Table 4. Quality-of-Life Scores

QLQ-C30[a]	Mean±SD Score: Ghrelin Group	Placebo Group	*P*
Global health status score			
Before	78±30	74±22	.51
After	52±18	26±13	< .0001
Functional scales			
Physical functioning			
Before	86±8	92±10	.62
After	78±20	72±18	.42
Role functioning			
Before	80±12	88±8	.43
After	68±16	70±15	.29
Emotional functioning			
Before	78±14	82±12	.26
After	70±18	68±14	.44
Cognitive functioning			
Before	88±11	90±10	.72
After	86±14	88±18	.67
Social functioning			
Before	84±20	82±22	.54
After	82±16	78±14	.52
Symptom scales/items			
Fatigue			
Before	12±6	14±8	.37
After	22±11	34±16	.082
Nausea/vomiting			
Before	5±6	4±7	.62
After	16±14	36±29	< .0001
Pain			
Before	8±6	7±9	.47
After	10±11	12±14	.59
Dyspnea			
Before	8±14	7±13	.68
After	8±12	7±14	.66
Insomnia			
Before	12±8	14±12	.75
After	20±12	19±14	.37
Loss of appetite			
Before	8±14	7±13	.43
After	26±14	54±22	< .0001
Constipation			
Before	7±13	8±12	.29
After	12±18	14±20	.21
Diarrhea			
Before	12±14	12±18	.69
After	22±18	26±22	.32
Financial difficulties			
Before	16±22	18±17	.58
After	18±24	16±21	.72

Abbreviations: SD, standard deviation.
[a] Before indicates before chemotherapy; After: after chemotherapy (day 8).

oral intake of calories decreased significantly to about 25% of the baseline level at day 8 after chemotherapy despite the use of a 5-HT3 antagonist.

Several observations suggest that ghrelin may play an important role in the delayed cisplatin-induced gastrointestinal effects. In rodents, a single cisplatin administration caused a transient decrease in plasma ghrelin concentration and prolonged suppression of both food intake and body weight loss.[22] Cotreatment with a 5-HT3 antagonist did not result in the recovery of ghrelin levels or dietary activity in that experiment. In our clinical study, we observed that chemotherapy that included cisplatin reduced plasma ghrelin levels to 67% and 57% of the baseline levels on days 3 and 8, respectively. In addition, there was a close relation between the extent of decline in plasma ghrelin, nutritional status, and adverse events of chemotherapy.[23] In the current trial, we demonstrated that the administration of synthetic ghrelin during chemotherapy successfully increased food intake and appetite. This effect may be explained by the effect on the GH/IGF-1 axis. The growth-promoting effect of GH is mediated, at least in part, by IGF-1.[24] However, serum GH and IGF-1 levels were stable in both groups, probably because of the rapid turnover of GH. Although this phenomenon was reported previous in earlier studies,[14,24] we should have measured GH and IGF-1 in a brief period.

5-HT3 antagonist also was administered in the current clinical study. Taken together, the acute and delayed effects of cisplatin on gastrointestinal functions may involve different mechanisms, and the delayed effects, which seemingly are not mediated through the 5-HT3 receptor, affect nutrition status in cancer patients more strongly than the acute effects.

Conversely, recent reports indicate that both the 5-HT2C receptor and the 5-HT2B receptor, but not the 5-HT3 receptor, mediate cisplatin-induced ghrelin suppression in rodents.[13,22] The 5-HT2B receptor is distributed mainly in gastrointestinal smooth muscle,[25] and the 5-HT2C receptor is localized in the central nerve system.[26] Vagal nerve function may regulate afferent and efferent signaling, which controls ghrelin secretion through these 5-HT2B and 5-HT2C receptors. However, in our previous study, ghrelin was administered to patients who had undergone gastrectomy and esophagectomy, which also included truncal vagotomy, and we observed significant effects on appetite and body weight increase.[10,11] Therefore, the association between ghrelin signaling and the vagal nerve remains unresolved.[27] In the literature, urinary 5-hydroxyindole acetic acid (5-HIAA), the major metabolite of 5-HT, increased rapidly and subsequently returned to baseline within the first 24 hours after cisplatin administration, and it was associated strongly with chemotherapy-induced emesis.[3,4,12,28] In the current study, serum 5-HT and 5-HIAA levels on days 3 and 8 of chemotherapy did not increase significantly compared

with baseline values (data not shown). Thus, because plasma ghrelin undergoes rapid turnover, our observation regarding 5-HIAA suggests that 5-HT does not directly control ghrelin secretion.

In other studies, substance P and neurokinin-1 (NK-1) receptor contributed to the delayed emetic symptoms associated with chemotherapy.[29] Accordingly, an NK-1 receptor antagonist could inhibit the binding of substance P to the NK1 receptor in the vomiting center.[29] Several studies have established that administration of such antagonists, such as aprepitant, together with the 5-HT3 receptor antagonist, lessens chemotherapy-induced nausea and vomiting in patients who are receiving emetogenic chemotherapy during the first 120 hours after initiation of chemotherapy.[30] Although aprepitant was not used commonly during the study period in our country, it is now used widely in clinical practice. Although the exact functional association between ghrelin and NK-1 receptor still is under investigation, their synergistic effect would be novel, and it would be interesting to resolve this issue in a clinical setting in the near future.

Exogenous ghrelin, as expected, successfully increased oral intake and nutritional status and also maintained QoL during chemotherapy. However, our ultimate objective is to ease the completion of chemotherapy and to enhance the overall antitumor effect. In this study, the required dose modifications in the second cycle of chemotherapy tended to be fewer in the ghrelin group (6 patients; 30%) than in the placebo group (10 patients; 50%). Specifically, modifications in the ghrelin group were because of 3 episodes of neutropenia, 2 episodes of thrombocytopenia, and 1 episode of nephrotoxicity; whereas the reasons for modifications in the placebo group included 6 episodes of neutropenia, 3 episodes of nephrotoxicity, and 1 episode of diarrhea. This suggests that ghrelin can prevent some adverse events directly in addition to its indirect effects through improvement of nutritional status. A larger cohort study is needed to verify this aspect of ghrelin administration.

Another clinical question to be answered is whether nutritional support during chemotherapy should be provided orally or intravenously.[31] Recently, we conducted a randomized trial to address this issue in patients with esophageal cancer who were receiving cisplatin-based chemotherapy. Various adverse effects of the chemotherapy, including hematologic toxicity, were observed less frequently in patients who received forced enteral nutrition than in those who received parenteral nutrition, although their total calorie intake was identical (unpublished data). This observation encourages the clinical application of ghrelin administration, which can physiologically increase oral food intake.

In terms of chemotherapy regimens, for this study, both the ACF regimen and the DCF regimen were used. Recently, intensive chemotherapy protocols involving multiple drugs are in fashion; however, to use such regimens, the adverse effects of the regimen components must be adequately managed. An appropriate nutrition supplement through oral food intake will be more important in the future.

