Table 6 continued

Risk factors	Categories	Univariate analysis	Multivariate analysis	
		P value	*P* value	Odds ratio (95 % CI)
Sivelestat use	Yes	0.3785		
	No			
Complications other than AF	Yes	<0.0001	0.0516	4.920 (1.924–16.710)
	No			
Sinus heart rate in POD 1 (bpm)	≥100	<0.0001	0.0004	18.401 (3.680–92.003)
	<100			
Hemoglobin in POD1 (g/dL)	<8	0.9764		
	≥8			
WBC on POD 1 (/μL)	≥10,000	0.2379		
	<10,000			
CRP on POD 1 (mg/dL)	≥10	0.8663		
	<10			
PaO_2 on POD 1 (mm Hg)	<100	0.5042		
	≥100			
Highest CVP on POD 1 (mm Hg)	<5	0.6214		
	≥5			
Highest fever on POD1 (°C)	≥37	0.0041	0.1261	3.053 (0.730–12.757)
	<37			

CI confidence interval, *BMI* body mass index, *NAC* neoadjuvant chemotherapy, *NACRT* neoadjuvant chemoradiotherapy, *Ut* upper third of the thorax, *Mt* middle third of the thorax, *Lt* lower third of the thorax, *R(L)BBB* right(left) bundle branch block, *PVC* premature ventricular contraction, *AV block* atrioventricular block, *AF* atrial fibrillation, *POD* postoperative day, *WBC* white blood cell, *CRP* C-reactive protein, *CVP* central venous pressure

[a] UICC TNM R0

Table 7 Incidence of postoperative atrial fibrillation in patients with two risk factors, colon conduit use for reconstructions and sinus tachycardia with a rate >100 bpm on POD1

Risk categories[a]	AF cases	(%)
Risk 0		
Without colon conduit, HR < 100 bpm	4/168	2.4
Risk 1–1		
Without colon conduit, HR ≥ 100 bpm	6/15	40.0
Risk 1–2		
With colon conduit, HR < 100 bpm	5/20	25.0
Risk 2		
With colon conduit, HR ≥ 100 bpm	4/4	100.0

POD postoperative day, *HR* heart rate, *AF* atrial fibrillation

[a] Risk 0 indicates the patients without colon conduit use for reconstruction after esophagectomy and with a heart rate <100 bpm on POD1. Risk 1–1 indicates the patients without colon conduit use for reconstruction but with tachycardia with a heart rate >100 bpm. Risk 1–2 indicates the patients with colon conduit use for reconstruction who had a heart rate <100 bpm. Risk 2 indicates the patients with both colon conduit use for reconstruction and tachycardia

demonstrated in the previous studies. In general, transthoracic esophagectomy produces an excessive inflammatory response, which may lead to the development of systemic inflammatory response syndrome and postoperative morbidities [24]. In particular, colonic reconstruction after esophagectomy is a more complex procedure, with increased morbidity compared with gastric transposition [31]. The blood loss is greater and the length of the operation is longer in the colonic reconstruction group, reflecting the complexity of the operation [31]. Therefore, it is suggested that esophagectomy with reconstruction using a colonic conduit is accompanied by much greater surgical stress, which might translate to a high incidence of postoperative AF.

In this study, sinus tachycardia with a rate >100 bpm on POD1 was the most significant risk factor for postoperative AF. The peak incidence of postoperative AF was on POD1 or 2, which is consistent with the findings of previous studies [2, 4]. A previous report in patients undergoing pulmonary surgery also showed similar results [14]. In our institute, esophageal cancer patients are consistently up and walking on POD1 or 2 after esophagectomy, and as a result, the greatest incidence of AF was found after walking. We suspect that the increases in heart rate during walking may have triggered the AF. We previously found that a perioperative physiotherapy and pulmonary rehabilitation program improves the exercise capacity and early

ambulation in patients undergoing esophagectomy [23]. Therefore, we consider early mobilization to be essential for patients that have undergone esophagectomy; however, it is also important to control the heart rate on POD1 and 2 in order to prevent AF after esophagectomy.

