

# Elevated Interleukin-6 and G-CSF in Human Pancreatic Cancer Cell Conditioned Medium Suppress Dendritic Cell Differentiation and Activation

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## Abstract

Although dendritic cell (DC) function is impaired in pancreatic cancer patients, the underlying mechanisms are unknown. This study analyzed the soluble factors released by pancreatic cancer cells responsible for inhibiting DC differentiation and activation. Medium conditioned by a highly metastatic human pancreatic cancer cell line BxPC-3 [BxPC-3 conditioned medium (BxCM)] was mainly used for the study. Both CD34<sup>+</sup> hematopoietic progenitor cell-derived and CD14<sup>+</sup> monocyte-derived immature DCs and mature DCs (mDCs) were inhibited by BxCM. Allostimulation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells by BxCM-treated mDCs was inefficient and resulted in production of lower levels of Th1 and Th2 cytokines. Antigen-specific T-cell activation capability was also reduced in BxCM-treated mDCs. Addition of exogenous interleukin-6 (IL-6) and granulocyte colony-stimulating factor (G-CSF), which were present in high amounts in BxCM, mimicked the inhibitory effect of BxCM on DC differentiation and maturation. IL-6 was able to suppress DC differentiation and G-CSF mainly acted on the suppressing allostimulatory capacity of DCs. In addition, pancreatic cancer patient sera were able to inhibit DC differentiation of CD14<sup>+</sup> monocytes obtained from healthy donors. Depleting IL-6 or G-CSF from BxCM could reverse the DC-inhibitory properties of BxCM. Furthermore, BxCM, IL-6, or G-CSF led to the activation of signal transducer and activator of transcription 3 (STAT3) in CD14<sup>+</sup> monocytes to different degrees. Blocking BxCM-induced STAT3 activation also reversed the inhibitory effect of BxCM on DC differentiation. Therefore, IL-6 and G-CSF in BxCM represent two main factors responsible for suppression of DC differentiation, maturation, and antigen presentation, and this suppression of DC functions may be due to the aberrant activation of STAT3 by BxCM. [Cancer Res 2007;67(11):5479–88]

## Introduction

Pancreatic cancer is an extremely aggressive malignant tumor characterized by extensive invasion and very early metastasis (1) with an overall 5-year survival rate of 0.4% (2). The lack of effective conventional therapy for pancreatic cancer makes immunotherapeutic approaches attractive alternatives. However, only limited success was achieved because of systemic and local immunologic

escape mechanisms deployed by the tumor cells (3). Dendritic cells (DCs) play an important role in immune surveillance protecting against infection and malignancy (4). Inhibition of DC maturation and functions is a common mode of evading immune surveillance by tumors (5, 6). Coculture with primary tumor cells/cell lines or tumor cell conditioned media inhibits DC functioning and/or differentiation/maturation *in vitro* in many cancers, such as prostate cancer, colon adenocarcinoma, and breast carcinoma (7). Moreover, a variety of soluble factors overexpressed either in the tumor microenvironment or by themselves have been implicated in the inhibition of DC functions, such as interleukin (IL)-10, vascular endothelial growth factor, transforming growth factor- $\beta$ , IL-6 (8), and macrophage colony-stimulating factor (M-CSF; refs. 7, 9). In pancreatic cancer patients, the number and function of circulating DCs have been found to be impaired (10); however, the detailed mechanism of the effect of pancreatic cancer on DCs are largely unknown (11).

Hematopoietic progenitor cells (HPC) and CD14<sup>+</sup> monocytes can be differentiated into immature DCs (iDCs; ref. 12). The iDCs have an enhanced Ag uptake capacity but have a relatively poor ability to activate T cells. The iDCs can be activated by several stimuli *in vitro*, including cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) monocyte conditioned medium (MCM) or CD40 receptor cross-linking (13) to become mature DCs (mDCs). The mDCs express high levels of antigen-presenting molecules (MHC class I/MHC class II/CD1) and costimulatory molecules and secrete high levels of inflammatory cytokines TNF- $\alpha$  and IL-12 and have a decreased antigen uptake capability but are very potent in activating T cells.

The purpose of this study was to determine the soluble factors released by pancreatic cancer cells and their effect on DC differentiation and maturation. Because BxPC-3 is a highly metastatic human pancreatic cancer cell line, we used BxPC-3 conditioned medium (BxCM) for the detailed part of the study. We also investigated the effects of conditioned medium from other human pancreatic cell lines and pancreatic cancer patient sera on DC differentiation and function. We further determined whether two soluble factors, IL-6 and granulocyte colony-stimulating factor (G-CSF), secreted by pancreatic cancer cells could be responsible for the DC differentiation and functional suppression. The possible signal transduction pathway involved in BxCM-induced suppression of iDC differentiation was also studied.

## Materials and Methods

### Antibodies and Reagents

FITC-labeled anti-CD1a, CD14, CD16, CD40, CD83, CD86, HLA-A, B, C, and HLA-DR, DP, DQ antibodies; phycoerythrin (PE)-labeled anti-CD8, CD4, CD11c, CD80, and HLA-DR antibodies; Alexa Fluor 288-labeled anti-CD11b antibody; and FITC- and PE-labeled isotype control antibodies mIgG1( $\kappa$ ) were obtained from BD PharMingen. FITC-labeled anti-CD1c antibody was

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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obtained from Miltenyi Biotec GmbH. All cytokines, anti-IL-6, and anti-G-CSF antibodies were purchased from R&D Systems.

### Pancreatic Cancer Cells and Conditioned Media

Pancreatic cancer cell lines Panc-1, Mia PaCa2, and BxPC-3 were obtained from American Type Culture Collection. To obtain the conditioned medium, cells were seeded at  $2 \times 10^6$  cells/75 cm<sup>2</sup> and cultivated until 60% confluence. Thereafter, the medium was replaced and the supernatants were harvested at 48 h of further incubation. IL-6 and G-CSF depletion from BxCM was done by using anti-IL-6/anti-G-CSF antibodies (2 µg/mL) and Sepharose A/G beads following regular immunoprecipitation techniques. The cytokine depletion was confirmed by Bio-Plex multiplex cytokine assay kit.

### Generation of DCs

**Monocyte-derived DCs.** Human peripheral blood mononuclear cells (PBMC) were isolated from 50 mL buffy coat according to standard protocols using Ficoll-Paque Plus (Amersham Biosciences). CD14<sup>+</sup> monocytes were purified by using anti-human CD14 antibody-conjugated microbeads magnetic separation (Miltenyi Biotec GmbH). CD14<sup>+</sup> monocytes were treated in the complete RPMI 1640 (10% FCS, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 50 µmol/L β-mercaptoethanol, 1 mmol/L sodium pyruvate, 100 IU/mL penicillin, and 100 µg/mL streptomycin) with either granulocyte M-CSF (GM-CSF; 1,000 units/mL) plus IL-4 (1,000 units/mL), GM-CSF plus IL-4 plus BxCM, or GM-CSF plus IL-4 plus MCM for 5 days to generate the iDC. For iDC activation, human recombinant TNF-α (rTNF-α; 20 ng/mL; R&D Systems) was added at day 5 and the culture was continued for 2 days to induce maturation of these cells into mDCs. Fresh medium replacement with all reagents was done on days 3 and 5.

**HPC-derived DCs.** CD34<sup>+</sup> HPC isolated from the bone marrow of healthy donors were purchased from Clonetics (Walkersville, Inc.) to generate iDCs as described previously (10) in the presence or absence of 10% BxCM. In brief, CD34 cells were cultured in RPMI 1640 containing 5% FCS, penicillin/streptomycin, 2 mmol/L L-glutamine, 50 mol/L 2-mercaptoethanol, 100 ng/mL GM-CSF, 25 ng/mL stem cell factor (SCF; R&D Systems), 2.5 ng/mL TNF-α, and 5% AB pooled human serum. After 6 days, cells were harvested and further cultured in the absence of AB serum but in the presence of GM-CSF and TNF-α for 6 additional days with a last medium change being done at day 10. Cells were collected at day 12.

To study the effects of IL-6 and G-CSF, CD14<sup>+</sup> monocytes were treated in the complete RPMI 1640 with GM-CSF and IL-4, GM-CSF and IL-4 plus IL-6 (20 ng/mL), GM-CSF and IL-4 plus G-CSF (20 ng/mL), or GM-CSF and IL-4 plus IL-6 (20 ng/mL) plus G-CSF (20 ng/mL) or GM-CSF and IL-4 plus BxCM (untreated or depleted of IL-6 or G-CSF), for 5 days and then with rTNF-α (20 ng/mL) for another 2 days.

