Inhibition of dendritic cell maturation by sera from patients with pancreatic cancer

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Inhibition of dendritic cell maturation by sera from patient with pancreatic cancer

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Abstract

Inhibition of dendritic cell maturation by sera from patients with pancreatic cancer

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Human pancreatic cancer is extremely difficult to treat and has a very poor prognosis. While it has been suggested that this type of cancer modulates immune system to evade immune surveillance, the underlying mechanism of how pancreatic cancers induce ineffective immune responses is still unclear.

The purpose of this study is to define the immunological parameters in patients with pancreatic cancer, to understand the underlying mechanisms of immune suppression by cancer, and to provide clues to improve the methods of immunolgical interventions. To gain insights of these immunosuppression in pancreatic cancer, PBMCs from healthy donors and pancreatic cancer patients were analyzed in their

difference of immune parameters. The proportion of immune surveillance related cells, such as dendritic cells (DCs), helper T cells, cytotoxic T cells and natural killer cells, were decreased in peripheral blood mononuclear cells of pancreatic cancer patients.

Immune cells were analyzed by FACS analysis and cell counting. The result reveled that $CD4^+$ T lymphocytes were remarkably reduced along with DCs and $CD8^+$ T lymphocytes. Certainly DCs play a key role in antigen uptake and processing followed by presentation of proper antigens to the effector cells. Recently, it has been reported that DCs was rarely found in pancreatic cancer tissues indicating that the lack of recruitment in the tumor or to an overall impaired DC development by cancer directly or indirectly through soluble cytokines.

To define the mechanism of how cancer inhibits DC generation, the following experiments were performed. DCs were cultured with sera derived from pancreatic cancer patients and its function and expression were observed. As a result, the ability to uptake antigen in immature DC and stimulus on allogeneic T cell in

mature DCs as well as the expression of DC associated markers were decreased respectively. Furthermore, the combinations of TGF-β, IL-10 and VEGF, well-known immunosuppressive factors secreted by pancreatic cancers, could replace the action of patient's serum in DC inhibition indicating that these three cytokines are the major inhibitory factors of serum. Expecially, IL-10 strongly inhibited T cell stimulation in mature DCs and induced early apoptosis in immature DCs generation. The results may provide a useful information for clinical application on cell-based immunotherapy.

Key words : pancreatic cancer, dendritic cell, immune suppression, inhibitory factors

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I. INTRODUCTION

Human pancreatic cancer is extremely difficult to treat and has a very poor prognosis. Most patients die within a year of diagnosis and the overall 5-year survival rate is only a few percent despites extensive clinical trials involving surgery, radiation, chemotherapy[1,2]. This is mainly due to the diagnosis at later stage of disease in most, if not all, cases. Thus, new strategies for early diagnosis and therapeutic approaches are essential for this deadly disease. An ineffective immune response may also contribute to the poor prognosis of patients with pancreatic adenocarcinoma. Until recently,

cancer, in general, was considered as a disease entity that immune system failed to recognize and react. Due to their immense proliferation with no apparent interception by immune system, cancer was considered as one of the most fatal disease that can not be cured by any means[3]. However, host immune systems have potency of handling immense burdens of pathogenic or nonpathogenic conditions as evidenced by organ transplantations where immune systems reject organ of weighting over several kilograms in few weeks. Thus, the question is how cancer evades such powerful immune systems. Several mechanisms of which cancers utilize to avoid or evade host immune recognition were proposed. First, tumor cells themselves may manifest low immunogenicity through defects in antigen processing and presentation, including low or absent expression of MHC class I or II, defects in antigen processing or transport, or low expression of distinct tumor-specific antigen[4]. Second, the mechanism of tumor cell inhibition on immune cell function is the production of soluble factors, such as Interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF). The effects of

these factors appear to be 2-folds: to inhibit effector function and to impair the development of immune cells by acting on earlier stages of immunopoiesis[5]. Third, cancers tend to modify their antigens by frequent mutations and adaptation.

To establish immune response against pathogens, cooperation and sequential interactions of various components of immune system are essential. These include antigen presenting cells, helper T cells, cytotoxic T cell, natural killer cells, B cells, monocytes and polymorphonuclear cells. Among these components, dendritic cells (DCs) play a key role in antigen uptake, processing and presentation of proper antigens to effector cells of immune system. Primary immune response is initiated by DCs. Immature DCs resides in epithelial and connective tissue compartments and after activation they migrate into draining lymph nodes and undergo the final maturation[6,7]. They are characterized by their ability to sample and present antigens to naive T cells in a MHC class I or class II restricted fashion, thus initiate antigen-specific immune responses.

Tumors may directly or indirectly influence the activity of DCs in means of escaping antitumor immune responses. It has been reported that both circulating and tumor infiltrating DCs are functionally impaired in tumor-bearing animals[8,9] and cancer patients[4,10]. Alternatively, cancer-derived cytokines have been found to be involved in suppressing effective antitumor DC functions[11,12]. Especially in pancreatic cancers, DCs were rarely found in the cancer tissues. The absence or paucity of DCs in pancreatic carcinoma could be related either to the lack of recruitment in the tumor or to an overall impaired DC development that could be due to the systemic action of factors such as VEGF, which is produced by most tumors[13]. Several immunomodulating cytokines produced by pancreatic cancers have been suggested for immunodeficiency found in these patients. The co-expression of TGF-β and IL-10 in pancreatic carcinoma tissue associated with significantly elevated levels of both cytokines in the sera of pancreatic carcinoma patients was also reported[14]. And roles of IL-10 in escape mechanisms were suggested since many tumors or tumor infiltrating cells express IL-10[15]. IL-10 has several immunosuppressive activities including the

inhibition of cytokine production by Th1 cells. However, the systemic and local immunological escape mechanisms need to be further clarified in order to define the contribution of each molecule to the immunosuppression and the collective mechanism of immunosuppression imposed by cancer.

The purposes of this study are to define the immunological parameters in pancreatic cancer patients, to understand the underlying mechanisms of immune suppression by serum derived from pancreatic cancers, and to provide clues to improve the methods of immunolgical interventions. For that, the overall parameters of adaptive immune system were examined numerically and proportionally on dendritic cells, T cells, NK cells in peripheral blood from pancreatic cancer patients and investigated whether the phenotypes and functions of DCs are affected by sera of pancreatic cancer patients or major immunomodulatory factors such as VEGF, TGF-β and IL-10. Moreover, the effect of tumor derived immunosuppressive factors highly produced by pancreatic cancer on monocyte-derived dendritic cell generation was examined. Finally, the methodology to prevent DC immunosuppression by cancer was provided. This

information is essential for the development of more effective immunotherapeutic approaches.

Ⅱ. MATERIALS AND METHODS

1. Reagents

Culture media used were RPMI-1640, M199 (Gibco BRL, Grand Island, NY, USA) and X-VIVO 10 medium (Bio-Whittaker, Walkersville, MD, USA) supplemented with penicillin, streptomycin (1,000 unit/mL; Gibco BRL, Grand Island, NY, USA), 2 mM L-glutamine, 20nM HEPES (Sigma, St. Louis, MO, USA). For RPMI-1640 and M-199, 10% fetal bovine serum (FBS, HyClone, Logan, Utah, USA) was added.

Growth factors for DCs generation were GM-CSF (200 ng/mL; LG. Co., Daejeon, Korea), IL-4 (1,000 unit/mL; BD Pharmingen, San Diego, CA, USA). TNF-α (1,000 unit/mL), IL-6 (100 unit/mL), IL-1β (100 unit/mL; BD pharmingen, San Diego, CA, USA), Prostaglandin E_2 (Sigma, St. Louis, MO, USA).