In conclusion, the current study demonstrated that short-term administration of exogenous ghrelin at the start of cisplatin-based chemotherapy stimulated food intake and minimized adverse events. We believe that ghrelin administration could increase the efficiency of chemotherapy, and we recommend the use of ghrelin in clinical practice.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

REFERENCES

1. Medical Research Council Oesophageal Cancer Working Group, Surgical resection with and without chemotherapy in oesophageal cancer: a randomized controlled trial. *Lancet.* 2002;359:1727-1733.
2. Kelsen DP, Ginsberg R, Pajak TF, et al. Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. *N Engl J Med.* 1998;339:1979-1984.
3. Cubeddu LX, Hoffmann IS, Fuenmayor NT, Malave JJ. Changes in serotonin metabolism in cancer patients: its relationship to nausea and vomiting induced by chemotherapeutic drugs. *Br J Cancer.* 1992;66:198-203.
4. Hesketh PJ, Van Belle S, Aapro M, et al. Differential involvement of neurotransmitters through the time course of cisplatin-induced emesis as revealed by therapy with specific receptor antagonists. *Eur J Cancer.* 2003;39:1074-1080.
5. Kojima M, Hosoda H, Date Y, et al. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature.* 1999;402:656-660.
6. Nakazato M, Murakami N, Date Y, et al. A role for ghrelin in the central regulation of feeding. *Nature.* 2001;409:194-198.
7. Van der Lely AJ, Tschop M, Heiman ML, Ghigo E. Biological physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocr Rev.* 2004;25:426-457.
8. Ariyasu H, Iwakura H, Yamada G, et al. Efficacy of ghrelin as a therapeutic approach for age related physiological changes. *Endocrinology.* 2008;149:3722-3728.
9. Akamizu T, Kangawa K. Translational research on the clinical applications of ghrelin. *Endocr J.* 2006;53:585-591.
10. Adachi S, Takiguchi S, Okada K, et al. Effects of ghrelin administration after total gastrectomy: a prospective, randomized, placebo-controlled phase II study. *Gastroenterology.* 2010;138:1312-1320.
11. Yamamoto K, Takiguchi S, Miyata H, et al. Randomized phase II study of clinical effects of ghrelin after esophagectomy with gastric tube reconstruction. *Surgery.* 2010;141:31-38.

12. Liu YL, Malik NM, Sanger GJ, Andrews PL. Ghrelin alleviates cancer chemotherapy-associated dyspepsia in rodents. *Cancer Chemother Pharmacol.* 2006;58:326-333.
13. Takeda H, Sadakane C, Hattori T, et al. Rikkunshito, an herbal medicine, suppresses cisplatin-induced anorexia in rats via 5-HT2 receptor antagonism. *Gastroenterology.* 2008;134:2004-2013.
14. Sobin LH, Wittekind C, eds. TNM Classification of Malignant Tumors. 6th ed. New York: John Wiley & Sons; 2002.
15. Shimakawa T, Naritaka Y, Asaka S, et al. Neoadjuvant chemotherapy (FAP) for advanced esophageal cancer. *Anticancer Res.* 2008;28:2321-2326.
16. Tanaka Y, Yoshida K, Sanada Y, et al. Biweekly docetaxel, cisplatin, and 5-fluorouracil (DCF) chemotherapy for advanced esophageal squamous cell carcinoma: a phase I dose-escalation study. *Cancer Chemother Pharmacol.* 2010;66:1159-1165.
17. National Cancer Institute. Common Terminology Criteria for Adverse Events (CTCAE), version 4.0. Bethesda, MD: US Department of Health and Human Services, National Institutes of Health; 2010. Available from: https://cabig-kc.nci.nih.gov/Vocab/KC/index.php/CTCAE. Accessed January 12, 2012.
18. Igaki H, Tachimori Y, Kato H. Improved survival for patients with upper and/or middle mediastinal lymph node metastasis of squamous cell carcinoma of the lower thoracic esophagus treated with 3-field dissection. *Ann Surg.* 2004;239:483-490.
19. Japanese Society for Esophageal Diseases. Guidelines for Clinical and Pathologic Studies on Carcinoma in the Esophagus. 9th ed. Tokyo, Japan: Kanehara & Company, Ltd.; 2001.
20. Aaronson NK, Ahmedzai S, Bergman B, et al. The European Organization for Research and Treatment of Cancer QLQ-C30: a quality-of-life instrument for use in international clinical trials in oncology. *J Natl Cancer Inst.* 1993;85:365-376.
21. Akamizu T, Shinomiya T, Irako T, et al. Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay. *J Clin Endocrinol Metab.* 2005;90:6-9.
22. Yakabi K, Sadakane C, Noguchi M, et al. Reduced ghrelin secretion in the hypothalamus of rats due to cisplatin-induced anorexia. *Endocrinology.* 2010;151:3773-3782.
23. Hiura Y, Takiguchi S, Yamamoto K, et al. Fall in plasma ghrelin concentrations after cisplatin-based chemotherapy in esophageal cancer patients [published online ahead of print July 20, 1022]. *Int J Clin Oncol.* 2011.
24. Skrtic S, Wallenius K, Gressner AM, Jansson AO. Insulin-like growth factor signaling pathways in rat hepatic stellate cells: importance for deoxyribonucleic acid synthesis and hepatocyte growth factor production. *Endocrinology.* 1990;140:5729-5735.
25. Kursar JD, Nelson DL, Wainscott DB, Cohen ML, Baez M. Molecular cloning, functional expression, and pharmacological characterization of a novel serotonin receptor (5-hydroxytryptamine 2F) from rat stomach fundus. *Mol Pharmacol.* 1992;42:549-557.
26. Wright DE, Seroogy KB, Lundgren KH, Davis BM, Jannes L. Comparative localization of serotonin 1A, 1C and 2 receptor subtype mRNAs in rat brain. *J Comp Neurol.* 1995;351:357-373.
27. Nonogaki K, Ohashi-Nozue K, Oka Y. A negative feedback system between brain serotonin systems and plasma active ghrelin levels in mice. *Biochem Biophys Res Commun.* 2006;341:703-707.
28. Wilder-Smith OH, Borgeat A, Chappuis P, Fathi M, Forni M. Urinary serotonin metabolite excretion during cisplatin chemotherapy. *Cancer.* 1993;72:2239-2241.
29. Cocquyt V, Van Belle S, Reinhardt RR, et al. Comparison of L-758,298, a prodrug for the selective neurokinin-1 antagonist, L-754,030, with ondansetron for the prevention of cisplatin-induced emesis. *Eur J Cancer.* 2001;37:835-842.
30. Steven G, Daniel C, Anish M, et al. Single-dose fosaprepitant for the prevention of chemotherapy-induced nausea and vomiting associated with cisplatin therapy: randomized, double-blind study protocol—EASE. *J Clin Oncol.* 2011;29:1495-1501.
31. Xiao HB, Cao WX, Yin HR, Lin YZ, Ye SH. Influence of L-methionine-deprived total parenteral nutrition with 5-fluorouracil on gastric cancer and host metabolism. *World J Gastroenterol.* 2001;7:698-701.