To study the effects of pancreatic patient sera on DC differentiation and maturation, CD14<sup>+</sup> monocytes were treated in the complete RPMI 1640 with GM-CSF and IL-4, and GM-CSF and IL-4 plus pancreatic cancer patient sera (15%) for 5 days and then with rTNF-α (20 ng/mL) for another 2 days. Sera from 13 patients with histologically confirmed pancreatic adenocarcinoma were used in the study. Informed consent was obtained from all individuals.

### Cell Surface Phenotyping

Phenotypic analysis of DC was done by using flow cytometry. At day 5 (iDCs) or day 7 (mDCs) of DC culture, cells were harvested and stained with labeled (FITC, PE, or Alexa Fluor 288) monoclonal antibody or appropriate isotypic controls on ice for 30 min. Cells were then washed and resuspended in 200 µL of cold PBS containing 5% fetal bovine serum and 2% para-formaldehyde. Stained cells were analyzed for single or double color immunofluorescence with a FACSCalibur. Data were acquired and analyzed using CellQuest software (Becton Dickinson).

### Allogeneic MLR

CD4<sup>+</sup> and CD8<sup>+</sup> T cells were selected from PBMCs using anti-human CD4 or anti-human CD8 magnetic beads (BD Biosciences). Labeled T cells were purified by passing through columns (Miltenyi Biotec GmbH) to reach a purity of ~96%.

The mDCs were irradiated (5,000 rad, 5 min), incubated with allogeneic CD4<sup>+</sup> or CD8<sup>+</sup> T cells at three different DC/T cell ratios of 1:10, 1:20, and 1:40, and cultured for 5 days in AIM V medium (Invitrogen Corp.) with [<sup>3</sup>H]thymidine (1 µCi/well; Amersham) for last 18 h. The cells were harvested by Filtermate Harvester (Packard Instrument Co.), and cell-associated radioactivity was determined by Top-Count NXT (Packard Instrument).

### Antigen-Specific CD8<sup>+</sup> T-Cell Activation

CD14<sup>+</sup> monocyte-derived mDCs (from HLA-A\*0201-positive donors) generated in the presence or absence of BxCM were loaded with 1 mg/mL influenza matrix protein (FMP) peptide GILGFVFTL (Sigma-Genosys) and 3 µg/mL β<sub>2</sub>-microglobulin (Sigma-Aldrich) for 2 h at room temperature. The peptide-pulsed mDCs were then used to stimulate autologous CD8<sup>+</sup> T cells at a DC/T cell ratio of 1:10. Enzyme-linked immunospot (ELISPOT) assay for T-cell IFN-γ production was done at 24 h after cell mixing. For T-cell proliferation assay, the mixed cells were continuously cultured for another 7 days with 25 units/mL recombinant IL-2 (Roche) added on days 3 and 5 and [<sup>3</sup>H]thymidine on day 6. T-cell proliferation was assessed 18 h after [<sup>3</sup>H]thymidine addition.

### Bio-Plex Cytokine Assay

The concentration of cytokines IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, GM-CSF, IFN-γ, and TNF-α were determined by using the Bio-Plex multiplex human cytokine Th1/Th2 Assay (Bio-Rad Laboratories) or a Biosource human 25-plex cytokine kit according to the manufacturer's protocols.

### Western Blot

To monitor the activation of signal transducer and activator of transcription 3 (STAT3), freshly isolated CD14<sup>+</sup> monocytes ( $2 \times 10^6$ ) were stimulated with GM-CSF and IL-4, 30% BxCM, 20 ng/mL G-CSF, 20 ng/mL IL-6, GM-CSF and IL-4 plus 30% BxCM, GM-CSF and IL-4 plus 20 ng/mL G-CSF, or GM-CSF and IL-4 plus 20 ng/mL IL-6 for 0, 5, 15, 30, and 60 min. Cells were lysed with 100 µL lysis buffer (Cell Signaling Technology). The lysates (100 µg) were then resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The levels of the phosphorylated STAT3 (p-STAT3) and total STAT3 proteins were detected using phospho-specific (Tyr<sup>705</sup>) antibodies for STAT3 and antibodies directed against STAT3 (Cell Signaling Technology) and were visualized using goat anti-mouse horseradish peroxidase conjugate antibodies (Cell Signaling Technology) and enhanced chemiluminescence (Amersham).

### Blocking STAT3 Activation Using Janus-Activated Kinase 2 Inhibitor Tyrphostin AG490

CD14<sup>+</sup> monocytes were treated with BxCM for 30 and 60 min, with or without pretreatment with 10 µmol/L AG490 for 2 h, proteins were extracted, and the level of p-STAT3 was determined using the Bio-Plex phosphoprotein kit. For studying the effect of blocking STAT3 activation on DC differentiation, CD14<sup>+</sup> monocytes were pretreated with 10 µmol/L AG490 for 2 h, and cells were washed thrice with PBS and resuspended in the regular DC differentiation cocktail (i.e., complete RPMI 1640 with GM-CSF and IL-4). After 5 days of treatment, cell surface markers were stained and analyzed as described above.

### Statistical Analysis

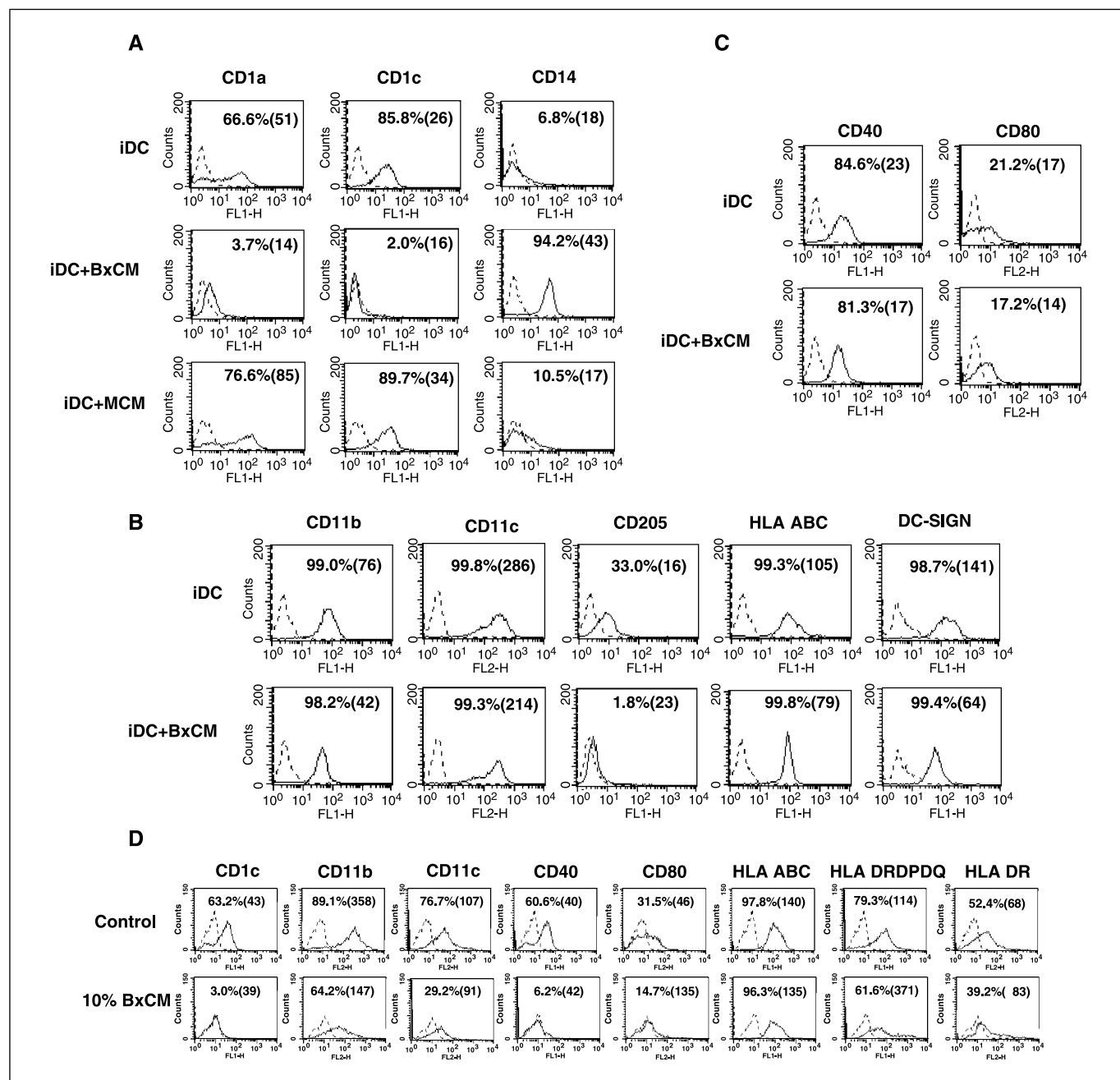
Quantitative results are shown as means ± SDs. The statistical analysis was done by Student's *t* test. *P* values <0.05 were considered significant.