Fluorochrome-labelled monoclonal antibodies were used to analyze phenotypes of cells in PBMC or cultured DCs. They are: Lin^--FITC/CD11c-PE, CD4-

FITC/CD8-PE, CD3-FITC/CD16+CD56-PE (BD pharmingen, San Jose, CA, USA), CD1a-PE, CD80-PE, CD83-FITC, CD86-FITC (BD pharmingen, San Jose, CA, USA).

2. Subjects

Peripheral blood was obtained from 8 normal healthy donors and 8 patients with pancreatic cancer. All patient samples were evaluated before chemotherapy to prevent variations in immune parameters by treatment. Patient blood samples containing EDTA tube were centrifuged 3000rpm at 4℃ for 10min to separate serum and cells. After centrifugation, peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy donors or patients by centrifugation over Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ, USA) gradients at 20℃, 900 x g for 30min. PBMCs were collected carefully, washed 3 times with PBS, and stained with MoAbs (Lin^-/CD11c, CD4/CD8, CD3/CD16+CD56) for flow cytometry. Data were analyzed with WINMdi program (ver. 2.8) to evaluate of immune parameter. The

collected sera were filtered through a 0.2μm filter (Pall Co., Ann Arbor, MI, USA). Isolated sera were stored at -70℃ and cells were used to test the inhibition of DC generation by serum derived pancreatic cancer patients.

3. Culture of monocyte derived dendritic cells

A. Preparation of peripheral blood mononuclear cells

PBMCs were isolated from buffy coat which was donated kindly from healthy volunteers by Ficoll-Hypaque gradient centrifugation. After centrifugation, PBMCs were collected and then washed with PBS.

B. Isolation of monocyte from peripheral blood mononuclear cells

Prepared PBMCs were resuspended with 500 $\mu\ell$ PBS containing 0.5% BSA and were incubated with magnetic bead conjugated $CD14^+$ antibody (200 $\mu\ell/2\times10^8$

PBMCs; Miltenyi Biotech, Auburn, CA, USA) at 4℃ for 15min. PBMCs were washed with cold PBS and resuspended with 3ml. PBMCs were processed through a MACS magnetic separation column (Miltenyi Biotec) to obtain purified $CD14^+$ cells.

C. Generation of monocyte derived dendritic cells

$CD14^+$ cells (2×10^6cells/mL) in 5ml RPMI 1640 supplement with 10% FBS (HyClone, Logan, Utah, USA) and 1% antibiotics were cultured in 6 well plate and incubated at 37℃ and 5% CO_2. On day1, 3, 5, GM-CSF (200 ng/mL), IL-4 (1,000 unit/mL) was added. On day 6, cytokine cocktail containing TNF-α (1,000 unit/mL), IL-1β (100 unit/mL), IL-6 (100 unit/mL), PGE_2 (10^{-7}M) was stimulated to generate the mature DCs.

4. Generation of monocyte derived DCs with sera from pancreatic cancer patients

The sera derived from pancreatic cancer patients which were stored at -70℃ were dissolved quickly and performed heat-inactivation to prevent undesirable effects at 55℃ for 30min. DCs were generated with same culture condition adding to 2% heat-inactivated serum derived from pancreatic cancer patient or healthy donor for control instead of 10% FBS.

5. Flow cytometry analysis

Cell staining was performed using the panel of the monoclonal antibodies listed in *Reagents*. 5×10^5 cells were stained with 10 ㎕ of monoclonal antibodies (MoABs). Cells were incubated for 15min at 4℃ with MoABs in PBS. After

incubation, single- and two-color flow cytometric analyses were performed, using a FACScan flow cytometer (Becton Dickinson). Data were analyzed with WINMdi program. Each analysis included at least 10,000 events.

6. Endocytosis assay

FITC-conjugated Dextran (Sigma, St. Louis, MO, USA) was added to the final concentration of 1mg/mL to the cultured DCs and incubation for 45min at 37℃. After incubation, DCs were washed 3 times with ice-cold PBS and analyzed by flow cytometry.

7. Allogeneic mixed leukocyte reaction

To test T cell stimulatory functions, DCs (1×10^4 cells/well) were added to allogeneic T cells (1×10^5 cells/well) and co-incubated for 4 days in RPMI1640 with 10% FBS and 1% antibiotics in 96-wells plate. After 4 days, proliferation was determined by addition of 50 $\mu\ell$ ^{3}H-thymidine (Amersham Bioscience) at 0.5 μCi/well final concentration for 16hrs to triplicate well. ^{3}H-thymidine incorporation was measured using a liquid scintillation counter (Beckman, Palo Alto, CA, USA).

8. Annexin V-fluorescein and propidium iodide staining

Annexin V-FITC Apoptosis Detection Kit Ⅰ (BD pharmingen, San Diego, CA, USA) was used to detect DC apoptosis. Cells were washed (1×10^6cells) with PBS and centrifuged at 200 g for 5 min. Cell pellets were resuspended in 100 $\mu\ell$ of staining solution (20 $\mu\ell$ of annexin V-fluorescein, and 20 $\mu\ell$ of propidium iodide (PI) in 1ml of HEPES buffer and incubated for 10-15 min at room temperature. Stained cells were

then analyzed on a flow cytometer using 488 nm excitation and 515 nm band pass filter for fluorescein detection and a filter 600 nm for PI detection.

Ⅲ. RESULTS

1. Expression of surface molecules in monocyte derived DCs generated at different culture conditions

Isolated $CD14^+$ cells were cultured at different media and matured with several combinations of cytokines. Monocyte-derived mature DCs cultured with RPMI-1640 had higher quantity of CD83, a typical mature DC marker, and CD86, a costimulatory molecule for T cell stimulation, expression than that of X-VIVO 10 or M199 media (Fig 1). Therefore, the next series of experiments were performed with RPIMI-1640 media.

To determine optimal cytokine combination for maturing DCs, different combinations of cytokines were added to immature DCs on Day 6 (Fig 2). After maturation for 72 hrs, the cocktail mixture of TNF-α, IL-1β, IL-6 and PGE_2 induced

the highest level of mature DC markers and costiumlatory molecules such as CD80, CD83, and CD86.

2. The variation of immune parameters in pancreatic cancer patients

To compare the immune cell population in the peripheral blood between healthy donors and pancreatic cancer patients, isolated PBMCs were stained with specific monoclonal antibodies against cell-type specific markers that can detect DCs (CD11c), NK cells (CD16, CD56) and T lymphocytes (CD4, CD8) (Fig 3). Here, PBMCs from 8 pancreatic cancer patients and 8 normal healthy volunteers were stained with various monoclonal antibodies and analyzed with flow cytometry to measure and compare the contents of immune cells. The patient group was composed of five men (62.5%) and three women (37.5%), with a median age of 58.85 ± 15.3 years (Table I). The percentage of $Lin^{-}CD11c^{+}$ which are DCs in healthy donors was