Intraoperative fluid therapy decisions may also influence the postoperative AF. Our surgical team and anesthetist kept the infusion rates between 6 and 8 mL/kg/h during esophagectomy, and the intraoperative urine output was kept between 0.5 and 1 mL/kg/h. Therefore, we consider that the water balance during the operation had no influence on the postoperative AF in our series of patients.

Prophylactic therapy may be performed for high-risk groups, including patients who undergo esophagectomy with reconstruction using a colon conduit and/or patients with sinus tachycardia after esophagectomy, to prevent the occurrence of postoperative AF. Indeed, the incidence of postoperative AF in patients with colon conduit use for reconstructions and tachycardia on POD1 was 100 %. The current commonly cited guidelines do not provide a specific recommendation for anticoagulation in the postoperative period for general thoracic surgeries [32]. Early attention to heart rate control and possible conversion back to normal sinus rhythm is a well-accepted principle of current patient care [32]. We consider landiolol hydrochloride to be a first-line therapy against postoperative AF [12]. Landiolol is a drug which acts as a highly cardioselective, ultrashort-acting β_1-selective β-blocker. It is often used as an anti-arrhythmic agent in Japan. Because of its extremely short half-life in the blood (<4 min), landiolol is outstanding in terms of its immediate efficacy and adjustability. Recent randomized controlled studies using landiolol have reported significant reductions in AF after cardiac surgery compared with placebo [12]. Another randomized controlled study clearly demonstrated that the mortality and the perioperative incidence of cardiovascular events could be reduced by the use of β-blockers in patients who underwent major noncardiac surgery [33]. Therefore, we may reduce not only the incidence of AF, but also other postoperative complications after esophagectomy, with the use of landiolol. While several medications have been used for postoperative AF prophylaxis without risk discrimination, a better understanding of the mechanisms responsible for postoperative AF could help in the design of novel prophylactic or therapeutic measures targeting those who are at the greatest risk. Clinical trials of the prophylactic use of landiolol to prevent postoperative AF are now planned in patients at high risk who undergo transthoracic esophagectomy.

Conflict of interest None.

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7 ペプチドワクチンを用いた膵癌治療

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Key words ：ペプチドワクチン，免疫療法，PEGASUS-PC，immunocheckpoint

はじめに

膵癌は唯一切除が治癒の可能性を有する治療法であるが，いまだに切除不能症例が切除可能症例を上回り，その予後は不良である[1]。また，切除例においても高率に再発をきたし，術後の治療が予後延長において重要となっている。ゲムシタビン塩酸塩は5-FUとの比較試験結果から，膵癌化学療法における標準治療となったが[2]，さらなる治療成績の向上を探索する目的で多くの薬剤とのhead-to-headの比較試験ならびにゲムシタビン塩酸塩との併用が試みられた。上皮成長因子受容体（Epidermal Growth Factor Receptor；EGFR）に対する分子標的治療薬であるエルロチニブとゲムシタビン塩酸塩との併用が，はじめてゲムシタビン塩酸塩単剤と比較して生存において優越性を示した薬剤である[3]。しかし，生存に関するHazard比は0.82であるが，その生存期間中央値は併用群6.2ヵ月，ゲムシタビン塩酸塩単独群5.9ヵ月[3]とわずかであり，副作用や費用対効果を考えると新たな標準治療との結論には至らないのが現状である。大腸癌の予後が新規抗悪性腫瘍薬の登場により著しく改善していることからも，膵癌の予後改善には新薬の開発が急務であることは想像に難くない。最近，FOLFIRINOX療法が報告され，ゲムシタビン塩酸塩を含まないregimenではじめて全生存期間に対する優越性を証明できたこと（Hazard ratio for death，0.57；95% confidence interval，0.45-0.73；$P<0.001$）[4]から，膵癌化学療法も大きく飛躍していくものと考えるが，その副作用は有熱性好中球減少症をはじめとする重篤なものが認められ，残念ながら，全膵癌患者に対し施行できる治療法ではないことが問題点である。一方，免疫療法は，抗癌剤や分子標的治療薬とは異なり，副作用の点からも開発が望まれている治療法である。