## Results

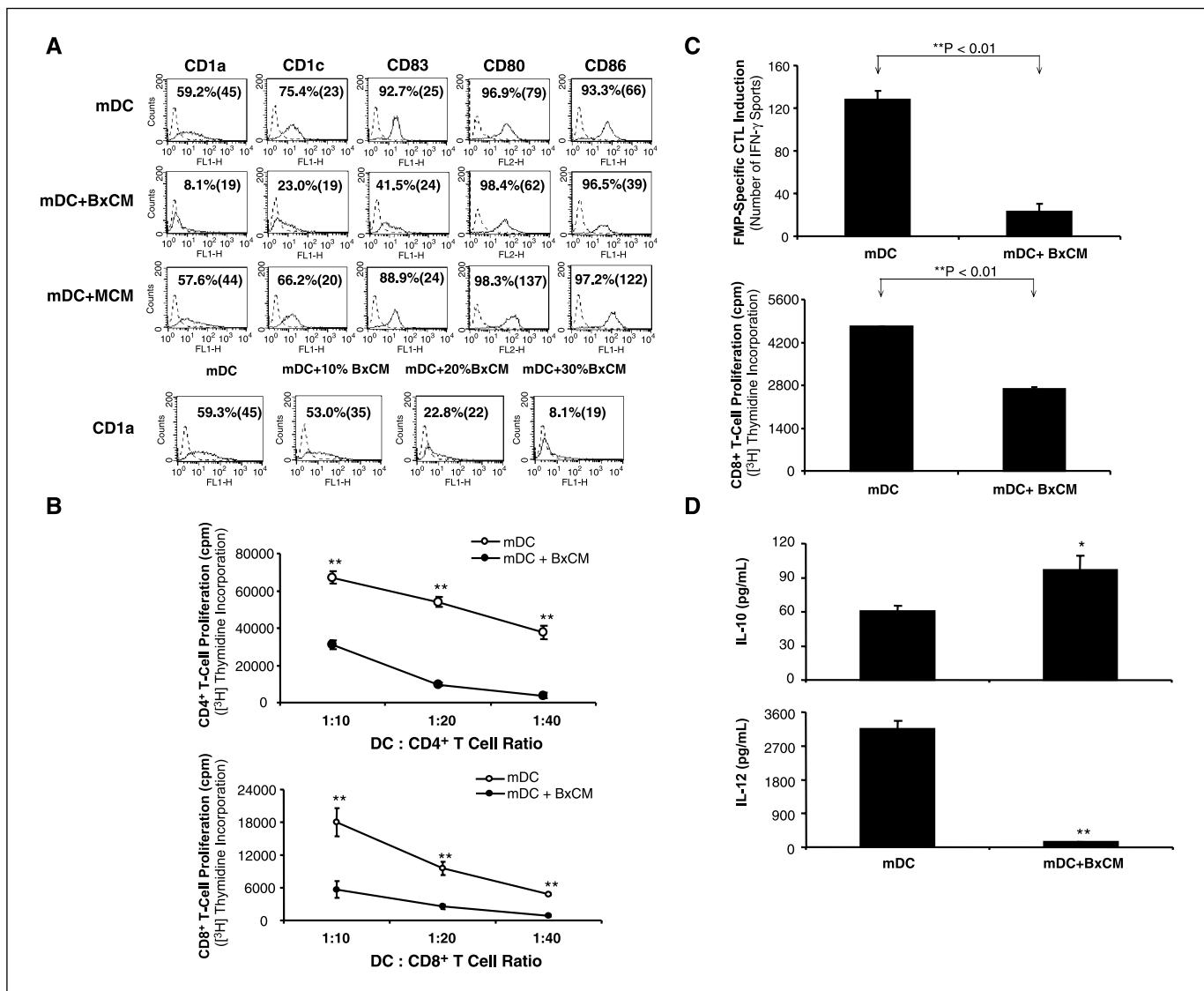
**BxCM inhibits phenotypic differentiation of DCs from CD14<sup>+</sup> monocytes.** Human PBMC-purified CD14<sup>+</sup> monocytes were cultured with or without addition of BxCM or MCM (negative control) for 5 days. In the presence of 30% BxCM, CD1a and CD1c expression in BxCM-treated iDCs remained low at 3.7% and 2.0%,

respectively (Fig. 1A) compared with that in normal iDCs. Moreover, CD14 expression remained high at 94.2% in BxCM-treated iDCs, whereas it was down-regulated to 6.8% in normal iDCs. MCM, the negative control medium, did not suppress the differentiation of CD14<sup>+</sup> monocytes to iDCs. In addition, BxCM-treated iDCs also had a decreased expression [mean fluorescence intensity (MFI)] of CD11b, CD11c, MHC class I, and CD209 (DC-

SIGN) and a decreased percentage of CD205<sup>+</sup> cells (Fig. 1B) compared with normal iDCs. There was no difference in the surface expression of CD40 and CD80 between the BxCM-treated and untreated iDCs (Fig. 1C). Therefore, BxCM could alter the characteristic phenotypic differentiation of CD14<sup>+</sup> monocytes to iDCs mainly by blocking both the up-regulation of CD1a and CD1c as well as the down-regulation of CD14.



**Figure 1.** Phenotypic analysis of BxCM on inhibiting CD14<sup>+</sup> monocyte-derived iDCs and CD34<sup>+</sup> HPC-derived DCs. CD14<sup>+</sup> monocytes were treated with normal medium, BxCM, or MCM for 5 d, cells were harvested, and the expression of various cell surface markers was analyzed by fluorescence-activated cell sorting (FACS). CD34<sup>+</sup> cells were cultured from days 0 to 6 with GM-CSF, TNF- $\alpha$ , SCF, and human AB serum and from days 6 to 12 with GM-CSF and TNF- $\alpha$  in the presence or absence of 10% BxCM. Cells were harvested and the expression of various cell surface markers was analyzed by FACS. The percentage of positive cells and MFIs (within parentheses) is denoted. Dotted histograms represent the fluorescence of the isotype control. **A**, major cell surface molecules affected by BxCM treatment. **B**, the inhibitory effect of BxCM on various cell surface markers. **C**, cell surface molecules unaffected by BxCM treatment. **D**, BxCM blocked the up-regulation of various DC markers during CD34<sup>+</sup> bone marrow-derived HPCs differentiation to DCs assayed at day 12. Results are representative of at least three independent experiments.



**Figure 2.** Effects of BxCM on the phenotype, T-cell stimulatory capacity in MLR and recall response to FMP and cytokine secretion pattern of CD14<sup>+</sup> monocyte-derived mDCs. CD14<sup>+</sup> monocytes were treated with normal DC differentiation medium, BxCM, or MCM for 5 d and activated by human rTNF- $\alpha$  for another 2 d. At the end of 7 d, expression of various cell surface antigens was analyzed by flow cytometry. The percentage of positive cells and MFIs (within parentheses) is denoted. Dotted histograms represent the fluorescence of the isotype control. *A*, inhibitory effects of BxCM on DC maturation by down-regulating DC surface phenotypic markers and dose-dependent inhibitory effect of BxCM on CD1a expression. For assessing the T-cell stimulatory capacity in MLR, mDCs were irradiated and incubated with allogeneic CD4<sup>+</sup> and CD8<sup>+</sup> T cells at three different DC/T cell ratios of 1:10, 1:20, and 1:40. *B*, [<sup>3</sup>H]thymidine incorporation (means of triplicates) in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Mature CD14<sup>+</sup> monocyte-derived DCs (from HLA-A\*0201-positive donors) generated in the presence or absence of BxCM were pulsed with FMP peptide (GILGFVFTL) and used to stimulate autologous CD8<sup>+</sup> T cells at a DC/T cell ratio of 1:10. *C*, ELISPOT for IFN- $\gamma$  production was done 24 h after mixing the cells and thymidine incorporation was used to assess the T-cell proliferation. *D*, supernatants from mDC cultures with or without BxCM treatments were assessed for IL-12 and IL-10 production using Luminex-based multicytokine assay. Columns, mean of replicates; bars, SD. \*\*, *P* < 0.01 compared with controls, *t* test.

**BxCM inhibits DC differentiation from hematopoietic CD34<sup>+</sup> cells.** We further examined the effect of BxCM on DC differentiation from hematopoietic CD34<sup>+</sup> cells. At day 12 of differentiation, reduced CD1c expression was observed in the presence of BxCM (from 63.2 to 3%; Fig. 1D). We also found lower expression of other markers, such as CD11b (from 89.1 to 64.2%), CD11c (from 76.7 to 29.2%), CD40 (from 60.6 to 6.2%), and CD80 (from 31.5 to 14.7%). These results indicate that BxCM is able to inhibit DC differentiation from two different progenitor cells CD14<sup>+</sup> and CD34<sup>+</sup> cells.