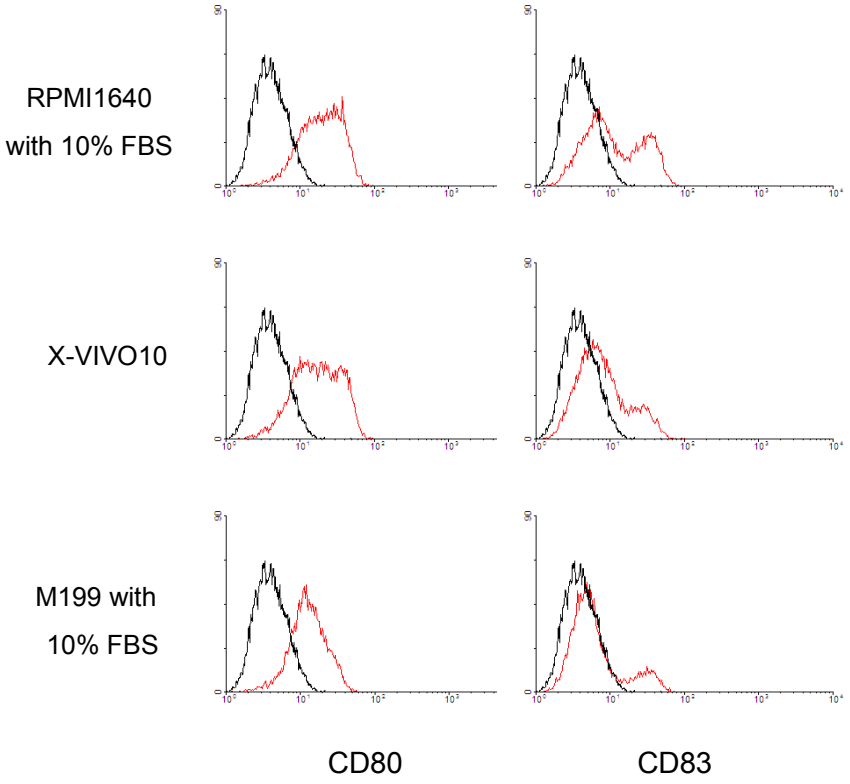


Fig 1. Phenotype of DCs generated in RPMI1640, M199 medium and serum-free X-VIVO10 medium. To define the optimal culture condition, mature DCs were generated by using different culture conditions, RPMI1640 with 10% FBS, X-VIVO10, M199 with 10% FBS. The characteristic phenotype of mature DCs was exhibited by these media. Among them, mature DCs were expressed costimulatory molecules (CD80, CD83) highly in RPMI1640 with 10% FBS condition. The cells were stained with FITC-conjugated monoclonal antibodies and analyzed by FACS.

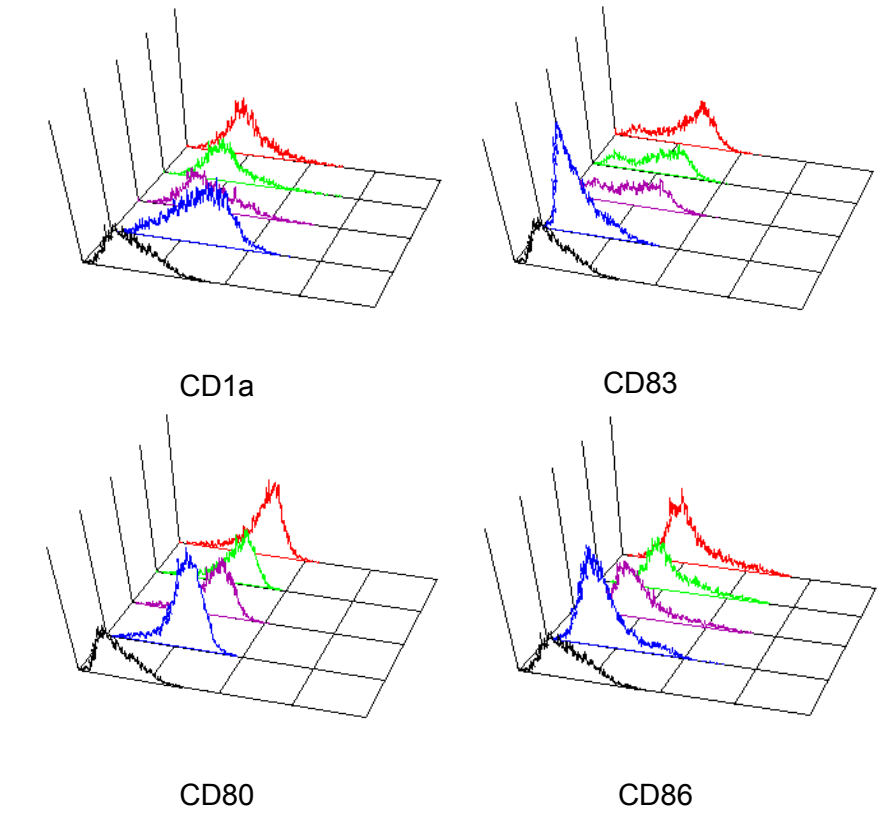


Fig 2. Phenotype of DCs generated in different maturation conditions. Immature DCs and mature DCs cultured in various conditions were stained and analyzed by FACS. Control sample stained with isotype matched monoclonal antibody was shown in black. Immature DC (blue line) expressed high levels of CD1a and CD80, but little CD83. Mature DCs exhibited different amounts of markers depending on their

about 1.6% of PBMCs, whereas it was decreased to 0.7% in PBMCs of pancreatic cancer patients (Fig. 4A). The number of Lin-CD11c^{+} in PBMCs of pancreatic cancer patients was significantly decreased about 2 folds comparing with healthy donors (Fig. 4B). While both CD4^{+} and CD8^{+} cells were dropped in number as well as proportion, reduction of CD4^{+} cells was more significant (64.75×10^{4}cells/mL to 18.69×10^{4}cells/mL and 34.53% to 13.87%). In CD16^{+}CD56^{+} cells, the percentage was reduced (19.15% to 11.65%), and the number of these cells was also decreased (36.72×10^{4}cells/mL to 18.16×10^{4}cells/mL) (Fig. 5).

3. The comparison of DCs between cultured with sera from healthy donors and pancreatic cancer patients

A. DCs generated in media supplemented with sera from pancreatic cancer patients are phenotypically different from DCs culture in media with sera derived from healthy donors

Table. 1 Characteristics of study subjects and their immunologic parameters

		Healthy donor	Pancreatic cancer patient
Number		**8**	**8**
Age (years)		**47.0 ±4.2**	**58.5 ± 15.3**
Sex (Male:Female)		**5 : 3**	**5 :3**
CD11C $^{+}$			
Mean ±SD	Percentage (%)	**1.56 ± 0.65**	**0.68 ± 0.27**
	Cell number	**2.84 ± 0.85**	**1.21 ± 1.07**
CD4^{+}			
Mean ±SD	Percentage (%)	**34.52 ± 5.19**	**13.87 ±**
	Cell number	**64.75 ±**	**18.69 ± 9.10**
CD8^{+}			
Mean ±SD	Percentage (%)	**22.26 ± 3.62**	**13.02 ± 5.59**
	Cell number	**41.80 ± 9.58**	**19.35 ± 8.08**
CD16^{+}CD56^{+}			
Mean ±SD	Percentage (%)	**19.15 ± 8.02**	**11.65 ± 5.32**
	Cell number	36.72 ± 16.88	18.16 ± 9.24