Ⅰ．T細胞の癌細胞識別

T細胞は異物となる抗原を特異的に認識して排除するが，抗原受容体であるT細胞受容体は直接抗原を認識しないことが抗体とは異なるところである。抗原の分解物質であるペプチドが，主要組織適合性複合体（major histocompatibility complex；MHC）に結合し，免疫応答を誘導する。腫瘍関連抗原においては細胞傷害性T細胞（Cytotoxic T lymphocyte；CTL）が認識できる腫瘍抗原ペプチドが報告され[5]，それまでは漠然としていた腫瘍抗原が明らかとなり，理論的根拠に立脚した腫瘍抗原を標的とした癌ワクチン療法が考案されることになった。標的となる腫瘍細胞に特異的に発現している内因性抗原である腫瘍関連抗原は樹状細胞に取り込まれ，プロテアソームによるプロセシング作用を受けてペプチド断片となり，主要組織適合抗原（MHC，ヒトではHLA）クラスⅠ分子の$\alpha 1$，$\alpha 2$ドメインに結合し，ゴルジ体を介して細胞表面へ表出し，MHC（HLA）－ペプチド複合体によりペプチドがCD8陽性T細胞に提示され，CD8陽性T細胞を活性化することにより抗原

特異的なCTLが誘導される。ペプチドワクチン療法では腫瘍特異的CTLを誘導しうるペプチドを同定し，それを癌患者に投与することで，樹状細胞に取り込まれ，上述の作用機序により腫瘍特異的CTLが誘導される。腫瘍抗原の同定に伴って特異的T細胞の頻度やサブセットなどが生命予後と関連すること[6]からも，T細胞が癌細胞の消去を担っていることが推測される。乳癌に対する癌ペプチドワクチンが術後補助療法として再発抑制効果が報告された[7]が，膵癌には期待されたような臨床効果は見られなかった[8]。その原因として癌細胞の免疫逃避機構の存在が示唆される。

Ⅱ．免疫逃避機構

化学発癌モデルやウイルス発癌モデルとは異なり，自然発癌において腫瘍細胞は宿主免疫監視機構をすり抜け発育している。一定以上の腫瘍細胞量にまで成長した腫瘍細胞はヘテロな集団であり，さまざまな免疫逃避機構が推測される[9,10]。腫瘍細胞は遺伝子変異を起こしやすく腫瘍関連抗原の発現も不安定であり，CTLが認識できない細胞が存在する。腫瘍抗原だけでなくHLA class Ⅰの発現が低下するため，HLA-ペプチド複合体が形成されず，CTLが腫瘍細胞を認識できない。このHLA class Ⅰの発現が低下する現象は多くの癌腫で報告されており[11~13]，膵癌でも同様の報告がされている[10]。また，HLA class Ⅰ発現の低下・消失は患者生存率が低下し，再発も多いことがほかの癌腫で報告されている[14,15]。さらに癌微小環境における免疫抑制因子の存在が危惧される。腫瘍細胞や周囲の間質細胞から産生されるIL-10に代表される免疫抑制性サイトカインやTGF-β[16]，制御性T細胞により[17]，CTLは免疫抑制状態となる。また，腫瘍抗原の長期に渡る持続的暴露がT細胞の機能不全・exhaustionに陥る[18]。