**BxCM inhibits the phenotypic maturation of CD14<sup>+</sup> monocyte-derived iDCs and its function.** After activation by TNF- $\alpha$  in the presence or absence of BxCM, all surface markers up-regulated in

mDCs were suppressed to different degrees in the presence of BxCM (Fig. 2A). CD83 was significantly suppressed by BxCM in mDCs (41.5%) compared with normal mDCs (92.7%). CD1a dramatically decreased from 59.2% to 8.1%. Consistently, none of the suppression effects exerted on BxCM-treated mDCs were observed in MCM-treated mDCs. Moreover, BxCM could block the phenotypic maturation of iDCs in a dose-dependent manner (Fig. 2A). Therefore, BxCM is able to suppress iDC maturation by suppressing CD83 expression as well as CD1a, CD1c, CD80, and CD86 expression in mDCs.

Antigen-presenting cell (APC) function of mDCs generated in the presence or absence of BxCM was evaluated by measuring their

capacity to induce the proliferation of allogeneic CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the MLR. As shown in Fig. 2B, at all three DC/T cell ratios (1:10, 1:20, and 1:40), BxCM-treated mDCs showed significantly less potency in stimulating both CD4<sup>+</sup> ( $P < 0.01$ ,  $t$  test) and CD8<sup>+</sup> ( $P < 0.01$ ,  $t$  test) allogeneic T-cell proliferation compared with normal mDCs. Cytokine secretion profiles by the activated T cells showed that CD4<sup>+</sup> T cells stimulated by BxCM-treated mDCs secreted significantly lower levels of Th1 type cytokines (IL-2, IL-12, IFN- $\gamma$ , and TNF- $\alpha$ ) as well as Th2 type cytokines (IL-10 and IL-13) compared with CD4<sup>+</sup> T cells stimulated by normal mDCs. Similarly, the levels of cytokines IL-2, IL-4, IL-13, IFN- $\gamma$ , and TNF- $\alpha$  secreted by the CD8<sup>+</sup> T cells stimulated by the BxCM-treated mDCs were much lower than those secreted by CD8<sup>+</sup> T cells stimulated by normal mDCs (Table 1). Thus, BxCM affects APC function of mDCs along with its effect on phenotypic maturation of mDCs.

**BxCM-treated DCs have reduced ability to activate Ag-specific autologous T cells.** We further examined Ag-specific T-cell response to confirm the suppressed ability of the BxCM-treated mDCs. CD14<sup>+</sup> monocyte-derived mDCs (from HLA-A\*0201-positive donors) generated in the presence or absence of BxCM were loaded with FMP peptide (GILGFVFTL). The peptide-pulsed mDCs were then used to stimulate autologous HLA-A\*0201 CD8<sup>+</sup> T cells. As shown in Fig. 2C, there was an increased production of IFN- $\gamma$  ( $P < 0.01$ ) by autologous CD8<sup>+</sup> T cells stimulated by normal mDCs compared with that by BxCM-treated mDCs. In addition, there was a significantly lower proliferation rate of CD8<sup>+</sup> T cells stimulated by the peptide-loaded BxCM-treated mDCs ( $P < 0.01$ ; Fig. 2C), which again confirmed that BxCM-treated mDCs reduced the ability of the mDCs to activate autologous T cells.

In analysis of the cytokine profiles produced by BxCM-treated mDCs, we found that there was a reduced production of IL-12 (a typical mDC cytokine) and a concomitant increased production of IL-10 (a known regulatory DC cytokine) in the supernatants from BxCM-treated mDCs (Fig. 2D). In supernatant of T cells activated by FMP peptide pulsed mDCs, there was an increase in the production of IL-4 (Th2 cytokine) and IL-17 (regulatory cytokine) by the BxCM-treated mDCs (data not shown). Because the IL-10-producing DCs with lower IL-12 production have been implicated in activation of T cells of regulatory nature in numerous studies,

BxCM-treated mDCs may have the property of eliciting a regulatory T cells (Treg) response to specific antigens.

**Conditioned media from different pancreatic cancer cell lines inhibit CD14<sup>+</sup> monocyte-derived phenotypic differentiation and maturation.** To determine the effect of conditioned media from other pancreatic cancer cells on iDC differentiation and maturation, we tested the phenotypic differentiation and maturation marker changes in the presence of conditioned media from Panc-1 and Mia PaCa2. Supplementary Table S1 showed that conditioned media from all three cell lines could suppress the phenotype of both iDCs (5 days) and mDCs (7 days) at different levels. There was a consistent decrease of CD1a and CD1c expression with all three different conditioned media treatments. Therefore, conditioned media from three pancreatic cancer cell lines have effects on suppression of DC differentiation and maturation, and BxCM shows a broad effect on down-regulating phenotypic markers of DC differentiation and maturation.

**Soluble factors IL-6 and G-CSF mimic the suppression effect of BxCM on DC differentiation.** Significant high levels of IL-6 (range, 269.3–1,249.7 pg/mL) and G-CSF (range, 215.3–918.5 pg/mL) were found in the BxCM. Furthermore, whereas the levels of both cytokines were relatively low in Panc-1 conditioned medium (17.8 and 28.4 pg/mL, respectively) compared with BxPC-3 cells, the Mia PaCa2 conditioned medium was moderately high in IL-6 (102.8 pg/mL) and was very high in G-CSF (583.0 pg/mL). IL-6 has been implicated in down-modulation of DC properties (8, 14, 15), and G-CSF has been reported to have an immunomodulatory role mainly on lymphocytes (16–18) as well as on CD14<sup>+</sup> monocytes (19–21) in *in vitro* and/or *in vivo* studies. We therefore hypothesized that the presence of high levels of IL-6 and G-CSF in BxCM could be responsible for the effect of BxCM on suppression of DC differentiation observed in our study. As shown in Fig. 3A, addition of IL-6 only showed a decrease in both CD1a and CD1c expression. However, in presence of both IL-6 and G-CSF, DC suppression effect was equivalent to the BxCM-treated group.

**Depletion of IL-6 and G-CSF from BxCM reverses the BxCM-mediated iDC phenotype changes.** The successful depletion of IL-6 or G-CSF from BxCM was confirmed and shown in Fig. 3B. The cytokine depleted BxCM was added during CD14<sup>+</sup> monocytes differentiation to iDCs. As shown in Fig. 3C, depletion of IL-6 and

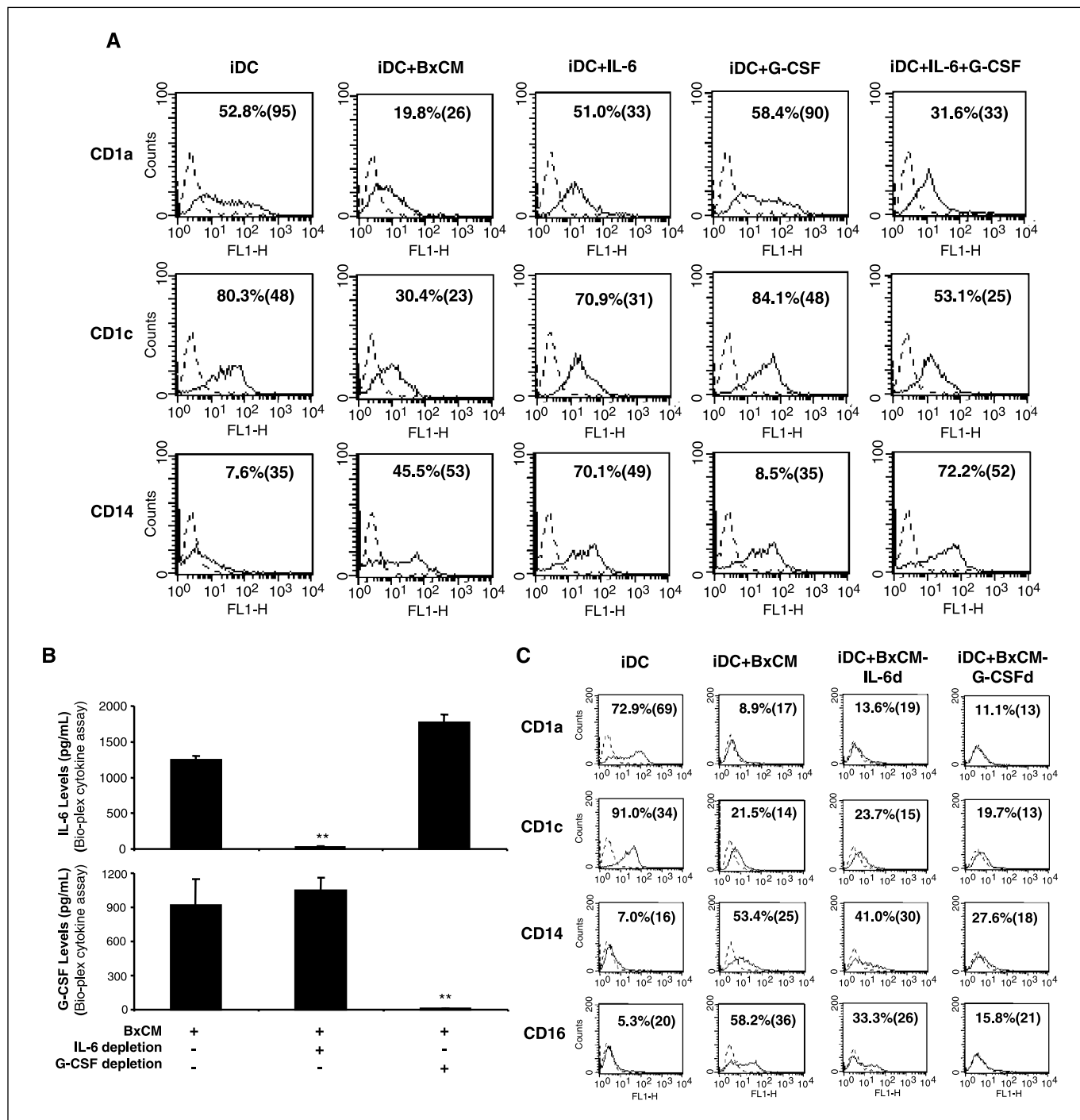
**Table 1.** Effects of BxCM on cytokine secretion and T-cell polarization by CD14<sup>+</sup> monocyte-derived DCs