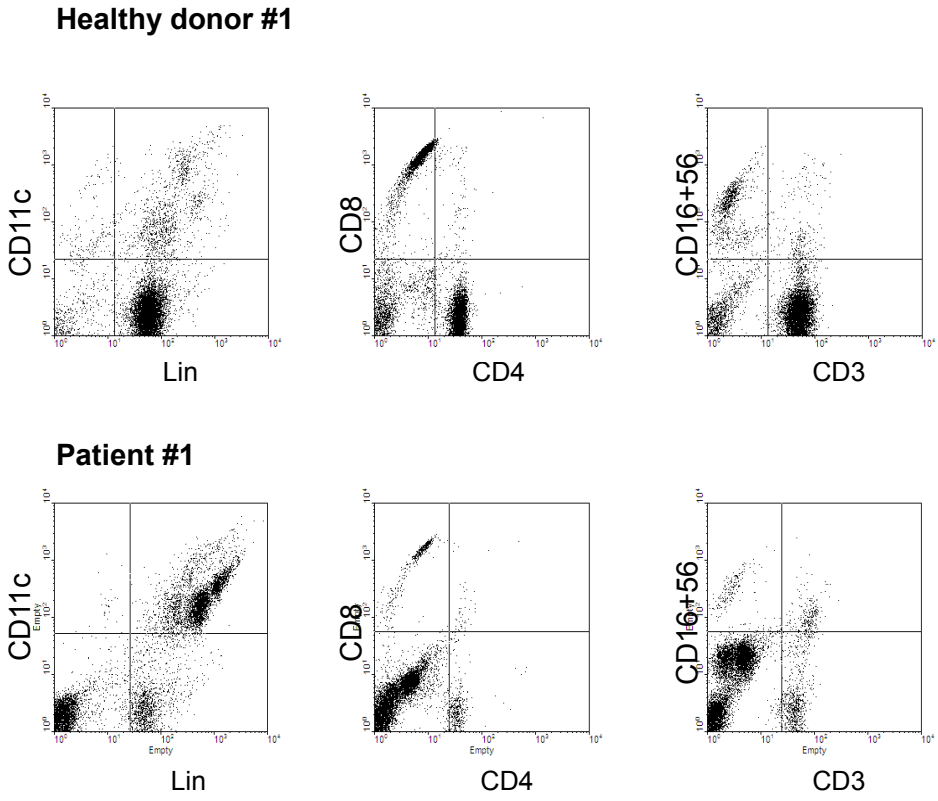


Fig 3. Representative data of PBMCs from healthy donors and pancreatic cancer patients. Isolated PBMCs were stained with antibodies against cell-type specific markers to measure immunological parameters. FITC conjugated antibodies were Lineage cocktail (Lin^-), CD4, CD3 and PE conjugated antibodies were CD11c, CD8, CD16+CD56. The proportion of each cell type was reduced in patient samples compared to control.

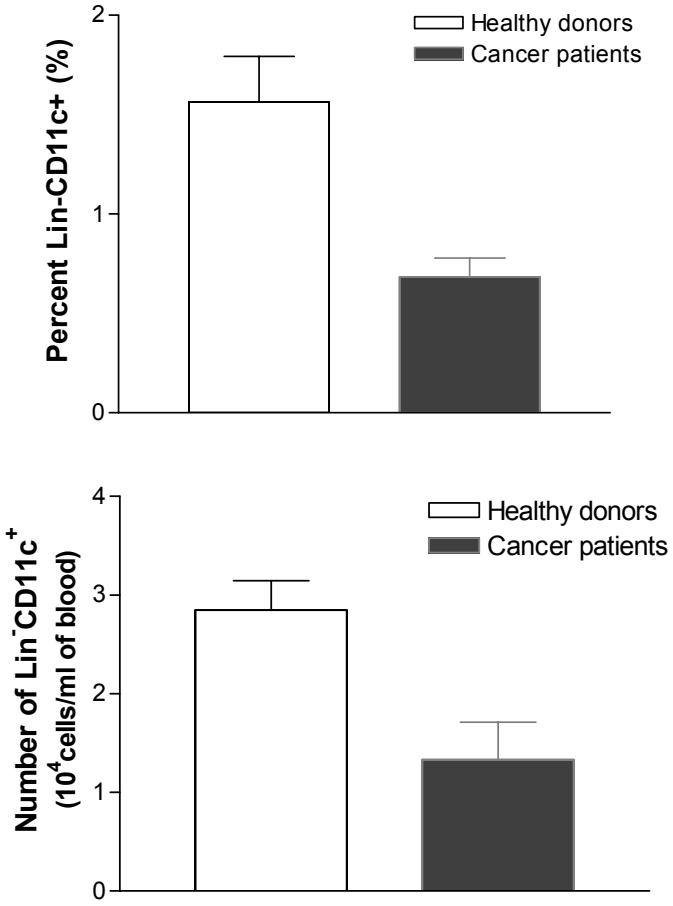


Fig 4. The proportion of DCs in peripheral blood from healthy donors and pancreatic cancer patients. The percentage of $Lin^{-}CD11c^{+}$ (DCs) cells was decreased in pancreatic cancer patients by half of the healthy donors and the number of $Lin^{-}CD11c^{+}$ cells was reduced by 61% (2.84x104cells/mL to 1.12x104cells/mL) in pancreatic cancer patients. The values are mean ± SD of 8 patients and 8 healthy donors.

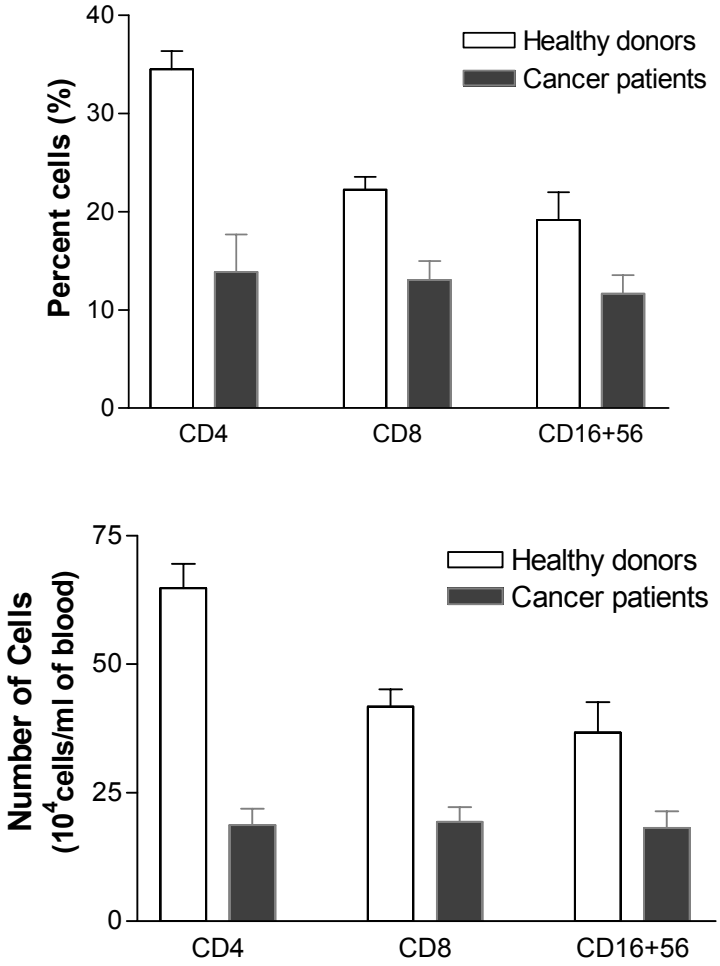
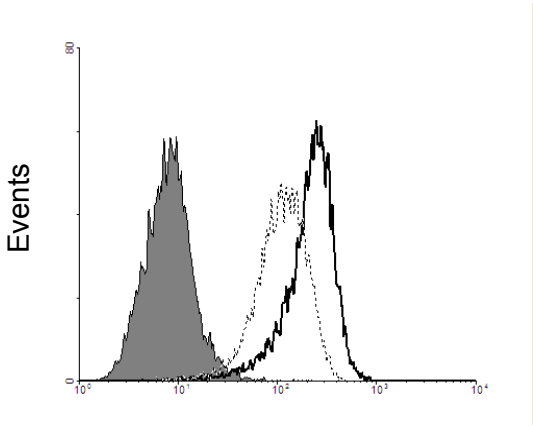


Fig 5. Comparison of peripheral blood mononuclear cell composition between pancreatic cancer patients and those from healthy donors. The overall immunological parameters were significantly reduced in pancreatic cancer patients. $CD4^+$ T lymphocytes were the most affected cell types in terms of proportion as well as number. $CD8^+$ cells were decreased also but lesser degree. The number and proportion of $CD16^+CD56^+$ cells were slightly decreased in pancreatic cancer

Mature DCs cultured with sera derived from healthy donors displayed typical DC phenotypes, whereas DCs cultured in the presence of a pancreatic cancer patients' sera exhibited a decrease in CD86 expression (Fig 6). These results suggest that T cell costimulatory activity of DCs generated with patient's sera is weaker than that of DCs cultured with sera of healthy donors.