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Phenotypic Analysis of Monocyte-derived Dendritic Cells Loaded with Tumor Antigen with Heat-shock Cognate Protein-70

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Abstract. *Background/Aim: The cross-presentation system of tumor antigen by monocyte-derived dendritic cells (mo-DCs) has been observed under appropriate conditions. Both CD14-negative and CD1a-positive phenotypes were critical in our previous study. This study compared the phenotype of mo-DCs and identified the conditions that favored T helper-1 (Th1) cytokine production after stimulation with the hsc70 and NY-ESO-1 p157-165 epitope fusion protein (hsc70/ESO p157-165). Materials and Methods: The mo-DCs were induced from healthy donors. Their surface markers and cytokine production were examined after stimulation with hsc70/ESO p157-165. Results: CD1a$^+$ and CD1a$^-$ mo-DCs were generated in half of the healthy donors. The concentration of fetal calf serum in the culture medium was critical for the induction of CD1a$^+$ DCs, which were able to produce interleukin-12 (IL-12), but not IL-10. Neutralizing IL-6 and IL-6R antibodies affected the expression of CD1a. Conclusion: Anti IL-6 analogs may be effective adjuvants for the development of mo-DC-based cancer vaccine.*

NY-ESO-1 is a promising target antigen for specific immune recognition of cancer because it has restricted expression in normal tissue but frequently occurs on human tumors (1-4). Clinical trials with this antigen have been conducted using the NY-ESO-1 peptide, full-length protein, and DNA (5, 6). These cancer vaccines are designed to enhance effector T-cell responses to tumor antigens. An appropriate antigen-presenting cell is required to induce favorable T-cell responses (7).

Dendritic cells (DCs) are the most potent antigen-presenting cells and they have been shown to play a critical role in the generation of immune responses. The unique features of antigen presentation by DCs have generated considerable interest in their use as therapeutic vehicles, especially for vaccination (8, 9). DCs-alone or in complexes with tumor antigens are expected to be a powerful tool in the development of cancer vaccines (10, 11). However, no consensus has yet been reached on the most appropriate DC population to be employed for immunization.

A fusion protein containing the human heat-shock cognate protein-70 (hsc70) and ESO p157-165, epitope of NY-ESO-1 was constructed, as part of the development of a new strategy to vaccinate cancer patients with tumor antigens (12-14). A previous study demonstrated that monocyte-derived (mo)-DCs capture and endogenously process the hsc70/ESO p157-165 fusion protein to major histocompatibility complex (MHC) class I molecules through the cross-presentation pathway (15, 16). However, this cross-presentation system could not always work. This study was conducted to define the appropriate conditions in order to use mo-DCs for vaccination after loading with the hsc70/ESO p157-165 fusion protein.

Materials and Methods

Expression and purification of the hsc70 and NY-ESO-1 p157-165 epitope fusion protein. The hsc70/ESO p157-165 fusion protein was manufactured as previously described (15). Briefly, human cDNA of

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hsc70 was generated by reverse transcription-polymerase chain reaction (RT-PCR) from the mRNA obtained from the peripheral blood mononuclear cells (PBMCs) of a healthy volunteer. The total mRNA was extracted from the PBMCs with an Isogen kit (Wako, Osaka, Japan). The mRNA was transcribed to cDNA with oligo (dT) 16 primer using AMV reverse transcriptase (Promega, Tokyo, Japan). The cDNA encoding hsc70 was amplified by LA Taq polymerase (Takara, Tokyo, Japan) using the primers AT GGATCC C ATG TCC AAG GGA CCT G (forward) and AT GGTACC TTA ATC AAC CTC TTC AAT G (reverse). The amplified cDNA was cloned into a pQE31 expression vector (Qiagen, Tokyo, Japan) at 5' BamHI and 3' KpnI restriction sites. The hsc70/ESO p157-165 fusion protein was generated by incorporating a mini-gene encoding NY-ESO-1 p157-165 in either the forward or reverse primers containing the 5' *BamHI* and 3' *Kpn*I restriction sites. *Escherichia coli* strain M15 was transformed by the constructed plasmids and grown in an Luria-Bertani (LB) medium, containing ampicillin (50 μg/ml) and kanamycin (20 μg/ml). Protein expression was induced by 0.1 M isopropyl-β-D-thiogalactoside (IPTG). The protein was solubilized in buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/ HCl, pH 8.0), the lysate was centrifuged of at 10,000 ×*g*, and the supernatant was applied to an Ni^{2+}-nitrilotriacetic acid (NTA) agarose column and extensively washed with buffer C (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 6.3). The Ni^{2+}-NTA resin-bound 6× His-tagged protein was re-folded rapidly by washing with 15 column volumes of urea-free Tris buffer (pH 7.5) and eluted with Tris buffer containing 200 mM imidazole. The eluate was extensively dialyzed against phosphate buffered saline (PBS) (pH 7.4) to remove imidazole and then concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore, Bedford, MA, USA). The fusion proteins were treated with Kurimover I and II (Kurita Incorporation, Tokyo, Japan) to remove the contaminating lipopolysaccharide (LPS). The level of LPS was determined by the Limulus ES-II test (Wako, Osaka, Japan).

Peptide. The human leukocyte antigen (HLA)-A0201 restricted NY-ESO-1 peptide p157-165 (SLLMWITQC) was identified by reactivity with cluster of differentiation 8 $(CD8)^+$ T-cell from patients with spontaneous NY-ESO-1 immunity. This epitope (ESO p157-165) was selected to analyze the $CD8^+$ T-cell response (17, 18). The peptide was synthesized by using the Multiple Peptide Systems, with a purity of >86%, as determined by reversed-phase high-performance liquid chromatography (HPLC).

Generation of dendritic cells from PBMCs. Mononuclear cells were isolated from the peripheral blood of healthy individuals by using Ficoll-Paque density gradient centrifugation after obtaining informed consent (19, 20). The $CD14^+$ monocytes were enriched by negative isolation using magnetic beads (Dynal, Oslo, Norway). Monocytes were seeded at a density of 1×10^6 cells/well in 24-well plates in 2 ml of RPMI 1640 medium with 2.5% or 10% fatal calf serum (FCS), 100 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (Leukine; Immunex, Seattle, WA, USA) and 50 ng/ml IL-4 (R&D Systems, Minneapolis, MN, USA). The culture was incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5 days. The harvested cells were characterized by flow cytometry and were then stimulated with hsc70/ESO p157-165 fusion protein or p157-165 peptide.

Flow cytometry. The cells were processed for double-staining using fluorescein isothiocyanate (FITC)-CD14, FITC-CD1a, phycoerythrin (PE)-CD83, PE-HLA-DR, PE-CD86 (B7.2) monoclonal antibodies (mAbs) (BD Pharmingen, San Diego, CA, USA). Fluorescence acquisition was carried out on a FACSCan (BD Biosciences, San Diego, CA, USA), and data analysis was carried out using the CellQuest software package (BD Biosciences).

