of the mean. Results are representative of four separate experiments

### The effects of TGF-β1 on the allostimulatory activity of BM-derived APC in primary MLR

When TGF-β1 was added for the last 3 days to 3–5 day cultures of whole BM, no significant effect was observed on the already low allostimulatory activity of these cells (Figure 2). However, when TGF-β1 was added to the APC-enriched population during the last 3 days of 11–17 day cultures, a pronounced inhibitory effect on their potent allostimulatory function was observed (Figure 3A). The effect was not seen if the TGF-β1 was withheld until the last 24 h of culture (data not shown).

Since it was conceivable that TGF-β1 'carried over' with pretreated stimulator cells could have contributed to the inhibitory effect observed, supernatants from TGF-β1-treated cultures were tested for inhibitory activity in MLR. They were found to have no effect on T cell proliferation in response to

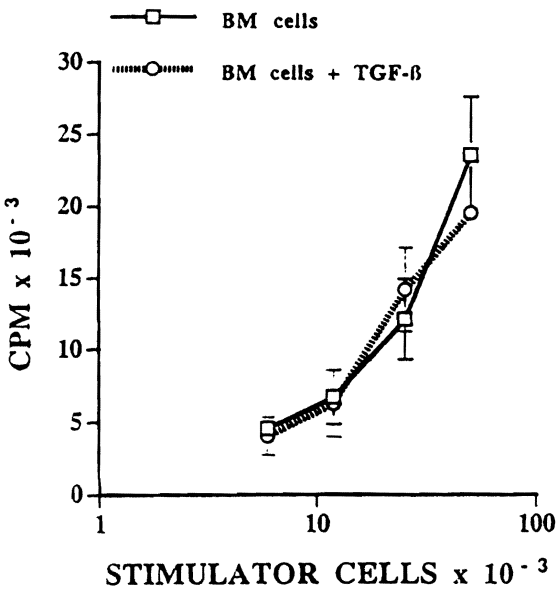


Figure 2 Primary MLR demonstrating the allostimulatory activity of GM-CSF-stimulated whole BM cells (3–5 day cultures) pretreated with TGF-β1. TGF-β1 treatment of the cells did not affect their allostimulatory activity. Vertical bars denote standard deviations of the mean. Results are representative of at least three experiments

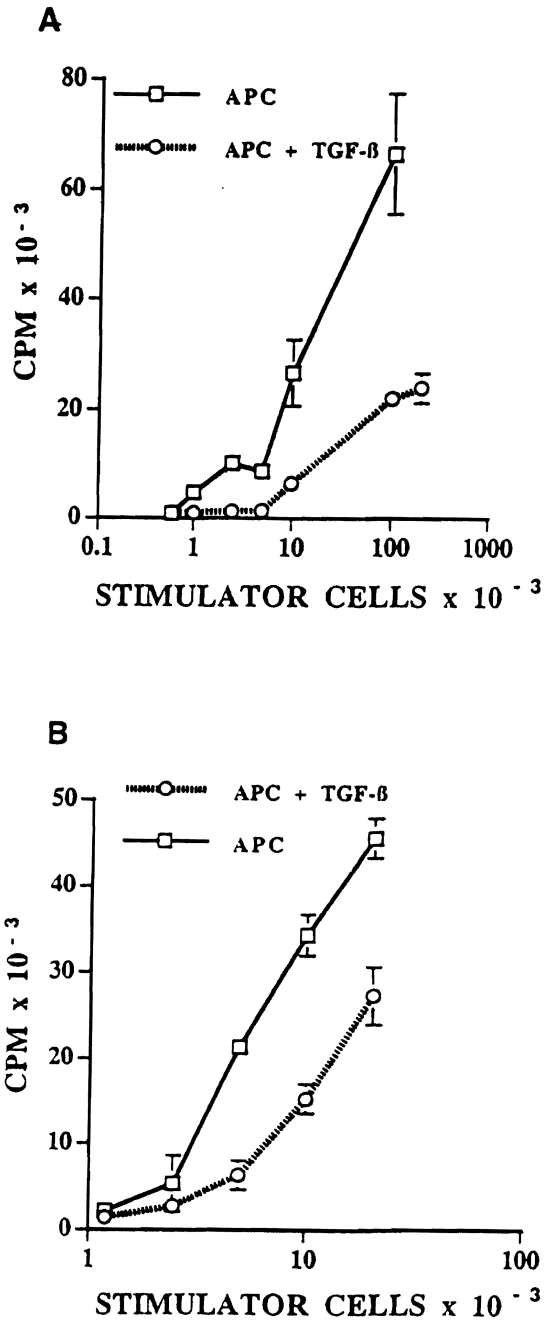


Figure 3 (A) MLR demonstrating the effect of TGF-β1 pretreatment of BM-derived APC (11–17 day cultures) on their allostimulatory activity in primary MLR. TGF-β1 treatment for the last 3 days of culture reduced the subsequent allostimulatory activity of the APC. Standard deviations are displayed as vertical bars. (B) PLT showing the effects of TGF-β1 pretreatment on the stimulatory activity of APC cultured with primed T lymphocytes in a secondary response. TGF-β1 reduced the allostimulation by the APC. Results are representative of at least three experiments

nor spleen cells (data not shown). This implies that the loss allostimulatory activity was a result of a direct effect of GF-β1 on the APC, rather than to a 'carry over' effect of GF-β1 on the responder T cells.