Ⅲ．腫瘍新生血管を標的とした癌ワクチン療法

免疫逃避機構を克服するには，癌細胞自体を標的にするのではなく膵癌細胞の増殖や転移に必須で[19,20]，かつHLA class Ⅰ発現が安定している腫瘍新生血管を標的とする新しい発想での免疫療法を施行することとした。VEGF-Aはほとんどの腫瘍で発現が上昇しており，VEGFR1およびVEGFR2の二つのレセプター型チロシンキナーゼと結合する[21]。VEGFR1およびVEGFR2を介したシグナル伝達を遮断することで，血管新生の阻害や癌細胞の増殖。転移を抑制することが期待できる[22]。また，VEGFR2はVEGFR1よりVEGF-Aによる血管内非細胞の増殖や血管透過性などの主要なシグナル伝達を強く担うレセプターであり，VEGFR2のシグナル伝達を遮断することは腫瘍新生血管の阻害ならびに腫瘍細胞の浸潤・転移を抑制できる可能性を示唆している。当科で施行した医師主導型第Ⅰ相臨床試験で用いたペプチドはVEGFR2由来エピトープペプチド（VEGFR2-169，Elpamotide，エルパモチド）であり，VEGFR2を特異的に認識し，最も強い腫瘍新生血管を傷害するCTLを誘導することができる[23]。また，担癌患者からも特異的CTLが誘導できることが明らかになっている。VEGFR1由来ペプチドも同定されており，ペプチドをパルスした細胞に対し細胞傷害活性を有することが確認され，VEGFR1を内因性に発現した細胞においても特異的活性化CTLの誘導が確認できている[24]。さらに，VEGFR2はFOXP3highCD4$^+$Treg細胞に選択的に発現しており[25]，VEGFR2を標的とすることでTreg細胞の抑制に期待ができる。

Ⅳ．当科で行った第Ⅰ相臨床試験

HLA-A-*2402を有する切除不能膵癌患者を対象に医師主導型第Ⅰ相臨床試験，「切除不能進行再発膵癌に対する腫瘍新生血管を標的としたHLA-A*2402拘束性エピトープペプチドとgemcitabine併用による第Ⅰ相

表1 局所皮膚反応と臨床効果

	局所皮膚反応	
	陽性	陰性
PR+SD	12	0
PD	3	3

参考文献26から改変転載

表2 免疫応答と臨床効果

	ペプチド投与量（mg）		
	0.5 (n=6)	1.0 (n=6)	2.0 (n=6)
局所皮膚反応（+/−）	5	4	6
CTL反応（+/−）	3	4	4
臨床効果			
PR/SD/PD	0/4/2	0/4/2	1/3/2
全生存期間（日）	233	207	344