Stimulation of DCs and measurement of cytokines. The harvested cells were incubated with GM-CSF and IL-4 for 5 days and exposed to p157-165 (5 μg/ml) or hsc70/ESO p157-165 fusion protein (350 μg/ml) in RPMI 1640 for 12 h. The cell culture supernatants were collected and then particulates were removed by centrifugation. The concentration of the cytokines (IL-10 and 12) in the supernatants was measured by an enzyme-linked immunosorbent assay (ELISA; Quantikine, R&D Systems, Minneapolis, MN, USA).

Neutralization of IL-6 for differentiation of DCs from monocytes. mo-DCs were generated as described above. Anti-human IL-6 and/or IL-6R neutralizing antibodies, at a concentration of 2.5 μg/ml (R&D Systems), were added to the cultures at day 0 and 3 (21).

Results

Phenotype of mo-DCs from healthy donors. The population of monocytes isolated from PBMCs exhibited a unique phenotype (Figure 1a). However, the mo-DCs from the healthy donor were differentiated into two distinct phenotypes (Figure 1b). After 5 days, culture in RPMI, containing 10% FCS with GM-CSF and IL-4, the expression of CD14 was down-regulated in cells from half of the donors. On the other hand, CD1a was expressed in those cases. The expression of CD83 was negative in cells from all donors.

IL-10 production of mo-DCs from each donor. IL-10 was measured in the supernatants of DCs stimulated for 12 h by hsc70/ESO p157-165 fusion protein or p157-165 (Figure 2). $CD14^+CD1a^-$ DCs secreted significant amounts of IL-10 in response to hsc70/ESO p157-165 fusion protein. However, there was less IL-10 secretion stimulated by hsc70/ESO p157-165 fusion protein in $CD14^-CD1a^+$ DCs from donors 1 and 3.

The expression of CD1a and production of cytokines from mo-DCs. $CD14^-CD1a^-$ DCs (donor 5) produced IL-10, but IL-12, which plays a prominent role in the induction of the T-helper 1 (Th1) immune response against cancer, was barely secreted. Meanwhile, $CD14^-CD1a^+$ DCs from donor 6 exhibited a reversed pattern of cytokine production for IL-10 and IL-12 (Figure 3). The expression of surface markers CD14 and CD1a of mo-DCs had an effect on the balance of Th1 and Th2 response.

Mo-DCs from the same donor exhibited different surface marker phenotype after induction under different conditions. $CD14^-CD1a^+$ DCs were generated from donor 7 following

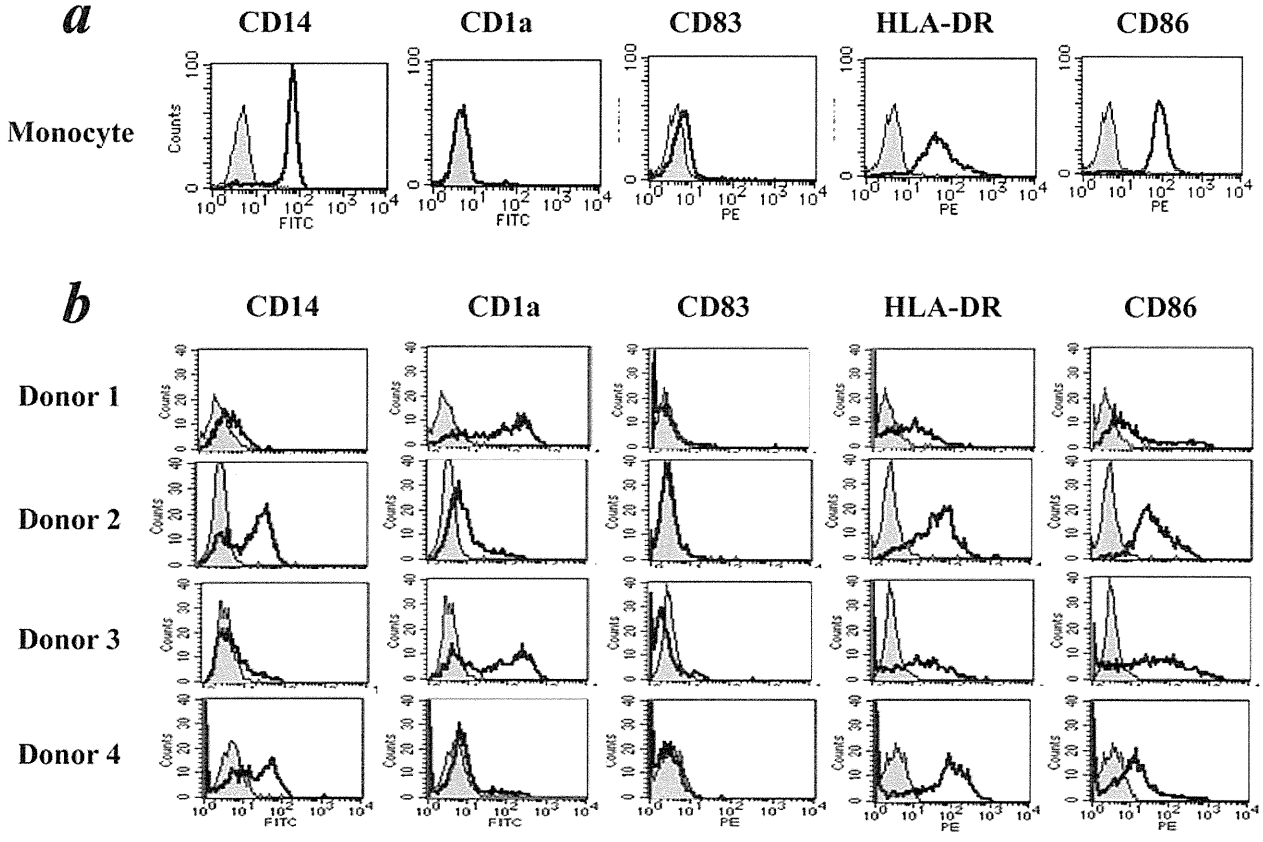

Figure 1. *a: Phenotype of monocytes isolated by magnetic beads from peripheral blood mononuclear cells. Cells were enriched and harvested immediately. b: Phenotype of monocyte-derived dendritic cells. Peripheral blood mononuclear cells were isolated from the peripheral blood of healthy individuals. The CD14+ monocytes enriched by negative isolation were incubated in RPMI plus 10% fetal calf serum, granulocyte macrophage colony-stimulating factor and interleukin-4, for 6 days. The phenotypes (CD14, CD1a, CD83, HLA-DR, CD86) of the harvested cells were analyzed by flow cytometry.*

culture in RPMI with 10% FCS. However, $CD14^+CD1a^-$ DCs were generated under culture conditions of RPMI with 2.5% FCS, using cells from the same donor. This phenotypic conversion changed the function of DCs, which exhibited IL-10 production rather than IL-12, in response to stimulation with hsc70/ESO p157-165 fusion protein (Figure 4).