### fects of TGF-β1 on the stimulatory activity of PC for primed T lymphocytes

ie APC-enriched GM-CSF-stimulated cells (11–17 days of lture) were tested for their ability to stimulate T cells that d been previously primed by a pooled panel of HLA antins. As in the primary MLR, TGF-β1 reduced the allostimulory capacity of the APC, again by about 50% (Figure 3B).

## iscussion

ere have been few reports of the effects of TGF-β on the nction of APC. It has been shown, however, that TGF-β nibits antigen presentation by peripheral blood mononuclear lls and cultured Langerhans cells, but not by freshly isolated ngerhans cells.[23] TGF-β has been implicated in endowing crophages with the capacity to induce antigen-specific mune deviation,[24] whereas it did not suppress tumor munity conferred by epidermal APC.[25]

In the present experiments, the allostimulatory capacity of ole BM cells was not affected by TGF-β1. As its allostimulory activity was initially much lower than that of the GM-SF-stimulated APC-enriched population, whole BM may ve contained insufficient numbers of TGF-β responsive PC to exhibit an effect. The antigen-presenting capability of latter cells, however, appears to be markedly diminished by posure to TGF-β1. The mechanism of this action is unclear. though the overall viability of the cells was not influenced, quantities of specific cell types may have been altered by GF-β1, even though no significant differences were detected flow cytometric analysis. Relatively small changes in the mbers of potent APC, such as dendritic cells, which were t detected by flow cytometry, may have profound effects the MLR. Although there was a trend towards fewer cells pressing the dendritic cell marker CD1a with TGF-β1 treatnt, this was not statistically significant. Mature dendritic lls are known to be potent stimulators of naive T cells.[26] erefore, relatively small changes in their numbers may have ofound effects on the MLR. Macrophages and activated B lls also possess the ability to stimulate T cells, although to nuch lesser degree than dendritic cells. The immunostimulary activity of each of these cells correlates with the level cell surface expression of MHC class II and costimulatory olecules.[13,14] Their comparative accessory function is not so ikingly different in the stimulation of primed T cell secondy allogeneic responses, probably due to a less stringent quirement for MHC class II involvement.[27] The effects of GF-β1 on the APC used in the PLT were nevertheless quite pressive. As suggested by the present results, TGF-β1 may ert its influence by means other than altering the expression MHC class II. Since TGF-β is known to inhibit cytokine oduction, it may attenuate the function of APC by suppression of one or more cytokine signals.

Costimulatory molecule expression was not changed significantly by TGF-β1 when considering the APC as a whole, cept for a trend towards lower B7-1 expression. This may ve contributed, in part, to the loss of allostimulatory capacity the APC. However, detailed analysis of individual cell types was not performed. Conceivably, TGF-β may increase costimulatory molecule expression on some cell types, while simultaneously decreasing expression of the same molecules on others. Such an effect could disrupt the second signal necessary for activating T cells.

TGF-β can induce autocrine secretion.[1,3,6] However, in our experiments, supernatants from washed APC did not affect the proliferation assays. Even so, the addition of APC secreting a continuously low level of TGF-β, or the presence of a latent form of TGF-β which later becomes activated in prolonged cultures, cannot be ruled out. TGF-β may have an effect on feedback received by the APC upon interaction with T cells. A third and a fourth signal in antigen presentation and T cell activation have been proposed.[28] Thus, T cell interaction with MHC class II molecules may activate APC, resulting in improved costimulatory activity. T cell secreted cytokines, such as IL-4, may also affect APC surface molecule expression and function. TGF-β may have an impact on signal transduction pathways involved in the processing of these third and fourth signals.

The ability of TGF-β (at concentrations substantially below those encountered in inflammatory conditions[29]) to inhibit the antigen-presenting capacity of accessory cells may have far-reaching clinical implications. It has been suggested that stimulation of TGF-β production by T cells may, in part, underlie the therapeutic efficacy of cyclosporin A.[2,30] TGF-β prolongs heart allograft and pancreatic islet xenograft survival in rats.[31,32] It also reduces the severity of experimentally induced allergic encephalomyelitis[33] and arthritis.[34] Moreover, it may be responsible for the postulated 'veto' function (inactivation/deletion of cytotoxic T cell precursors) of a donor BM-derived population that can promote the induction of transplant tolerance in primates.[35] Augmentation of the acceptance of solid organ transplants with BM infusion is being attempted in several centers.[15,16,36] Cytokines such as TGF-β produced constitutively or as the result of gene transfer[37] may influence significantly the allostimulatory activity of the transplanted cells and the host–donor immunologic interaction. Further studies, particularly observations in animal models, will indicate the potential clinical significance of TGF-β for the therapy of allograft rejection.

### Acknowledgements

We thank Mr Del McCaslin and Ms Frances Chambers for skilled technical assistance and Ms Shelly Conklin for secretarial support. The work was supported, in part, by National Institutes of Health Grant DK 29961-13 and the Competitive Research Fund of the University of Pittsburgh Medical Center.

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