参考文献26から改変転載

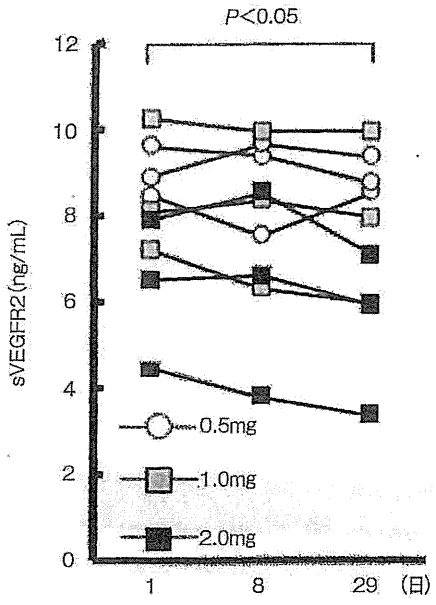

図1 ペプチドワクチン投与によるsVEGFR2抑制効果
参考文献26より改変転載

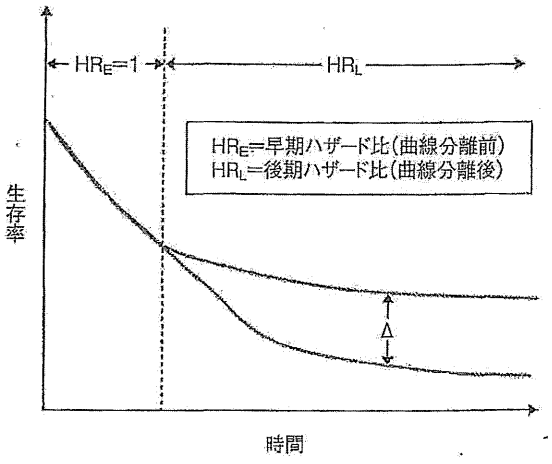

図2 癌ワクチン療法における生存曲線分離効果遅延

臨床試験」（ClinicalTrials. gov ID：NCT00622622）を施行した。種々の理由からdrop outした3名を除き，評価対象患者は18名で主要評価項目は安全性とした。注射部位反応やCTL反応解析などの免疫反応，臨床的反応を副次的評価項目とし推奨投与量を決定することとした。VEGFR2-169を0.5 mg，1.0 mg，2.0 mgの各コホート6名とし，週1回の投与とした。Gemcitabineは1,000 mg/m^{2}とし，通常投与と同じ3週投薬1週休薬とした。免疫学的解析ではVEGFR2-169特異的CTLが11例（61%）で誘導され，注射部位反応も15例（83%）に認められた（**表1**）。副作用は許容範囲内であり，投与量を規定する毒性は認めなかった。臨床的効果はペプチド投与部位の局所皮膚反応が陽性であった症例15例のうち12例（80%）がpartial responseまたはstable diseaseであったが，陰性であった症例3例すべてprogress diseaseであった（**表1**）。さらに2 mg投与群の生存期間が最も長かった（**表2**）。以上の結果から推奨投与量は2 mg/bodyとした[26]。また，ほかの第Ⅰ相臨床試験においても，重篤な副作用は認めず，血清sVEGFR2濃度はエルパモチド投与により有意に低下し，濃度依存性を示す傾向があった（**図1**）[27]。

V. 第Ⅱ/Ⅲ相臨床治験の意義—PEGASUS-PC 試験—

この医師主導型第Ⅰ相臨床試験の結果にて，pivotal に第Ⅱ/Ⅲ相臨床治験（PEGASUS-PC 試験）へと発展した。実薬と偽薬の割付比率は 2：1 で，153 例が登録された。主要評価項目である全生存期間では，実薬群と偽薬群で統計学的有意差は認められなかった。しかし，注射部位反応によるサブグループ解析を行ったところ，実薬群で皮膚反応を強く認めた 10 例の生存期間中央値が 16.0 ヵ月であるのに対し，偽薬ならびに実薬で皮膚反応のない群の生存期間中央値が約 8 ヵ月であった。すなわち，皮膚反応が一つのバイオマーカーになり得る可能性とペプチドワクチンにより生存期間が延長する responder が存在し，エルパモチドにより誘導された VEGFR-2 特異的 CTL の効果と考えられた。

PEGASUS-PC 試験では，全症例における生存期間の有意な延長は認めなかったが，PEGASUS-PC 試験は日本で初めてのペプチドワクチンによる膵癌に対する質の高い治験であるだけでなく，後の免疫療法の臨床試験のあり方の礎になると考えられる。とくに，免疫治療の特徴を評価する解析法要，すなわち，従来の抗悪性腫瘍薬とは全く異なった新しい解析方法で行われた点がほかの試験とは異なる斬新な試験ということができよう。通常，有効性は主に Kaplan-Meier 法により生存割合を算出し，log-rank 検定あるいは Willcoxon 検定によって治療群間の比較検定を行うことで，主要評価項目である生存期間あるいは無病生存期間の差により評価される。しかし，癌ペプチドワクチンにおいては，抗原特異的免疫応答を介した薬理薬効から遅発性の効果発現が想定されている（**図 2**）。このことを考慮し，観察期間後期に重み付けを置く Harrington-Fleming 法[28]による解析が行われ，独立行政法人医薬品医療機器総合機構（PMDA）がこれを許可したことは免疫療法の新時代を感じさせるものである。その反面，ペプチドワクチン療法に代表される免疫療法は，治療効果が得られるまでの時間が十分に得られない間に癌の進行による全身状態の悪化ならびに癌死亡に至ることが，免疫療法の有効性の証明を阻んでいるものと考える。

VI. ペプチドワクチンの効果発現の特性

ペプチドワクチンは生体の免疫反応を介した効果であるため，従来の抗腫瘍薬とは違った観点から評価をしなければならない。米国 FDA ではすでにガイダンスが発行されているが，日本には癌ワクチン療法のガイダンスはなく，日本バイオセラピィ学会が 2012 年 12 月に「がん治療用ペプチドワクチンガイダンス」を発行した（http://jsbt.org/guidance/）。統計検定においても，Harrington-Fleming 法[28]のような薬剤の特性に応じたハザード比の変化に対応した検定が必要である。また，腫瘍縮小効果は RECIST（Response Evaluation Criteria in Solid Tumours）での評価が一般的であるが，病勢進行（PD）の場合遅延性の効果については評価ができない。そこで PD 基準を修飾した irRC（immune-related response criteria）が提唱された[29,30]。irRC が生存期間延長効果と相関するかについては，今後のさらなる臨床研究が必要である。