The expression of CD1a and IL-6 antagonists. IL-6 affects the differentiation of monocytes into DCs and macrophages. The addition of IL-6 and/or IL-6R antibodies during the generation of mo-DCs up-regulated the expression of CD1a. The expression of CD1a was remarkable following blocking of both IL-6 and IL-6R (Figure 5). Although the DCs generated in medium containing 2.5% FCS were almost all $CD1a^-$ cells, the expression of CD1a was positive following the addition of neutralizing IL-6 and IL-6R antibodies on day 0 and 3. The function of the DCs differed according to their phenotypic features and they produced IL-12 in response to the activation by hsc70/ESO p157-165 fusion protein (Figure 6).

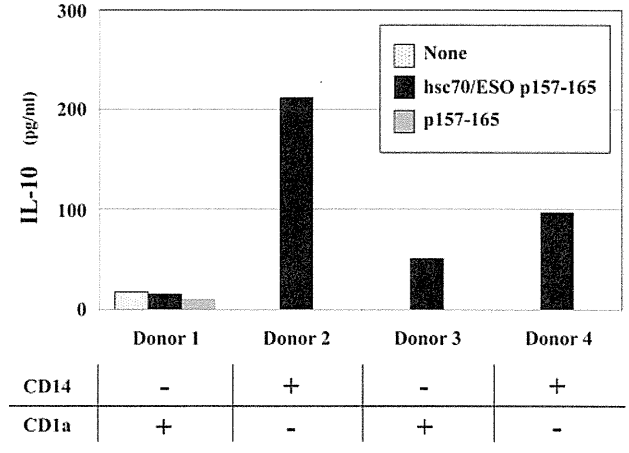

Figure 2. *Interleukin-10 (IL-10) production of dendritic cells (DCs). $CD14^-CD1a^+$ DCs and $CD14^+CD1a^-$ DCs were stimulated by hsc70/ESO p157-165 fusion protein or p157-165 in RPMI medium for 12 h. The IL-10 in the supernatants of pulsed DCs was measured by an enzyme-linked immunosorbent assay.*

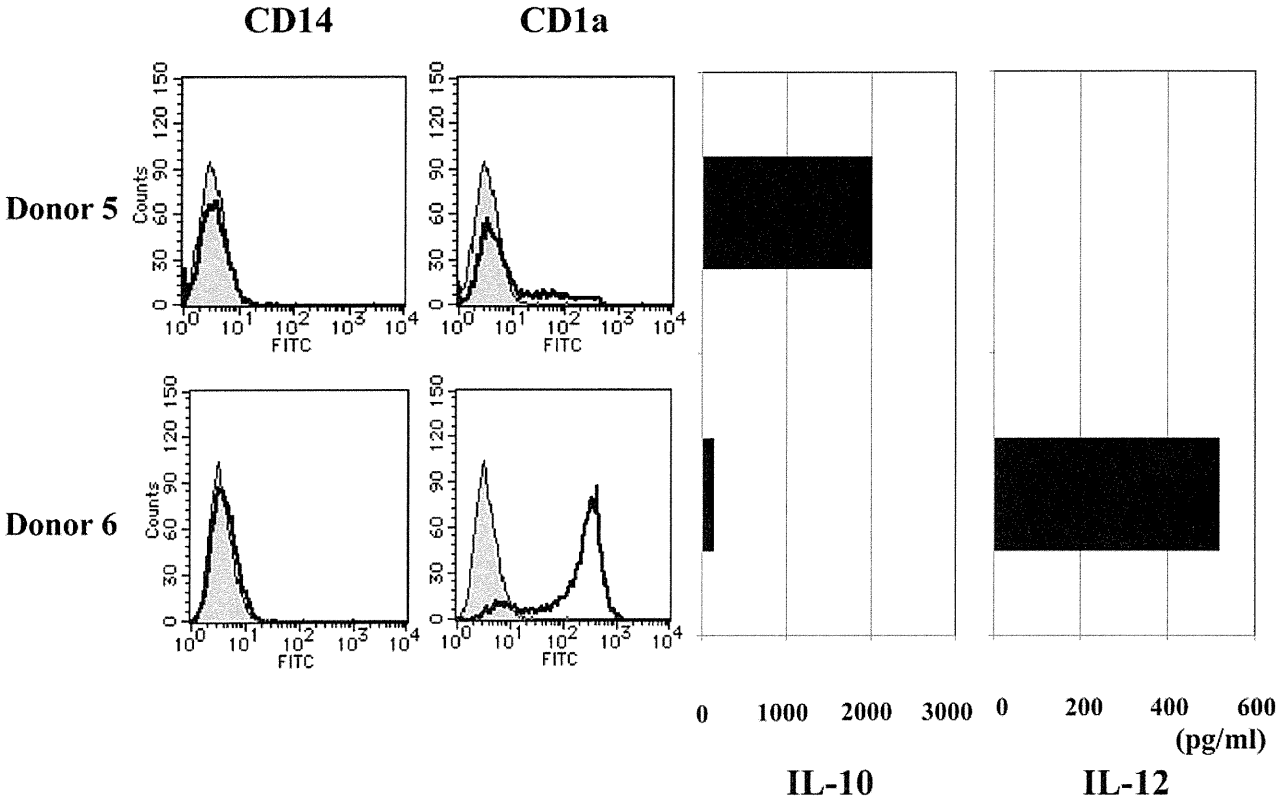

Figure 3. *Interleukin-10 (IL-10) and interleukin-12 (IL-12) production of dendritic cells (DCs). $CD14^{-}CD1a^{+}$ DCs and $CD14^{+}CD1a^{-}$ DCs were activated, as described in Materials and Methods by the hsc70/ESO p157-165 fusion protein. IL-10 and IL-12 in the supernatants of cells were measured by an enzyme-linked immunosorbent assay.*