	Cytokine secreted by CD4 <sup>+</sup> T cells (pg/mL)		Cytokine secreted by CD8 <sup>+</sup> T cells (pg/mL)	
	mDC	mDC + BxCM	mDC	mDC + BxCM
IL-2	2,302.0 $\pm$ 160.1	762.9 $\pm$ 110.5	721.5 $\pm$ 79.4	215.3 $\pm$ 107.3
IL-4	1,178.2 $\pm$ 127.7	913.8 $\pm$ 86.8	1,215.5 $\pm$ 38.5	770.4 $\pm$ 1.8
IL-5	15.2 $\pm$ 7.3	2.6 $\pm$ 0.4	<0.2	<0.2
IL-10	62.9 $\pm$ 4.4	18.6 $\pm$ 7.1	10.9 $\pm$ 3.9	10.5 $\pm$ 3.5
IL-12	22 $\pm$ 1.7	9.2 $\pm$ 9.6	18.4 $\pm$ 10.2	19.6 $\pm$ 8.5
IL-13	96.0 $\pm$ 7.1	27.7 $\pm$ 10.3	20.5 $\pm$ 0.0	9.2 $\pm$ 9.5
IFN- $\gamma$	334.5 $\pm$ 15.9	171.6 $\pm$ 28.3	307.4 $\pm$ 16.1	181.7 $\pm$ 21.1
TNF- $\alpha$	704.8 $\pm$ 72.5	305.7 $\pm$ 74.5	702.1 $\pm$ 15.5	370.8 $\pm$ 19.3

NOTE: TNF- $\alpha$ -matured mDCs were incubated with allogeneic CD4<sup>+</sup> or CD8<sup>+</sup> T cells at DC/T cell ratio of 1:10. Supernatants from CD4<sup>+</sup> T-cell and mDC cocultures or CD8<sup>+</sup> T-cell and mDC cocultures were collected after 24 h and tested for IL-2, IL-4, IL-5, IL-10, IL-13, IFN- $\gamma$ , and TNF- $\alpha$  production by Bioplex cytokine assay. The mean  $\pm$  SD values shown are the quantities of the cytokines in picogram per milliliter.

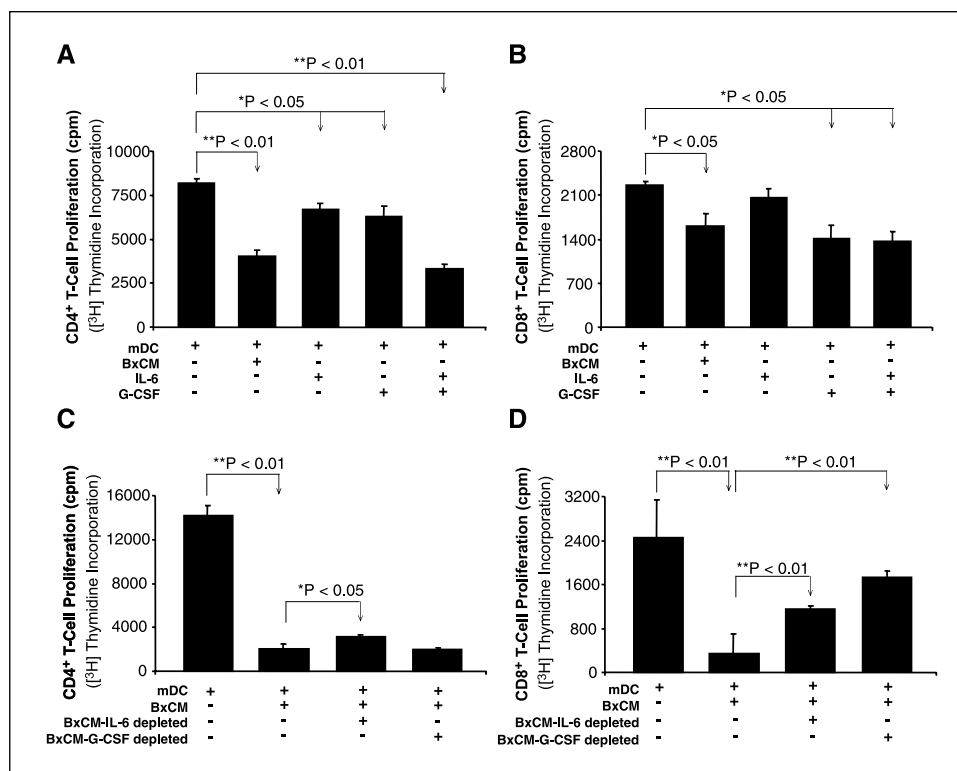
G-CSF had a slight effect on BxCM-mediated down-regulation of CD1a and CD1c but had a profound effect on reversal of CD14 and CD16 expression caused by BxCM. These data clearly point out that IL-6 and G-CSF in BxCM may mediate the changes in DC phenotype.

**G-CSF inhibits the APC function of DCs.** For allogeneic CD4<sup>+</sup> T-cell proliferation (Fig. 4A), there was a slight inhibition in the allostimulatory activity of IL-6-treated mDCs ( $P < 0.05$ , *t* test) and G-CSF-treated mDCs ( $P < 0.05$ , *t* test) compared with that of normal mDCs. However, the treatment with the combination of



**Figure 3.** Effects of rIL-6 or rG-CSF or IL-6/G-CSF-depleted BxCM on CD14<sup>+</sup> monocyte-derived iDC phenotype and function. CD14<sup>+</sup> monocytes were treated with normal medium, BxCM, or normal medium with addition of IL-6 (20 ng/mL) or normal medium with addition of G-CSF (20 ng/mL) or normal medium with addition of IL-6 (20 ng/mL) plus G-CSF (20 ng/mL) or BxCM depleted of IL-6/G-CSF for 5 d and then tested for the cell surface expression of cell surface markers by FACS. **A**, effect of addition of IL-6 or G-CSF or IL-6 plus G-CSF on phenotype changes of iDC. **B**, confirmation of depleted IL-6 and G-CSF in BxCM by Bio-Plex multiplex cytokine assay. Columns, mean quantities of the cytokines (pg/mL); bars, SD. \*\*,  $P < 0.01$  compared with controls, *t* test. **C**, effect of IL-6- or G-CSF-depleted BxCM on phenotype changes of iDC.

**Figure 4.** Effects of addition of rIL-6 or rG-CSF or IL-6/G-CSF-depleted BxCM on CD14<sup>+</sup> monocyte-derived mDC allostimulatory function. CD14<sup>+</sup> monocytes were treated with normal medium or BxCM or normal medium with addition of IL-6 (20 ng/mL) or normal medium with addition of G-CSF (20 ng/mL) or normal medium with addition of IL-6 (20 ng/mL) plus G-CSF (20 ng/mL) or BxCM depleted of IL-6/G-CSF for 5 d and then for another 2 d with human rTNF- $\alpha$  for iDC activation. The TNF- $\alpha$ -matured cells were irradiated and incubated with allogeneic CD4<sup>+</sup> and CD8<sup>+</sup> T cells at three different DC/T cell ratios of 1:10, 1:20, and 1:40. [<sup>3</sup>H]Thymidine incorporation (means of triplicates) in CD4<sup>+</sup> T cells was determined as shown in (A) and (C) and CD8<sup>+</sup> T cells as shown in (B) and (D). Columns, mean; bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with mDCs or BxCM-treated mDCs,  $t$  test.