B. Endocytosis and induction of T lymphocyte proliferation by dendritic cells are inhibited by sera from pancreatic cancer patients

To confirm this possibility, the functional activities of these DCs were compared. Antigen uptake ability of immature DCs was assessed by FITC-conjugated Dextran endocytosis assay. DCs cultured with sera derived from pancreatic cancer patient displayed 50% reduction in endocytic capability compared to immature DCs cultured with sera from healthy donors (Fig 7). Next, antigen presentation activity was conducted by allogeneic Mixed Leukocytes Reactions (MLR). For that, DCs and T



····· : Immature DCs cultured with 2% cancer serum

——— : Immature DCs cultured with 2% normal serum

Fig 6. Effect of pancreatic cancer sera on FITC-Dextran endocytosis of immature DCs. Monocytes from normal PBMCs were cultured with GM-CSF and IL-4 in the presence of either sera from cancer patients or normal. The endocytosis of immature DCs was measured at Day 6. Immature DCs cultured with 2% sera derived pancreatic cancer patient (dotted line) were decreased the phagocytosis compared to that of immature DCs cultured with 2% normal sera. DCs (2×10^6 cells) were incubated with FITC conjugated Dextran (1mg/mL) for 1hr at 37℃. Background uptake at 4℃ was served as negative control. After incubation, collected DCs were washed with PBS and fixed with 1% paraformaldehyde. The result was analyzed by FACS.

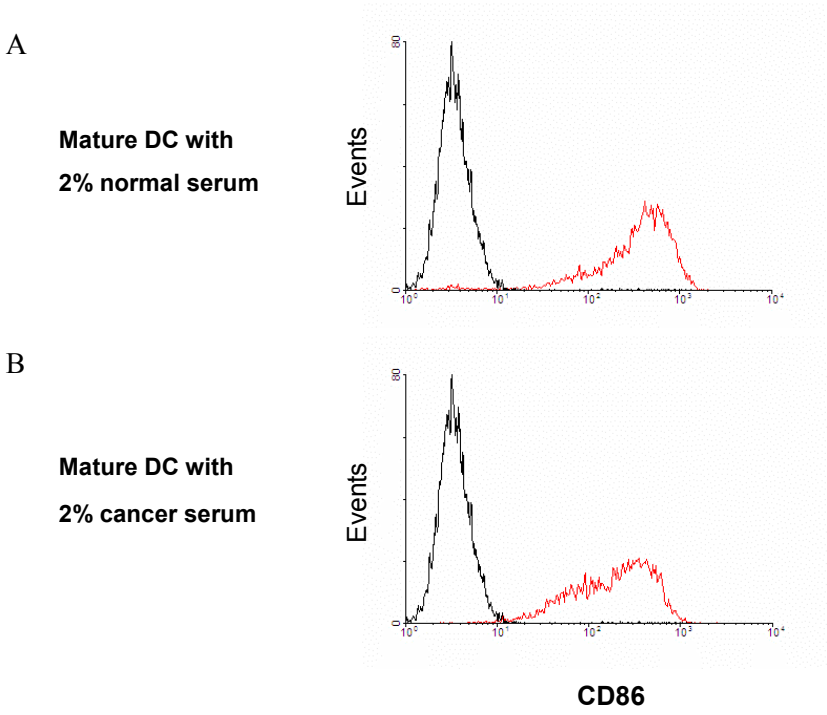


Fig 7. The phenotype of mature DCs cultured in the presence of sera from healthy donors (A) or pancreatic cancer patients (B). To generate mature DCs, immature DCs were cultured with TNF-α, IL-1β, IL-6 and PGE_2 in the presence of either 2% sera from healthy donors or 2% sera from cancer patients. Costimulatory molecule, CD86, on DCs was analyzed by FACS. Mature DCs cultured with serum from a pancreatic cancer patient were reduced in the expression of costiumlatory molecule (CD86).

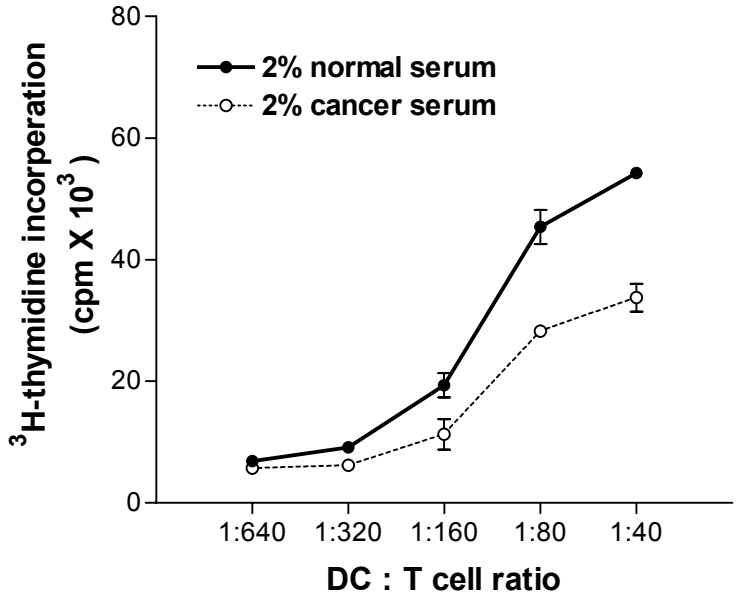


Fig 8. The capability of inducing allogeneic T cell stimulation of DCs cultured in 2% sera from healthy donors or pancreatic cancer patients. Mature DCs generated in the presence of serum from a healthy donor provided a potent stimulation to allogeneic T lymphocytes which was 30% stronger than DCs cultured with serum from a pancreatic cancer patient. Value is the mean ±SD obtained by triplicate cultures.

cells collected from different donors were used. In agreement with the previous phenotypic data, mature DCs cultured with sera derived pancreatic cancer patients induced significantly less proliferation of allogeneic T lymphocyte than that of mature DCs cultured with sera derived from healthy donors (Fig 8) .

4. Suppression of endocytosis by dendritic cells generated with mixture of inhibitory factors

It has been reported that cancer sera contain mixture of immunosuppressive cytokines[5]. TGF- β and VEGF were highly expressed in pancreatic cancer patients. However, it is unclear whether singular or combinations of these cytokines are responsible for the inhibitory effect of patient's sera on DC function and phenotype observed. To verify the cytokine-induced inhibition of DC function and phenotype, various combinations of TGF-β, IL-10 and VEGF were applied to the DC generation.