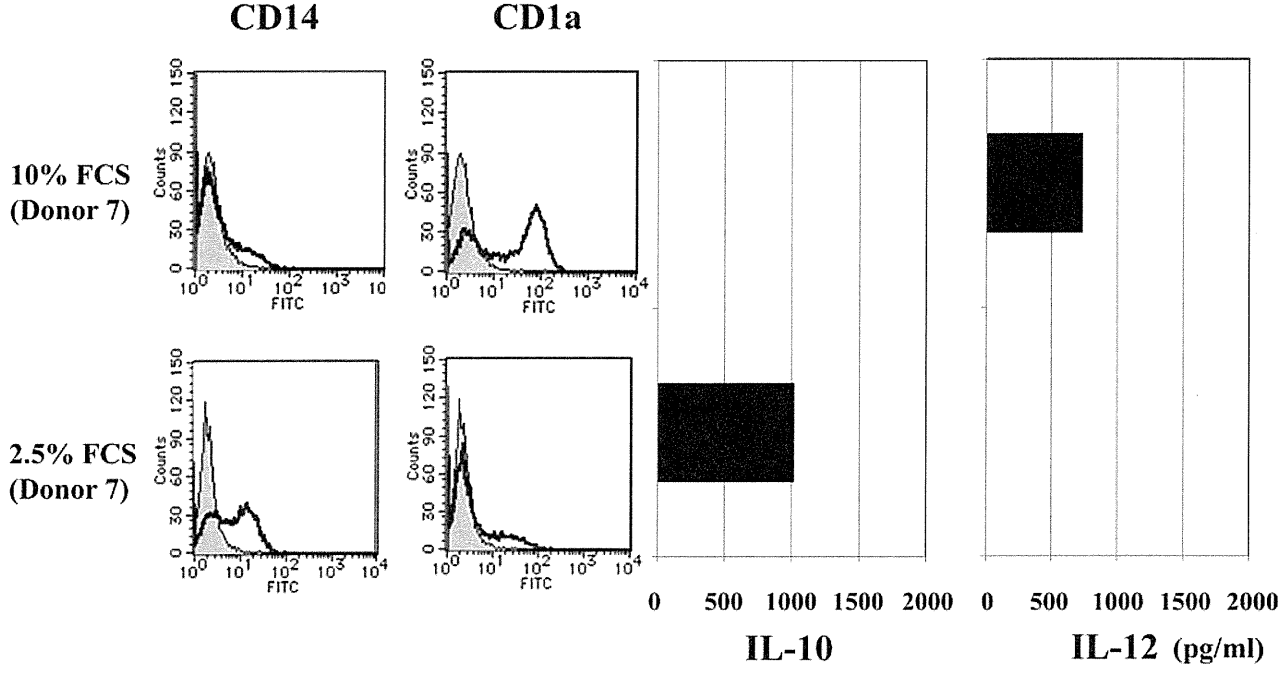

Figure 4. *Cytokine production of dendritic cells (DCs) in different culture medium. The percentage of fetal calf serum (FCS) in RPMI medium for generation of monocyte-derived DCs was changed from 10% to 2.5%.*

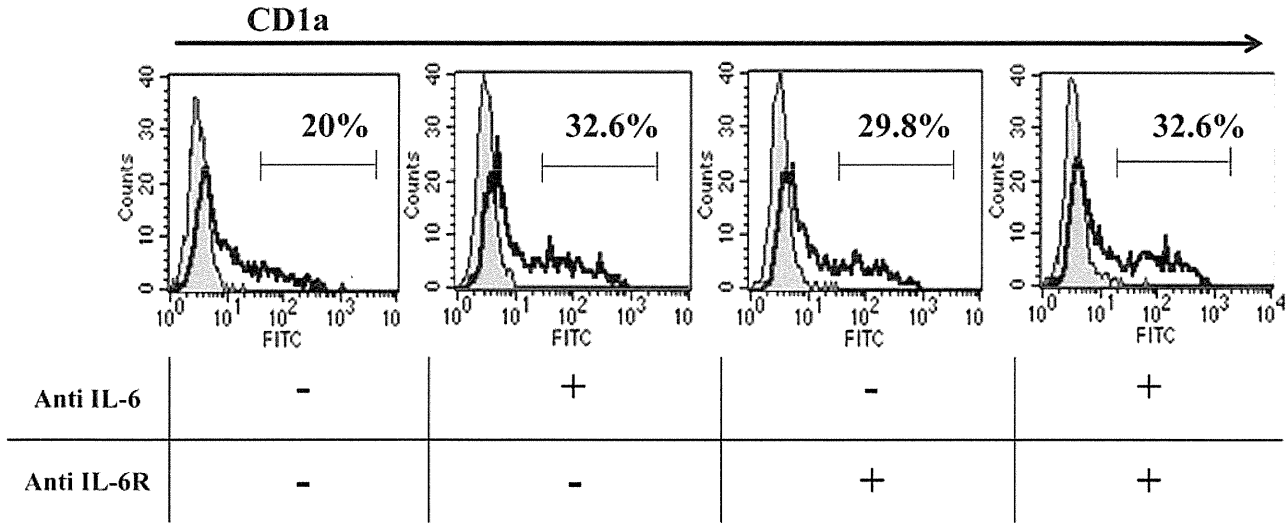

Figure 5. *The population of CD1a$^+$ dendritic cells (DCs) after neutralizing Interleukin-6 (IL-6) and/or IL-6R. Antibodies to human IL-6 and IL-6R were added to the culture on day 0 and 3 of the generation of DCs from monocytes, in a medium with 2.5% fatal calf serum (FCS). The CD1a expression of harvested DCs was analyzed by flow cytometry on day 6.*

Discussion

NY-ESO-1 is a prototype cancer/testis antigen that is expressed in a variety of human malignancies, but not in normal tissues, except for the testis. Spontaneous immune responses involving an antibody, as well as CD4$^+$ and CD8$^+$ T-cells directed against a broad range of MHC class I- and class II-restricted NY-ESO-1 peptides are observed in patients with advanced NY-ESO-1-expressing tumors (22). Therefore, NY-ESO-1 is thought to be a favorable target for use as a cancer vaccine. The initial trials of peptide vaccination against NY-ESO-1 were particularly effective in generating CD8$^+$ T-cell responses. However, the clinical outcome remains unsatisfactory.

The major role of heat shock proteins (HSPs) is to act as a molecular chaperone, binding immature peptides during their synthesis and assisting in their folding (23-25). The peptides are thought to be degraded in the cytoplasm and are then transferred to the endoplasmic reticulum by binding to HSP70 or HSP90, but not by natural diffusion (26). In addition, HSPs are thought to bind to a diverse array of antigenic peptides in tumor cells, and that the tumor-derived HSP-antigenic peptide complexes can be purified for vaccination against cancer (27).

A mini-gene encompassing the NY-ESO-1 cytotoxic T-lymphocyte (CTL) epitope p157-165 (ESO p157-165) was genetically fused to the human heat shock cognate protein-70 (hsc70), and the resulting fusion proteins were expressed in *E. coli*. mo-DCs captured and endogenously processed the hsc70/ESO p157-165 fusion protein to MHC class I molecules through the cross-presentation pathway. Finally, NY-ESO-1-specific CTL were generated by *in vitro* stimulation with hsc70/ESO p157-165 fusion protein on mo-DCs.

DCs play a crucial role in the initiation of antigen-specific immune responses, exhibiting a variety of specializations that contribute to their efficiency as antigen-presenting cells (9, 28). One major population of DCs is myeloid DCs which include specific subtypes, including Langerhans cells, interstitial DCs and mo-DCs that have unique phenotypic features. The CD14$^+$ monocytes are the most common source of DCs and can be enriched by negative isolation from PBMCs and incubated in RPMI with 10% FCS, GM-CSF and IL-4 to generate DCs (29, 30). However, not all DCs generated or cultured under the same conditions are equivalent (31). They appear to be derived from multiple lineages and, depending on their origin, site of residence, or the type of maturation stimulus received, they program different T-cell outcomes (32). The generated mo-DCs exhibit various expression levels of CD1a, CD14, CD83, human leucocyte antigen-DR (HLA-DR) and CD86 according to the culture conditions and individuals from which they are sourced. mo-DC subsets are defined by their phenotypic features and have a functional diversity of cytokine production that regulates the polarization of naïve T-cells to Th1 or Th2 (33-36). However, this diversity creates difficulties in their clinical application in cancer immunotherapy using DCs. The optimal phenotypic features of DCs and appropriate conditions for clinical applications must be determined.