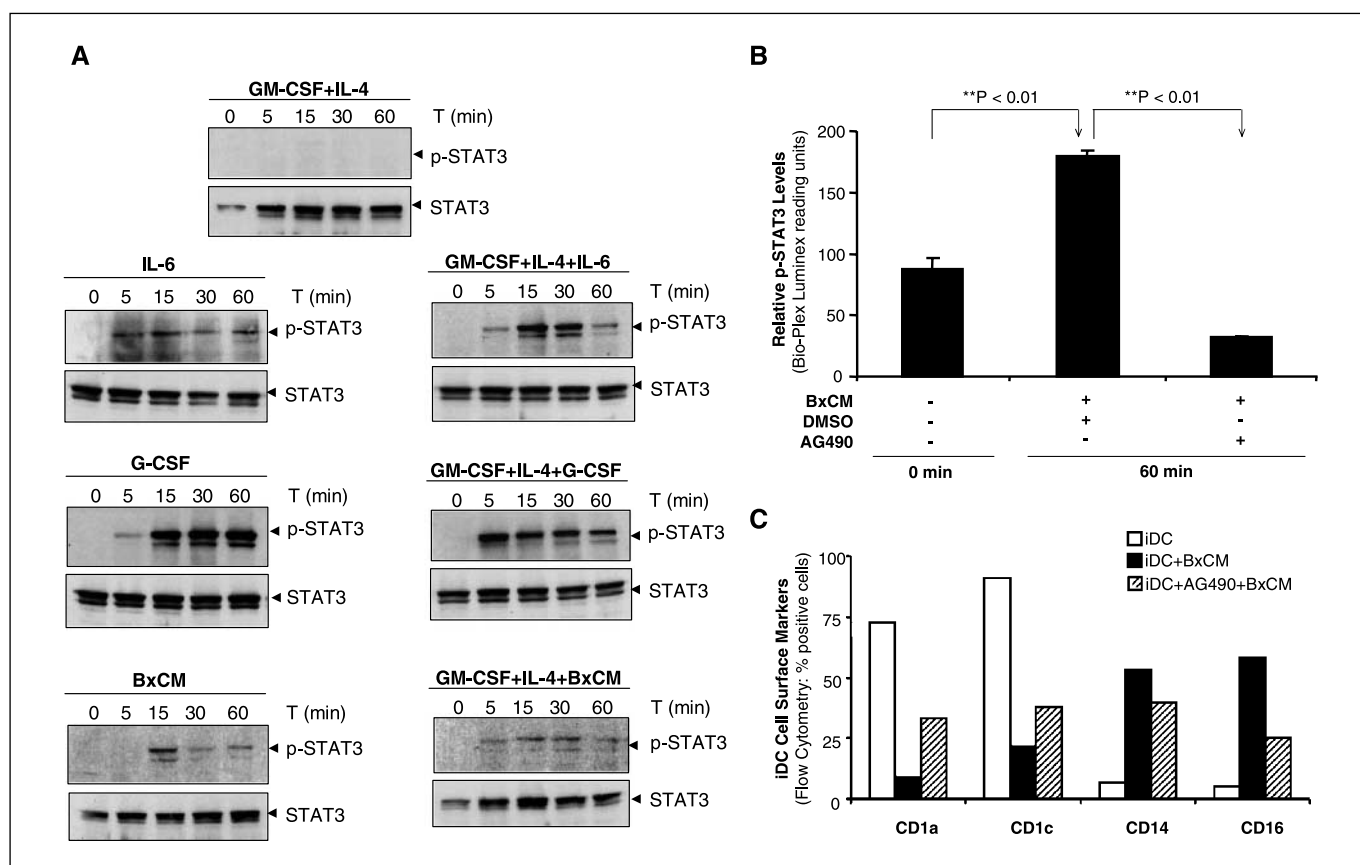
IL-6 and G-CSF ( $P < 0.01$ ,  $t$  test) suppressed the allostimulatory capacity of mDCs to a similar low level as that of BxCM-treated mDCs ( $P < 0.01$ ,  $t$  test). For allogeneic CD8<sup>+</sup> T-cell proliferation (Fig. 4B), suppressed allostimulatory effects were seen with the combination of IL-6 and G-CSF-treated mDCs ( $P < 0.05$ ,  $t$  test) to the same extent as BxCM-treated mDCs ( $P < 0.05$ ,  $t$  test); G-CSF alone was also able to decrease the allostimulatory ability significantly ( $P < 0.05$ ,  $t$  test), whereas IL-6 alone could not. Analysis of cell surface expression of MHC class I expression on these various mDC populations revealed that G-CSF alone could reduce the MFI for MHC class I from 276 to 190 in mDCs, whereas IL-6 treatment alone could not (Supplementary Table S2). Thus, the pronounced inhibitory effect of G-CSF on CD8<sup>+</sup> T-cell proliferation could be due to the effect on MHC class I, the primary antigen-presenting molecule for CD8<sup>+</sup> T cells.

**Depletion of G-CSF from BxCM reverses the inhibitory effect of BxCM on the APC function of DCs.** For CD4<sup>+</sup> T-cell allostimulation shown in Fig. 4C, only IL-6-depleted BxCM-treated DCs could reverse the suppression effect of BxCM-treated DC to a marginal but significant extent ( $P < 0.05$ ). For CD8<sup>+</sup> T cells, however (Fig. 4D), both IL-6 and G-CSF-depleted BxCM-treated DCs showed active allostimulatory function. Therefore, because BxCM-treated mDCs had a significantly lower allostimulatory capacity than the normal mDCs ( $P < 0.01$ ), the allostimulation was significantly higher in both the IL-6-depleted BxCM-treated DCs ( $P < 0.01$ ) and the G-CSF-depleted BxCM-treated DCs ( $P < 0.01$ ) compared with that in BxCM-treated DCs. These data indicate that IL-6 and/or G-CSF may play an important role in the allostimulatory function of mDCs.

**BxCM activates STAT3 during DC differentiation.** Abnormal induction of STAT3 is reported to be involved in DC-regulatory function by tumor conditioned medium (14, 22, 23). To determine

the STAT3 activation status in BxCM-treated DC during DC differentiation, we examined the effects on STAT3 phosphorylation by BxCM, IL-6, or G-CSF either alone or in combination with GM-CSF plus IL-4 (cytokines in normal DC differentiation medium) by western blot. As shown in Fig. 5A, there was no STAT3 activation in CD14<sup>+</sup> monocytes cultured with iDC differentiation medium (GM-CSF plus IL-4). However, there was consistent STAT3 activation in the cells after supplement of IL-6, G-CSF, or BxCM. Because IL-6 and G-CSF both are known activators of STAT3 (14, 24), the observed activation of STAT3 by IL-6 and G-CSF in turn indicates that BxCM-induced STAT3 activation may due to high IL-6 and G-CSF in BxCM. We also found that Panc-1 conditioned medium induced a strong activation of STAT3 in DC within 5 min similar as that by BxCM treatment (data not shown). This indicates that STAT3 activation may be responsible for BxCM-induced aberrant iDC differentiation.

**Blocking BxCM-induced STAT3 activation by Janus-activated kinase 2 inhibitor tyrostatin AG490 reverses the inhibitory effect of BxCM on DC differentiation.** CD14<sup>+</sup> monocytes were treated with BxCM for 60 min with or without pretreatment with AG490 [Janus-activated kinase 2 (JAK2) inhibitor], a previously reported STAT3 activation inhibitor, for 2 h. BxCM treatment increased p-STAT3 by 2-folds after 60 min compared with control cells ( $P < 0.01$ ) as shown in Fig. 5B. When cells were pretreated with AG490, the increased level of p-STAT3 was significantly inhibited ( $P < 0.01$ ). Furthermore, although BxCM treatment decreased CD1a expression on iDCs, prior blocking with AG490 partially reversed this decrease (8.9–33%). Similarly, the down-regulated CD1c expression by BxCM from 91% to 21.5% could be reversed to 38% by prior AG490 treatment. STAT3 inhibitor pretreatment could also down-regulate high CD14 and CD16 expression by BxCM treatment. This reveals that blocking



**Figure 5.** The relevance of STAT3 activation by BxCM, IL-6, and G-CSF in the BxCM-mediated blocking of DC differentiation from CD14<sup>+</sup> monocytes. **A**, CD14<sup>+</sup> monocytes were treated with normal medium or BxCM or IL-6 (20 ng/mL) or G-CSF (20 ng/mL) alone or in different combinations for 0, 5, 15, 30, and 60 min. The treated cells were then collected. Whole-cell proteins were extracted and used for Western blot with anti-p-STAT3 and anti-STAT3 antibodies. **B**, CD14<sup>+</sup> monocytes were treated with BxCM for 30 and 60 min with or without pretreatment with the JAK2 inhibitor tyrphostin AG490, cells were collected, whole-cell proteins were extracted, and the level of p-STAT3 was detected by Bio-Plex phosphoprotein assay kit. **C**, CD14<sup>+</sup> monocytes were treated with either normal or BxCM for 5 d with or without pretreatment with AG490, cells were harvested, and expression of various cell surface markers was analyzed by flow cytometry.