As shown in Fig 9A, uptake of FITC conjugated Dextran was severely impaired in immature DCs cultured with inhibitory cytokines mixture. This result was displayed similar pattern with immature DCs cultured with sera derived from pancreatic cancer patients. The endocytosis activity of immature DCs cultured in the presence of inhibitory cytokines mixture reduced about 40% in mean fluorescence intensity (MFI) compared with that of immature DCs which were cultured in absence of inhibitory mixtures (Fig 9B).

5. Reduction of T cell stimulation by mature dendritic cells cultured with mixture of inhibitory factors

An allogeneic MLR was used to compare the T cell stimulatory activity of control DCs and DCs exposed to the mixture of inhibitory factors. Mature DCs cultured under normal conditions showed strong T cell stimulation. Mature DCs

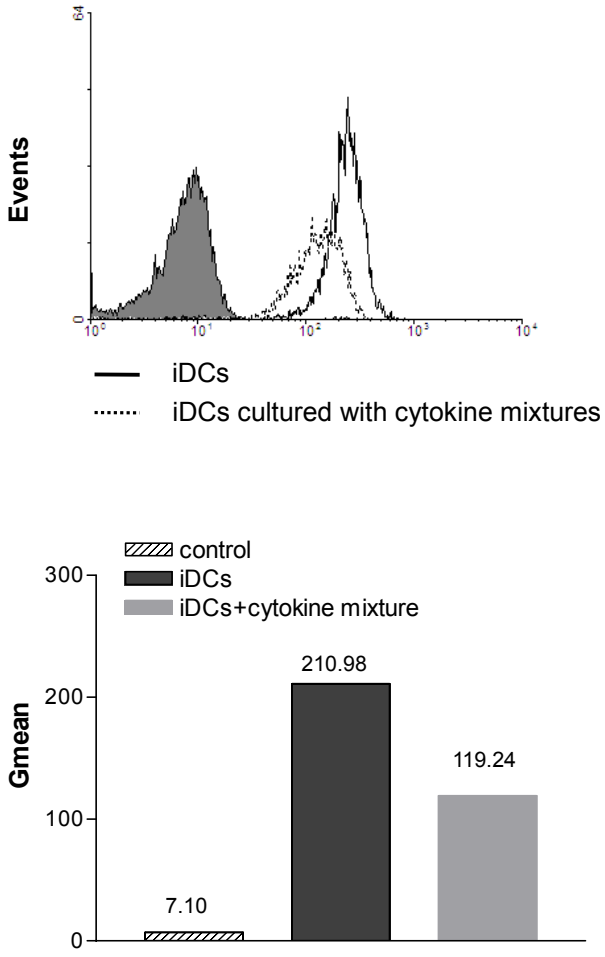


Fig 9. Effect of IL-10, VEGF and TGF-β on maturation of DCs. Immature DCs were generated from monocytes in the presence or absence of cytokines mixture (IL-10, VEGF and TGF-β). Endocytic activity was measured at Day 6 using FITC-Dextran and analyzed by FACS (A). The cytokine concentrations were IL-10 (10ng/mL), VEGF (10ng/mL), TGF-β (10ng/mL) (dotted line). The phagocytosis of DCs treated with cytokine cocktail was greatly reduced than that of immature DCs. iDC; immature DCs

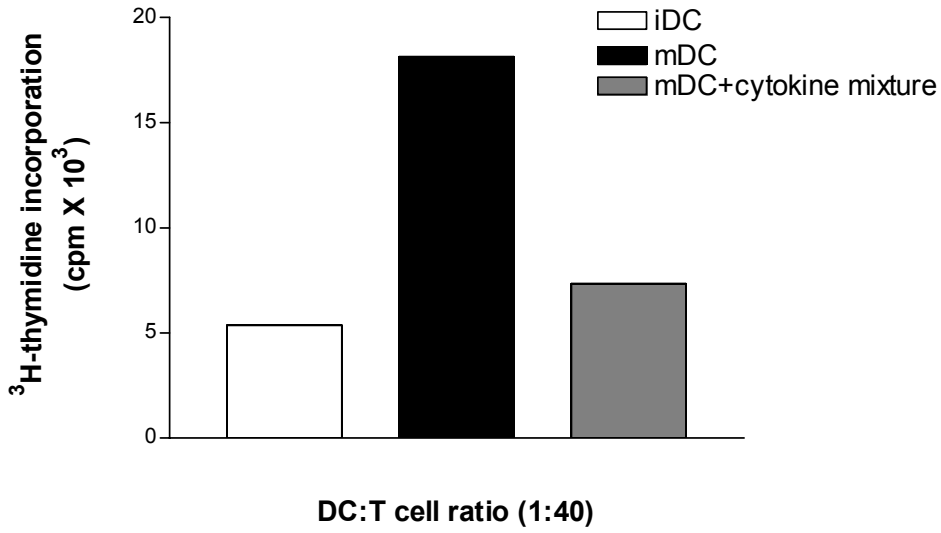


Fig 10. Allostimulatory capacity of mature DCs generated in the presence or absence of mixed cytokines mixture (IL-10, VEGF and TGF-β). PBMCs cells were used as responder cells at a concentration of $1x10^6$ cells/well. The stimulator cells ($2.5x10^4$cells/well) were co-cultured with responder cells as 1:40 ratio. ^{3}H-thymidine incorporation was measured using a β-counter. This representative data is shown among the repeated four trial. mDC; mature DCs

generated in the presence of inhibitory factors, however, reduced T cell stimulation by at least two folds than that of control (Fig 10). Therefore, these data clearly indicate that these cytokine combinations are the major inhibitory cytokines inhibiting both DCs phenotype and function.

6. Inhibition of the function of mature dendritic cells by IL-10

To determine which cytokine(s) affects most on the function of DCs, mature DCs were cultured with either IL-10, TGF-β and VEGF alone or combinations. Mature DCs generated with IL-10 alone were significantly impaired in their capacity to T cell stimulation (Fig 11). But IL-10 did not displayed any synergistic effect with other cytokines. Allogeneic PBMCs were used as responder cells at a concentration of $1x10^6$ cells/well. The stimulator cells ($2.5x10^4$cells/well) were co-cultured with responder cells as 1:40 ratio.

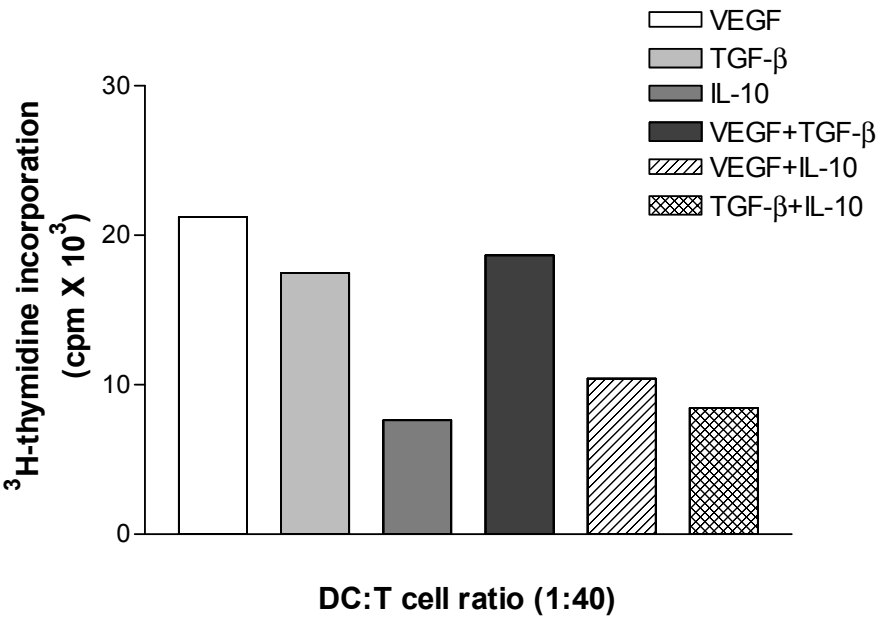


Fig 11. Contribution of singular or combinatorial cytokines in inhibiting DCs function. Allogeneic MLR was performed to measure T cell stimulatory capacity of DCs that were generated with various inhibitory cytokines combinations. Mature PBMC cells were used as responder cells at a concentration of $1x10^6$ cells/well. The stimulator cells ($2.5x10^4$cells/well) were co-cultured with responder cells as 1:40 ratio. T cell stimulation was measured by ^{3}H-thymidine incorporation with using β-counter. Results from one of three experiments are shown.