CD1a is one of the common DC subset markers (37, 38). The proportion of CD1a$^+$ and CD1a$^-$ DCs varies in individuals.

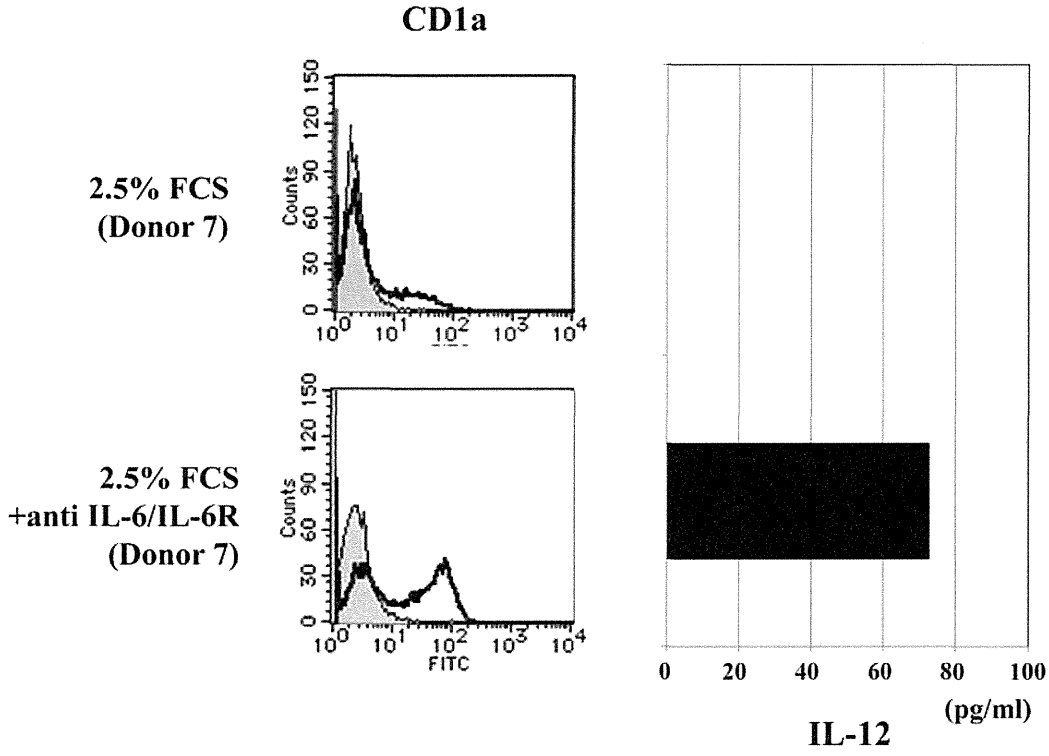

Figure 6. *Interleukin-12 (IL-12) production of monocyte-derived dendritic cells (mo-DCs) following culture with antibodies to interleukin-6 (IL-6) and IL-6R. The DCs generated in RPMI plus 2.5% fetal calf serum (FCS) and IL6/IL-6R antibodies up-regulated their expression of CD1a. The IL-12 production from DCs cultured with and without neutralizing IL-6 antibodies is shown.*

$CD1a^+$ DCs are able to secrete more IL-12 in response to stimulation with LPS than do $CD1a^-$ DCs. On the other hand, the IL-10 production of $CD1a^+$ DCs is less, or similar to that of $CD1a^-$ DCs (39). The present study generated two types of DCs, $CD1a^+$ and $CD1a^-$, under the same conditions. Interestingly, there were different patterns of CD1a expression under the different conditions during induction, even when using cells from the same donor. This strongly suggests that the conditions of DC culture were critical for induction of the appropriate antigen-presenting cells *in vivo*.

Humoral factors in the serum also affect the differentiation of immature DCs (40, 41). Culture medium with 2.5% FCS converted the phenotype of DCs from $CD1a^+$ to $CD1a^-$. This conversion also changed the cytokine production from IL-12 to IL-10. This is a critical conversion associated with the polarization of Th1 and Th2 cells. A major question was whether this conversion was reversible. IL-6 inhibits the differentiation of monocytes to DCs by promoting their differentiation toward macrophages (42, 43). On the other hand, an antagonist of IL-6 can drive monocytes to form immature DCs. Therefore, the present study compared the effect of anti-IL-6 agents on CD1a expression. Cells treated with both antibodies to IL-6 and IL-6R, recovered CD1a expression and secreted IL-12. These results suggest that anti-IL-6 analogs may be used as an effective adjuvant for the development of a mo-DC-based cancer vaccine with the hsc70/ESO p157-165 fusion protein.

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References

1 Caballero OL and Chen YT: Cancer/testis (CT) antigens: potential targets for immunotherapy. Cancer Sci *100*: 2014-2021, 2009.

2 Jager E, Stockert E, Zidianakis Z, Chen YT, Karbach J, Jager D, Arand M, Ritter G, Old LJ and Knuth A: Humoral immune responses of cancer patients against Cancer-Testis antigen NY-ESO-1: correlation with clinical events. Int J Cancer *84*: 506-510, 1999.

3 Scanlan MJ, Gure AO, Jungbluth AA, Old LJ and Chen YT: Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunol Rev *188*: 22-32, 2002.

4 Gnjatic S, Nishikawa H, Jungbluth AA, Gure AO, Ritter G, Jager E, Knuth A, Chen YT and Old LJ: NY-ESO-1: review of an immunogenic tumor antigen. Adv Cancer Res *95*: 1-30, 2006.