BxCM-induced STAT3 activation by prior treatment with AG490 reverses BxCM-induced inhibition of DC differentiation. Therefore, this shows a critical role of STAT3 activation by BxCM treatment in mediating the inhibition of DC differentiation.

**Sera from pancreatic cancer patients inhibit DC differentiation.** CD14<sup>+</sup> monocytes were used to differentiate into iDCs in the presence of 15% serum from five pancreatic cancer patients. After 5 days of differentiation, CD1a, CD1c, and CD80 were drastically reduced in iDC in the presence of patients' sera (Supplementary Fig. S1A). In addition, there was a reduced expression of CD1a, CD1c, CD40, CD80, CD83, and MHC class II (Supplementary Fig. S1B) on mDCs in the presence of patients' sera. Our results convincingly prove that pancreatic cancer patients have soluble factors released in their sera, which could effectively block DC differentiation and maturation.

To further elucidate the nature of the soluble factors present in the sera of these pancreatic cancer patients, we examined the levels of IL-6 and G-CSF in these sera (Supplementary Fig. S1C). The average serum IL-6 level in these patient sera was  $6.7 \pm 3.8$  pg/mL (range, 0.9–14.8 pg/mL) and that for G-CSF was  $4.5 \pm 4.6$  pg/mL (range, 1.0–13.2 pg/mL). When we tried to correlate the IL-6 and G-CSF levels in these patients' sera and the capability of inhibiting DC phenotypic differentiation, we found that those sera that had

higher level of IL-6 (such as patients 1 and 3 who showed 14.9 and 8.7 pg/mL IL-6 in sera) reduced the level of CD1c expression more (from 85.6% in iDC to 3.3% and 6.4%, respectively) than the sera that had lower levels of the cytokine (such as patient 13 who showed 4.8 pg/mL IL-6 reduced from 85.6% to 12.6%). These data again prove the role of enhanced IL-6 and G-CSF in reduced DC differentiation found commonly in most pancreatic cancer patients.

## Discussion

Current study shows that pancreatic cancer cells, represented by pancreatic cancer cell line BxPC-3, release high levels of IL-6 and G-CSF that inhibit DC differentiation and maturation from CD14<sup>+</sup> monocytes and CD34<sup>+</sup> HPCs. The inhibitory effects of BxCM are shown both phenotypically in down-regulating DC differentiation and maturation markers and functionally in inhibiting allostimulatory activity to CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes as well as antigen-specific CD8<sup>+</sup> T-cell activation. Addition of IL-6 and G-CSF in the normal culture medium shows a DC-inhibitory effect similar to BxCM treatment. In addition, depletion of IL-6 and G-CSF from BxCM significantly reverses its DC-inhibitory effects. Pancreatic cancer patient sera containing high levels of IL-6 and G-CSF also



significantly block the phenotypic differentiation and maturation of DCs from CD14<sup>+</sup> monocytes. Furthermore, BxCM treatment induces STAT3 activation in CD14<sup>+</sup> monocytes. Consequently, blocking BxCM-induced STAT3 activation by AG490 effectively reverses its inhibitory effect on DC differentiation from CD14<sup>+</sup> monocytes, suggesting a critical role of STAT3 in the BxCM-induced inhibition of DC differentiation.

In our study, many monocyte-derived DC-specific differentiation and maturation markers were found to be down-regulated in the presence of BxCM, especially CD1a. CD1a down-regulation is suggested to be an important mechanism used by tumor cells to suppress the antitumor responses (25). The down-regulation of CD1a in both iDCs and mDCs by pancreatic patient sera definitely suggests a similar mechanism operating in pancreatic cancer. Other DC cell surface markers involved in adhesion and homing (CD11b and CD11c), antigen presentation (CD1c, HLA ABC, and CD205), and DC-SIGN on iDCs were also down-regulated by BxCM treatment. BxCM-treated CD14<sup>+</sup> cells did not lose the CD14 marker over 5 days of DC differentiation process. This phenomenon clearly indicates that these cells did not undergo differentiation and were still monocytes (26, 27). The fact that BxCM treatment affects the expression of many surface molecules during iDC differentiation suggests an immune inhibitory mechanism that may be operative in pancreatic cancer and is substantiated by our data showing the down-regulation of most of these markers and/or others (eg CD1c, CD11b, and CD80) in iDC generated in the presence of pancreatic patient sera.

We found that CD4<sup>+</sup> and CD8<sup>+</sup> T-cell allostimulatory function of mDCs was decreased after treatment with BxCM. Because both CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been shown to play a central role in immune surveillance, interference with the activation of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells is likely the strategy for tumor cells to evade the antitumor immunity from the host (28, 29). Further, the BxCM-treated DCs reduced their ability to activate antigen-specific T cells by secreting lower IL-12 and higher IL-10. The BxCM-treated mDC-activated T cells secrete lower levels of IFN- $\gamma$  and higher levels of IL-4 and IL-17. These data suggest a tolerogenic DC (TDC) phenotype (11). The down-regulation of the costimulatory molecules CD40, CD80, and CD83 and MHC class II in mDCs generated in the presence of pancreatic patients' sera also points toward a similar DC phenotype.

Although previous studies have shown that cancer conditioned medium can inhibit DC functions, roles of individual components in the conditioned medium have not been clearly established, especially in pancreatic cancer. We found that inhibitory effects of BxCM on iDC phenotype could be reproduced by addition of only recombinant IL-6 (rIL-6) or both IL-6 and G-CSF in the culture medium. Depleting IL-6 and G-CSF from BxCM also reversed DC-inhibitory effects of BxCM, which also clearly points toward the role of these cytokines in inhibiting DC differentiation. Elevated plasma IL-6 has been associated with greater chances of surgical site infection following pancreaticoduodenectomy in pancreatic cancer patients (30). In consistency with previous studies (31–33), we found that IL-6 levels were very high ( $6.7 \pm 3.8$  pg/mL; range, 0.9–14.8 pg/mL) in the sera from 13 pancreatic cancer patients. It is an important correlation of our findings with the clinical significance of elevated IL-6 in pancreatic cancer patients.

The most interesting aspect of this study is the involvement of G-CSF in inhibiting DC functional differentiation and maturation. The immunomodulatory effect of G-CSF on T cells is believed to be

mediated exclusively through other effector cells, such as monocytes, by down-regulating costimulatory molecules (16), increasing IL-10 production (20), and decreasing secretion of IL-12 and TNF- $\alpha$  (19, 34) or selective mobilization of type 2 DC skewing T-cell differentiation toward a Th2 phenotype (35). G-CSF depletion from BxCM clearly reversed the inhibitory effect of BxCM on DC differentiation. Because G-CSF favors the *in vitro* differentiation of peripheral blood monocytes into TDCs through the release of IL-10 and IFN- $\gamma$  (21, 35), our experimental findings support the notion that G-CSF could be an important cytokine to inhibit antitumor immune activation by DCs in pancreatic cancer patients. Aberrant expression of G-CSF has been reported in many cancers (36–38); however, little is reported about G-CSF-producing pancreatic cancer (39–41). Furthermore, G-CSF treatment ameliorated autoimmune spontaneous type 1 diabetes in nonobese diabetic mice by activating TDCs and Treg cells (42). Using G-CSF-mobilized stem cell grafts reduced rejection of allogeneic stem cell transplantation (43). These data showed that G-CSF treatment causes DCs with a reduced ability to stimulate effector T cells whether in an autoimmune context or a transplantation scenario. We have also found that G-CSF-treated DCs had a significantly lower ability to stimulate allogeneic CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and the inhibitory effect was more pronounced in CD8<sup>+</sup> cells. Together with this observation and the fact that G-CSF reduced MHC class I expression on mDCs, it is our understanding that G-CSF may play a very active role in inhibiting the TNF- $\alpha$ -induced DC maturation process. As CD8<sup>+</sup> cytotoxic T cells are the major effector cells for any successful immunotherapy for killing tumor cells, controlling the level of G-CSF in any effort for pancreatic cancer immunotherapy may improve the treatment outcome.