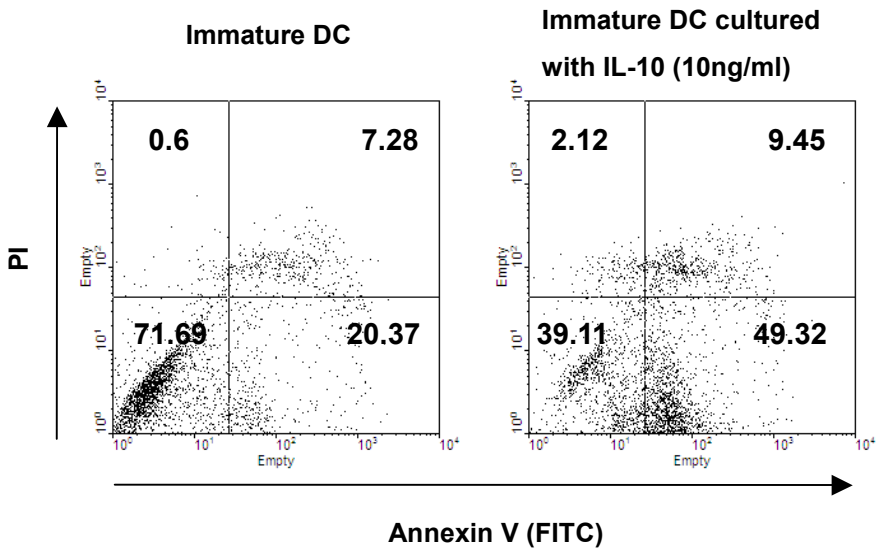


Fig 12. Induction of DCs apoptosis by IL-10. To examine the apoptosis induced by IL-10, DCs were cultured in the presence or absence of IL-10 (10ng/ml). In the presence of IL-10, late apoptosis (UR, PI-positive Annexin V-positive) and necrosis (UL, PI-positive only) proportion were slightly increased (0.66 to 2.12 and 7.28 to 9.45), whereas early apoptosis (LR, Annexin V-positive only) fraction was significantly increased about 2.5 folds from 20.37 to 49.32.

7. Induction of DC apoptosis by IL-10

The effect of IL-10-mediated DCs suppression was examined by DC recovery after culture. IL-10 severely reduced the DC output after maturation possibly via induction of apoptosis during culture. To prove this hypothesis, apoptosis was monitored by Annexin V and PI staining. DCs were cultured with GM-CSF, IL-4 and GM-CSF, IL-4 and IL-10 from Day 0 to 6. As shown in Fig 12, IL-10 induced the apoptosis in significant fraction of DCs. The proportion of necrotic and late apoptotic cells were increased slightly, whereas the portion of early apoptotic cells was increased over 2 times by IL-10.

V. DISCUSSION

A number of previous studies have demonstrated that tumor induced the general immune deficiencies. These include the reduction of both number and functions of immunological parameters. Of these, the functional defects of dendritic cell differentiation have been implied as an important mechanism of tumor escape from immune surveillance[16]. In case of pancreatic adenocarcinoma that is one of the worst prognoses among cancers, significant decrease of DCs in tumoral area has been reported [17]. However, it is uncertain whether this decrease of DCs in tumor is a direct consequence of defects in DC function or in development in situ. Furthermore, contributions by other immune-associated cells in pancreatic cancer patients have not been thoroughly studied.

I have examined 1) the general immunologic parameters in pancreatic cancer patients in relation to immunosuppression, 2) whether DCs generated from patients'

sera are defective bona fide, 3) which cytokine of patients' sera plays the major inhibitory role in DC maturation.

To establish optimal condition for DC generation from peripheral blood monocytes, three different media were examined. The data clearly indicated that RPMI-1640 with 10% fetal bovine serum gave the best output in terms of DC phenotypes. The costimulatory factor CD80, which is the ligand for CD28 on T lymphocytes, and CD83, one of the DC markers, are highly expressed on DCs cultured in RPMI-1640 with10% FBS in comparison with serum free media, X-VIVO 10, or M199 with 10% FBS.

To define the optimal condition for generating fully mature DCs, three different protocols have been applied to immature DCs. The results showed that inflammatory cytokine cocktail, which is a mixture of TNF-α, IL-1β, IL-6 and PGE_2, is superior than that of TNF-α alone or TNF-α with PGE_2.

As shown by Liyanage et al.[18], general reduction of immune cell contents has been considered the major cause of immune deficiency in cancer patients. The results

of this study support the idea that, in patients suffering from pancreatic carcinoma, the numbers of immune surveillance-related cells were decreased compared to that of healthy donor's. The levels of $Lin^{-}CD11c^{+}$ cells (DCs), $CD8^{+}$ lymphocytes (cytotoxic T cells) and most prominently $CD4^{+}$ lymphocytes (helper inducer cell) were decreased significantly. Moreover, the ratio of CD4/CD8 lymphocytes was decreased by 30-50% in PBMCs of patient with pancreatic carcinoma as compared with that of controls. In addition, the results indicated that there is a significant reduction of DCs in peripheral blood from 1.6% in healthy donors to 0.7% in patients. Thus, these results suggest that adaptive arm of immune systems are severely impaired quantitatively. Furthermore, $CD16^{+}CD56^{+}$ NK cells or $CD3^{+}CD16^{+}CD56^{+}$ NKT cells were significantly reduced suggesting that the components of innate immune system are affected by pancreatic cancer.

Since DCs are the key player in induction of immune response against cancer, this reduction in DC number may be critically associated with cancer-induced immune suppression in patients. It has been suggested that pancreatic cancer could induced the

inactivation of DCs that mediate antigen specific T lymphocyte stimulation in immune system[19]. It is becoming increasingly evident that this contributes significantly to the escape of host immune surveillance in pancreatic cancer particularly[16]. However, the underlying mechanism that leads to this immune evasion is unclear. Decreased immune function in cancer patients is well-characterized, and tumor cells have developed a variety of mechanisms to avoid anti-tumor immune responses[20]. One mechanism for inhibition of immune cell function by tumors is the production of soluble factors, such as IL-10, TNF, TGF-β, and VEGF[21-27]. Furthermore, these cytokines are known to affect DC generation and/or function. VEGF is produced by most tumors, and its production is closely associated with poor prognosis[28]. TGF-β is another immunosuppressive cytokine that are known to be involved in carcinogenesis as well as in defects of host immune system[29]. The association of the level of IL-10 and immunosuppression in sera of patients with pancreatic carcinoma and gastrointestinal cancer was also reported recently[30,31].