5 Jager E, Nagata Y, Gnjatic S, Wada H, Stockert E, Karbach J, Dunbar PR, Lee SY, Jungbluth A, Jager D, Arand M, Ritter G, Cerundolo V, Dupont B, Chen YT, Old LJ and Knuth A: Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. Proc Natl Acad Sci USA *97*: 4760-4765, 2000.
6 Karbach J, Gnjatic S, Bender A, Neumann A, Weidmann E, Yuan J, Ferrara CA, Hoffmann E, Old LJ, Altorki NK and Jager E: Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51: association with survival. Int J Cancer *126*: 909-918, 2010.
7 Old LJ: Cancer vaccines: an overview. Cancer Immun 8 Suppl *1*: 1-4, 2008.
8 Banchereau J and Steinman RM: Dendritic cells and the control of immunity. Nature *392*: 245-252, 1998.
9 Mellman I and Steinman RM: Dendritic cells: specialized and regulated antigen processing machines. Cell *106*: 255-258, 2001.
10 Albert ML, Sauter B and Bhardwaj N: Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature *392*: 86-89, 1998.
11 Liu WM, Dennis JL, Fowler DW and Dalgleish AG: The gene expression profile of unstimulated dendritic cells can be used as a predictor of function. Int J Cancer *130*: 979-990, 2011.
12 Kawabata R, Wada H, Isobe M, Saika T, Sato S, Uenaka A, Miyata H, Yasuda T, Doki Y, Noguchi Y, Kumon H, Tsuji K, Iwatsuki K, Shiku H, Ritter G, Murphy R, Hoffman E, Old LJ, Monden M and Nakayama E: Antibody response against NY-ESO-1 in CHP-NY-ESO-1 vaccinated patients. Int J Cancer *120*: 2178-2184, 2007.
13 Mizukami S, Kajiwara C, Ishikawa H, Katayama I, Yui K and Udono H: Both CD4+ and CD8+ T-cell epitopes fused to heat shock cognate protein 70 (hsc70) can function to eradicate tumors. Cancer Sci *99*: 1008-1015, 2008.
14 Udono H, Saito T, Ogawa M and Yui Y: Hsp-antigen fusion and their use for immunization. Methods *32*: 21-24, 2004.
15 Susumu S, Nagata Y, Ito S, Matsuo M, Valmori D, Yui K, Udono H and Kanematsu T: Cross-presentation of NY-ESO-1 cytotoxic T lymphocyte epitope fused to human heat shock cognate protein 70 by dendritic cells. Cancer Sci *99*: 107-112, 2008.
16 Udono H, Yamano T, Kawabata Y, Ueda M and Yui K: Generation of cytotoxic T lymphocytes by MHC class I ligands fused to heat-shock cognate protein 70. Int Immunol *13*: 1233-1242, 2001.
17 Gnjatic S, Nagata Y, Jager E, Stockert E, Shankara S, Roberts BL, Mazzara GP, Lee SY, Dunbar PR, Dupont B, Cerundolo V, Ritter G, Chen YT, Knuth A and Old LJ: Strategy for monitoring T-cell responses to NY-ESO-1 in patients with any HLA class I allele. Proc Natl Acad Sci USA *97*: 10917-10922, 2000.
18 Jager E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jager D, Arand M, Wada H, Noguchi Y, Stockert E, Old LJ and Knuth A: Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J Exp Med *187*: 265-270, 1998.
19 Freudenthal PS and Steinman RM: The distinct surface of human blood dendritic cells, as observed after an improved isolation method. Proc Natl Acad Sci USA *87*: 7698-7702, 1990.
20 O'Doherty U, Steinman RM, Peng M, Cameron PU, Gezelter S, Kopeloff I, Swiggard WJ, Pope M and Bhardwaj N: Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. J Exp Med *178*: 1067-1076, 1993.
21 Chomarat P, Banchereau J, Davoust J and Palucka AK: IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. Nat Immunol *1*: 510-514, 2000.
22 Gnjatic S, Atanackovic D, Jager E, Matsuo M, Selvakumar A, Altorki NK, Maki RG, Dupont B, Ritter G, Chen YT, Knuth A and Old LJ: Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. Proc Natl Acad Sci USA *100*: 8862-8867, 2003.
23 Fourie AM, Sambrook JF and Gething MJ: Common and divergent peptide binding specificities of hsp70 molecular chaperones. J Biol Chem *269*: 30470-30478, 1994.
24 Srivastava PK, Udono H, Blachere NE and Li Z: Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetics *39*: 93-98, 1994.
25 Suto R and Srivastava PK: A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. Science *269*: 1585-1588, 1995.
26 Blachere NE, Li Z, Chandawarkar RY, Suto R, Jaikaria NS, Basu S, Udono H and Srivastava PK: Heat shock protein-peptide complexes, reconstituted *in vitro*, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. J Exp Med *186*: 1315-1322, 1997.
27 Gong J, Zhang Y, Durfee J, Weng D, Liu C, Koido S, Song B, Apostolopoulos V and Calderwood SK: A heat shock protein 70-based vaccine with enhanced immunogenicity for clinical use. J Immunol *184*: 488-496, 2010.
28 Ohno S, Takano F, Ohta Y, Kyo S, Myojo S, Dohi S, Sugiyama H, Ohta T and Inoue M: Frequency of myeloid dendritic cells can predict the efficacy of Wilms' tumor 1 peptide vaccination. Anticancer Res *31*: 2447-2452, 2011.
29 Sallusto F and 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 *179*: 1109-1118, 1994.
30 Becker S, Warren MK and Haskill S: Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. J Immunol *139*: 3703-3709, 1987.
31 Hikino H, Kasono K, Kanzaki M, Kai T, Konishi F and Kawakami M: Granulocyte/macrophage colony-stimulating factor and interleukin-4-induced dendritic cells. Anticancer Res *24*: 1609-1615, 2004.
32 Shinozaki Y, Wang S, Miyazaki Y, Miyazaki K, Yamada H, Yoshikai Y, Hara H and Yoshida H: Tumor-specific cytotoxic T cell generation and dendritic cell function are differentially regulated by interleukin 27 during development of anti-tumor immunity. Int J Cancer *124*: 1372-1378, 2009.
33 Ekkens MJ, Shedlock DJ, Jung E, Troy A, Pearce EL, Shen H and Pearce EJ: Th1 and Th2 cells help CD8 T-cell responses. Infect Immun *75*: 2291-2296, 2007.
34 Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R and Liu YJ: Reciprocal control of T helper cell and dendritic cell differentiation. Science *283*: 1183-1186, 1999.
35 Sen D, Forrest L, Kepler TB, Parker I and Cahalan MD: Selective and site-specific mobilization of dermal dendritic cells and Langerhans cells by Th1- and Th2-polarizing adjuvants. Proc Natl Acad Sci USA *107*: 8334-8339, 2010.

36 Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S and Liu YJ: The nature of the principal type 1 interferon-producing cells in human blood. Science *284*: 1835-1837, 1999.
37 Lebre MC and Tak PP: Dendritic cell subsets: their roles in rheumatoid arthritis. Acta Reumatol Port *33*: 35-45, 2008.
38 Liu YJ: Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell *106*: 259-262, 2001.
39 Cernadas M, Lu J, Watts G and Brenner MB: CD1a expression defines an interleukin-12 producing population of human dendritic cells. Clin Exp Immunol *155*: 523-533, 2009.
40 Mohamadzadeh M, Berard F, Essert G, Chalouni C, Pulendran B, Davoust J, Bridges G, Palucka AK and Banchereau J: Interleukin 15 skews monocyte differentiation into dendritic cells with features of Langerhans cells. J Exp Med *194*: 1013-1020, 2001.
41 Wertel I, Bednarek W, Stachowicz N, Rogala E, Nowicka A and Kotarski J: Phenotype of dendritic cells generated from peripheral blood monocytes of patients with ovarian cancer. Transplant Proc *42*: 3301-3305, 2010.
42 Hildenbrand B, Lorenzen D, Sauer B, Hertkorn C, Freudenberg MA, Peters JH, Nesselhut T, Unger C and Azemar M: IFN-γ enhances T(H)1 polarisation of monocyte-derived dendritic cells matured with clinical-grade cytokines using serum-free conditions. Anticancer Res *28*: 1467-1476, 2008.
43 Mitani H, Katayama N, Araki H, Ohishi K, Kobayashi K, Suzuki H, Nishii K, Masuya M, Yasukawa K, Minami N and Shiku H: Activity of interleukin 6 in the differentiation of monocytes to macrophages and dendritic cells. Br J Haematol *109*: 288-295, 2000.

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