Aberrant activation of the transcription factor STAT3 plays a pivotal role in regulation of antitumor immunity specifically the differentiation of DCs in various cancers (14, 22, 23). IL-6 has been shown to exert an immunoregulatory role by inducing STAT3 activation in murine fibrosarcoma (14). G-CSF is known to exert its proliferative and granulocytic differentiating functions through STAT3 induction (24). Keeping in line with these observations, we found that the activation of STAT3 by BxCM, IL-6, or G-CSF may be responsible for the inhibition of DC phenotypic differentiation. Other pathways activated by IL-6 and G-CSF and/or other factors/cytokines present in BxCM that could also play a role in modulating DC phenotype and function are warranted for further study.

In summary, we have found that conditioned media from three different pancreatic cancer cell lines inhibit DC differentiation. IL-6 and G-CSF in the conditioned medium cooperate to exert the inhibitory effect on DC differentiation, maturation, and Ag presentation functions. The aberrant activation of STAT3 is found to be responsible for the inhibitory effect on DC differentiation. Taken together, our data suggest important immunomodulatory targets in pancreatic cancer, which may help to design immunotherapeutic regimens for pancreatic cancer patients.

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## References

1. Keleg S, Buchler P, Ludwig R, Buchler MW, Friess H. Invasion and metastasis in pancreatic cancer. *Mol Cancer* 2003;2:14.
2. Bramhall SR, Allum WH, Jones AG, et al. Treatment and survival in 13,560 patients with pancreatic cancer, and incidence of the disease, in the West Midlands: an epidemiological study. *Br J Surg* 1995;82:111–5.
3. Laheru D, Jaffee EM. Immunotherapy for pancreatic cancer—science driving clinical progress. *Nat Rev Cancer* 2005;5:459–67.
4. Palucka AK, Laupeze B, Aspod C, et al. Immunotherapy via dendritic cells. *Adv Exp Med Biol* 2005;560:105–14.
5. Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* 2001;166:678–89.
6. Kierscher SM, Luo J, Dubinett SM, Roth MD. Tumors promote altered maturation and early apoptosis of monocyte-derived dendritic cells. *J Immunol* 2000;164:1269–76.
7. Pinzon-Charry A, Maxwell T, Lopez JA. Dendritic cell dysfunction in cancer: a mechanism for immunosuppression. *Immunol Cell Biol* 2005;83:451–61.
8. Hegde S, Pahne J, Smola-Hess S. Novel immunosuppressive properties of interleukin-6 in dendritic cells: inhibition of NF- $\kappa$ B binding activity and CCR7 expression. *FASEB J* 2004;18:1439–41.
9. Menetrier-Caux C, Montmain G, Dieu MC, et al. Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood* 1998;92:4778–91.
10. Yanagimoto H, Takai S, Sato S, et al. Impaired function of circulating dendritic cells in patients with pancreatic cancer. *Clin Immunol* 2005;114:52–60.
11. Bellone G, Carbone A, Smirne C, et al. Cooperative induction of a tolerogenic dendritic cell phenotype by cytokines secreted by pancreatic carcinoma cells. *J Immunol* 2006;177:3448–60.
12. Winzler C, Rovere P, Zimmermann VS, et al. Checkpoints and functional stages in DC maturation. *Adv Exp Med Biol* 1997;417:59–64.
13. Reis e Sousa C. Dendritic cells in a mature age. *Nat Rev Immunol* 2006;6:476–83.
14. Park SJ, Nakagawa T, Kitamura H, et al. IL-6 regulates *in vivo* dendritic cell differentiation through STAT3 activation. *J Immunol* 2004;173:3844–54.
15. Chomarati P, Banchereau J, Davoust J, Palucka AK. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol* 2000;1:510–4.
16. Pan L, Delmonte J, Jr., Jalonon CK, Ferrara JL. Pretreatment of donor mice with granulocyte colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-versus-host disease. *Blood* 1995;86:4422–9.
17. Sloan EM, Kim S, Maciejewski JP, et al. Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes *in vitro* and *in vivo*. *Blood* 2000;95:2269–74.
18. Reyes E, Garcia-Castro I, Esquivel F, et al. Granulocyte colony-stimulating factor (G-CSF) transiently suppresses mitogen-stimulated T-cell proliferative response. *Br J Cancer* 1999;80:229–35.
19. Mielcarek M, Martin PJ, Torok-Storb B. Suppression of alloantigen-induced T-cell proliferation by CD14<sup>+</sup> cells derived from granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells. *Blood* 1997;89:1629–34.
20. Mielcarek M, Graf L, Johnson G, Torok-Storb B. Production of interleukin-10 by granulocyte colony-stimulating factor-mobilized blood products: a mechanism for monocyte-mediated suppression of T-cell proliferation. *Blood* 1998;92:215–22.
21. Rutella S, Bonanno G, Pierelli L, et al. Granulocyte colony-stimulating factor promotes the generation of regulatory DC through induction of IL-10 and IFN- $\alpha$ . *Eur J Immunol* 2004;34:1291–302.
22. Wang T, Niu G, Kortylewski M, et al. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat Med* 2004;10:48–54.
23. Nefedova Y, Huang M, Kusmartsev S, et al. Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer. *J Immunol* 2004;172:464–74.
24. McLemore ML, Grewal S, Liu F, et al. STAT-3 activation is required for normal G-CSF-dependent proliferation and granulocytic differentiation. *Immunity* 2001;14:193–204.
25. Coventry B, Heinzel S. CD1a in human cancers: a new role for an old molecule. *Trends Immunol* 2004;25:242–8.
26. Zhou LJ, Tedder TF. CD14<sup>+</sup> blood monocytes can differentiate into functionally mature CD83<sup>+</sup> dendritic cells. *Proc Natl Acad Sci U S A* 1996;93:2588–92.
27. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor  $\alpha$ . *J Exp Med* 1994;179:1109–18.
28. Boon Tvan der Bruggen P. Human tumor antigens recognized by T lymphocytes. *J Exp Med* 1996;183:725–9.
29. Pardoll DM, Topalian SL. The role of CD4<sup>+</sup> T cell responses in antitumor immunity. *Curr Opin Immunol* 1998;10:588–94.
30. Kimura F, Shimizu H, Yoshidome H, et al. Increased plasma levels of IL-6 and IL-8 are associated with surgical site infection after pancreaticoduodenectomy. *Pancreas* 2006;32:178–85.
31. Ebrahimi B, Tucker SL, Li D, Abbruzzese JL, Kurzrock R. Cytokines in pancreatic carcinoma: correlation with phenotypic characteristics and prognosis. *Cancer* 2004;101:2727–36.
32. Martignoni ME, Kunze P, Hildebrandt W, et al. Role of mononuclear cells and inflammatory cytokines in pancreatic cancer-related cachexia. *Clin Cancer Res* 2005;11:5802–8.
33. Bartsch R, Woehrer S, Raderer M, Hejna M. Serum interleukin-6 levels in patients with gastric MALT lymphoma compared to gastric and pancreatic cancer. *Anticancer Res* 2006;26:3187–90.
34. Zeng D, Dejbakhsh-Jones S, Strober S. Granulocyte colony-stimulating factor reduces the capacity of blood mononuclear cells to induce graft-versus-host disease: impact on blood progenitor cell transplantation. *Blood* 1997;90:453–63.
35. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C. Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. *Blood* 2000;95:2484–90.
36. Chakraborty A, White SM, Guha S. Granulocyte colony-stimulating factor promotes  $\beta_1$ -integrin-mediated adhesion and invasion of bladder cancer cells. *Urology* 2006;68:208–13.
37. Savarese TM, Mitchell K, McQuain C, et al. Coexpression of granulocyte colony stimulating factor and its receptor in primary ovarian carcinomas. *Cancer Lett* 2001;162:105–15.
38. Kyo S, Kanaya T, Takakura M, Inoue M. A case of cervical cancer with aggressive tumor growth: possible autocrine growth stimulation by G-CSF and IL-6. *Gynecol Oncol* 2000;78:383–7.
39. Uematsu T, Tsuchie K, Ukai K, et al. Granulocyte-colony stimulating factor produced by pancreatic carcinoma. *Int J Pancreatol* 1996;19:135–9.
40. Ohtsubo K, Mouri H, Sakai J, et al. Pancreatic cancer associated with granulocyte-colony stimulating factor production confirmed by immunohistochemistry. *J Clin Gastroenterol* 1998;27:357–60.
41. Fukushima N, Sasatomi E, Tokunaga O, Miyahara M. A case of pancreatic cancer with production of granulocyte colony-stimulating factor. *Am J Gastroenterol* 2001;96:258–9.
42. Kared H, Masson A, Adle-Biassette H, et al. Treatment with granulocyte colony-stimulating factor prevents diabetes in NOD mice by recruiting plasmacytoid dendritic cells and functional CD4(+)CD25(+) regulatory T-cells. *Diabetes* 2005;54:78–84.
43. Bensinger WI, Storb R. Allogeneic peripheral blood stem cell transplantation. *Rev Clin Exp Hematol* 2001;5:67–86.