VII. 複数のペプチドワクチンによる治療

PEGASUS-PC 試験では実薬群の中に強い皮膚反応を示す症例があり，生存期間が延長する可能性が示唆された。そこで，使用するペプチドの種類を増やすことで免疫能の改善する症例が増えることが期待される。Stage Ⅲ大腸癌では RNF43 と TOMM34 由来の HLA-A24 拘束性ペプチドと経口抗癌剤である UFT/LV を投与したところ，RNF43 と TOMM34 の双方に対する CTL 反応陽性群の生存期間中央値が 36.1 ヵ月であるのに対し，CTL 反応陰性群の生存期間中央値は 9.5 ヵ月と短かった（$p=0.0079$）[28]。標準治療不応進行食道癌では TTK，LY6K，IMP3 由来の HLA-A24 拘束性ペプチドを投与したところ，三つの抗原に対する CTL 陰性群に

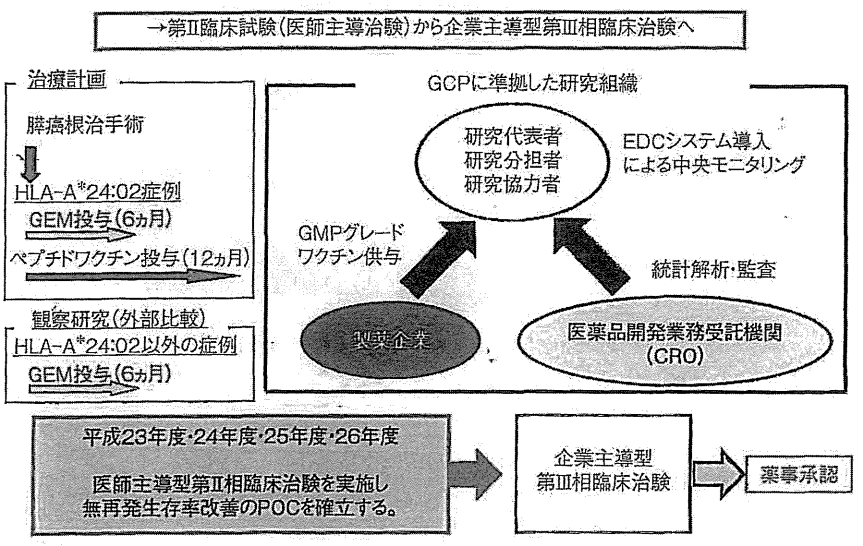

図3 膵癌に対する術後再発予防のための2方向性ペプチドワクチン療法の開発

比較して，陽性抗原数が増えるにつれ生存期間が延長し，TTK，LY6K，IMP3の3抗原すべてに反応を示した群のみに1年以上の長期生存例が見られた[31]。膵癌においても，腫瘍新生血管と腫瘍細胞を直接標的とする2方向性のワクチン療法として，VEGFR1，VEGFR2，KIF20Aに対する3種類のペプチドワクチンを混合したC01を用いた標準療法不応膵癌に対するプラセボ対照ランダム化第III相臨床治験（COMbined PEptide ThErapy for Pancreatic Cancer；COMPETE-PC試験）が全国40施設で実施された。しかし，対象症例が標準治療不応の進行膵癌であるため，生存期間に対する優越性を証明することはできず，中間解析において無効中止となった。これらの治験結果を詳細に層別化に基づく解析を進めることで，ペプチドワクチンの対象症例を絞り込めるものと考えられることから，詳細な報告が待たれる。

Ⅷ．医師主導治験：膵癌に対する術後再発予防のための2方向性新規ペプチドワクチン療法の開発

本来，免疫療法は再発予防に最も適した治療法である。そこで，現在われわれはC01ワクチンを用いた膵癌切除後の再発予防を目的とした多施設共同第II相臨床治験を医師主導型治験として実施している。本研究の結果を基に，企業主導型第III相治験へ展開し，薬事承認を目指すことを目的としている（**図3**）。