I addressed the question whether this reduction of DC number in pancreatic cancer patients is due to cancer environment, i.e., cancer sera or cancer-derived

immunomodulatory cytokines. This was assessed by generating DCs from normal monocytes in the presence or absence of 1) sera from pancreatic cancer patients or 2) immunomodulatory cytokines or mixture of cytokines.

DCs cultured with 2% sera derived from pancreatic cancer patients displayed defects in both phenotype as well as function. For phenotypic analysis, DC-associated markers, such as CD80, CD83 and CD86, were examined. For functional analysis, antigen uptake capability by endocytosis of FITC-Dextran and antigen presentation capability by allogeneic MLR were assessed. Mature DCs were cultured in presence of sera derived from pancreatic cancer patients. The expression of costimulatory molecule (CD86) on mature DCs was severely reduced. In functional test of immature DCs, endocytosis by DCs cultured with sera from pancreatic cancer patients was decreased significantly compared to that of DCs cultured in the presence of sera derived from healthy donors. In addition, mature DCs generated with sera derived from cancer patients did not stimulate T cell as much as DCs cultured with sera from healthy donors. These data indicate that sera from cancer patients contain soluble factors

inhibiting DC generation and/or DC function. Previous studies reported that TGF-β, IL-10 and VEGF were significantly increased in the sera derived from pancreatic carcinoma patients compared to that of healthy donors[32,33].

To identify the nature of immunosuppressive activity in cancer patient's sera, three major immunomodulatory cytokines known to be highly secreted in pancreatic cancer were screened. When DCs were generated in the presence of these inhibitory factors, the mixture of inhibitory factors has been shown to reduce functions of endocytosis in immature DCs as well as of T cell stimulation in mature DCs. This result mirrored the inhibitory effects of cancer patient's serum on DCs. Among those cytokines, IL-10 had a strongest inhibition on DCs. Moreover, IL-10 induced early apoptosis in the generation of monocyte derived dendritic cells, thereby affecting the recovery as well as function of DCs in culture. The physiological significance of increased level of IL-10 in the sera of pancreatic cancer patients is unclear. Tumor cells may produce IL-10 by themselves[34,35] for either to promote the tumor growth or to disturb host immune system by modulating hematopoiesis as well as adaptive immune

responses or both. Studies of syngeneic mouse tumor model systems have shown that IL-10 when overexpressed by tumor cells and this cytokine modulate the antitumor immune response with significant benefits for survival and growth of the tumor cells in host[14]. In humans, IL-10 was reported to inhibit proliferation and effector functions of T cells and to prevent the antigen-presenting functions of DCs[36,37]. In agreement with these reports, I have demonstrated that IL-10 significantly affected the generation of DCs from monocytes, the survival of DCs and antigen presentation function.

It has been demonstrated that (1) monocyte-derived DCs are both phenotypically and functionally intact when they are generated in the absence of sera from cancer patients. However (2) DCs cultured with sera of pancreatic cancer patients displayed functional defects. These data imply that, since sera from pancreatic cancer patients contain many of these inhibitory cytokines, culturing DCs in the presence of sera from pancreatic cancer patients must be avoided. This information may provide basic protocols of DC-based immunotherapy that can be applied to clinics.

VI. CONCLUSION

In these experiments, optimal culture condition was defined for DC generation in RPMI-1640 supplemented with 10% FBS and cytokine cocktail for maturation. The portion of immune surveillance related cells such as DCs, helper T cells, cytotoxic T cells and NK cells were decreased in peripheral blood mononuclear cells of pancreatic cancer patients. DCs were cultured with sera derived from pancreatic cancer patients and their function and marker expression were observed. The ability to uptake antigen in immature DC and to stimulate allogeneic T cell by mature DCs as well as the expression of DC associated markers were decreased significantly.

The inhibition on DCs generation was represented in vitro with mixture of inflammatory cytokines in the place of serum derived pancreatic cancer patients. The mixture of inhibitory factors displayed similar results as the one cultured in the presence of sera derived from pancreatic cancer patients. DCs cultured in the cytokine mixture displayed defects in phagocytosis and T cell stimulation. Especially, IL-10

strongly inhibited T cell stimulation in mature DCs and induced early apoptosis in immature DC generation.

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ABBREVIATION LIST

Abbreviations	Full names
DC	Dendritic cell
iDC	Immatured dendritic cell
mDC	Matured dendritic cell
GM-CSF	Granulocyte macrophage-colony stimulating factor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
MoAB	Monoclonal antibody
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cell
PGE_2	Prostaglandin E2
PI	Propidium iodide
TGF-β	Tumor growth factor-beta
TNF-α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor

Abstract (in Korean)

췌장암 환자의 혈청에 의한 수지상세포 분화 억제

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구 태 석

췌장암은 예후가 극히 불량한 암으로 흔히 p16, p53, K-ras 와 같은 유전자의 변화가 흔히 발견된다. 췌장암 세포로부터 발현된 면역억제 인자들에 의하여 수지상 세포의 기능을 억제하여 면역 감시체계로부터 벗어날 수 있다는 연구 결과가 일부 보고 되고 있으나, 근본적인 기전에 대해서는 현재까지 연구가 이루어지지 않고 있다.

따라서 본 연구에서는 췌장암 환자의 혈액 중 면역반응에 관여하는 세포들의 종양에 의한 억제 현상을 이해하기 위하여 췌장암 환자의 혈액에서의 항원제공 세포인 수지상세포 (DC)와 T 림프구 (CD4, CD8)와 자연독살세포 (CD15+56)와 같이 면역반응에 관여하는 제반 세포를 유세포

분석을 통하여 정상인과 비교, 분석하였다. 췌장암 환자의 경우에 있어서 수지상 세포뿐 아니라 면역체계에 관여하는 제반 세포의 수가 정상인에 비하여 감소된 것을 확인할 수 있었다.

췌장암 환자의 혈청에서 특이적으로 분비가 증가된 면역억제 인자와 수지상세포의 기능 결손에 대하여 알아보기 위하여 췌장암 환자 유래의 혈청을 첨가한 상황에서 수지상세포의 분화를 유도하였다. 정상인의 혈청을 이용하여 분화를 시킨 수지상 세포에 비하여 특이적 세포표면 단백질의 발현이 감소하였고, 기능상에 있어서도 현저히 감소된 것을 알 수 있었다. 이러한 사실을 바탕으로, 췌장암 환자의 혈청에서 특이적으로 증가한 면역억제 인자들 중 VEGF, TGF-β, IL-10 재조합 단백질을 첨가하여 수지상세포 분화를 유도한 결과, 췌장암 환자의 혈청을 첨가하여 분화시킨 수지상세포에 나타난 기능상의 저하가 재현되었으며, T 림프구를 자극하는 기능이 IL-10 을 첨가하여 분화시킨 수지상세포의 경우에 상당히 저하된 것으로 나타났다. 또한 수지상 세포의 분화과정에 있어서 IL-10 에 의한 세포사멸이 유도된 것을 알 수 있었다.

췌장암 환자의 면역관련 세포들의 수적 감소와 췌장암 환자의 혈청에 의한 수지상세포의 기능 저하는 췌장암 세포로부터 분비되는 면역억제인자에 의한 것임을 알 수 있었으며, 이상의 결과는 임상적으로 췌장암 환자의 세포 기반 치료법에 유용한 정보를 제공할 것이다.

핵심되는 말 : 췌장암, 수지상세포, 면역감시관련 세포, 면역 억제인자