Ⅸ．ペプチドワクチン療法のbreak through

進行癌患者ではすでに免疫抑制状態に陥り，細胞傷害性T細胞なども疲弊している可能性がある。進行癌におけるT細胞の不適切な抗原刺激はT細胞の活性を抑制すること，すなわちanergyが示唆されていたが，このT細胞の抑制メカニズムとしてImmune checkpointが重要であることが明らかとなってきた。すなわち，Immune checkpointを介した不適切な細胞傷害性Tリンパ球の活性化により抗腫瘍効果を失う一方（**図4**），Immune checkpointに対する抗体でImmune checkpointをblockすると細胞傷害性Tリンパ球の抗腫瘍活性は回復することが明らかになった。これまでに，メラノーマ患者にImmune checkpointであるPD-1とCTLA-4の抗体を単独投与する第I相試験の結果，PD-1とCTLA-4に対する2種類の抗体を1 mg/kgまたは3 mg/kg投与する同時併用投与のcohortでは，40～53％のObjective Responseを認め，その効果は持続した[32]。

これらの効果により，FDAは2011年にCTLA-4抗体を転移性メラノーマに対する標準治療として承認し

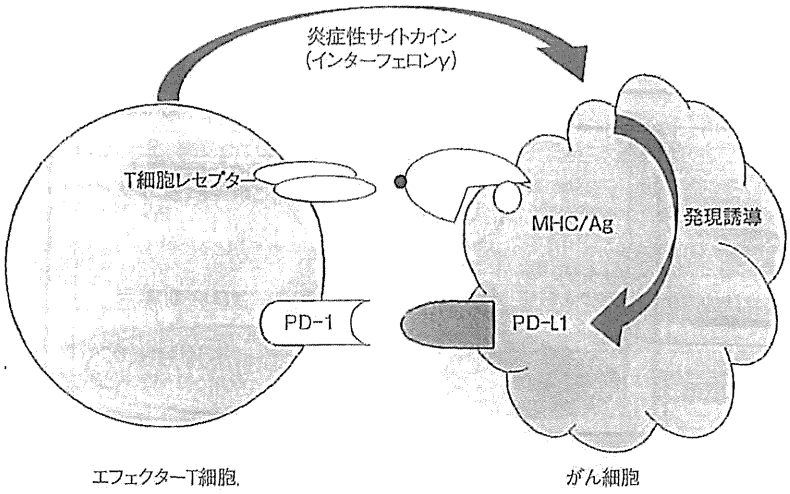

図4 PD-1/PD-L1 チェックポイントによるがん細胞の免疫抑制

た。さらに，CTLA-4 と PD-1 抗体の併用や抗癌剤との併用，対象癌種の拡大にて immune checkpoint を標的とする治療開発は急速に発展すると考えられる。

おわりに

膵癌遠隔転移症例を対象に FOLFIRINOX 療法が無作為化比較試験でゲムシタビン塩酸塩に対し有意に生存期間を延長することが報告され[4]，Nanoparticle albumin-bound paclitaxel (nab-paclitaxel) も今後の有望な薬剤となってくると考えられる。FOLFIRINOX 療法はゲムシタビン塩酸塩を含まない regimen であり，臨床の現場ではその効果に期待がもたれるが，feasibility の検証結果，とくに好中球減少などの有害事象の検討が待たれるところである。しかし，膵癌は診断時に栄養状態不良な症例や高齢者も多く含まれるため，このような toxic regimen が非適応の症例も多数存在し，副作用の軽微な治療法の開発も非常に重要である。ペプチドワクチン療法は副作用の少ない治療法であり，今後，有効なペプチドワクチンの同定だけでなく，quality assessment や quality control が必要である。また，T 細胞反応，Treg・myeloid-derived suppressor 細胞・NK 細胞・樹状細胞の解析，腫瘍関連抗原に対する抗体などが検討されているが[33,34]，免疫療法において有効性を示唆する biomarker の更なる開発が必要である。また，ペプチド投与により有効に強力な CTL が誘導できるワクチンアジュバントの開発も必要である。腫瘍周辺の微小環境は免疫療法の場としては有利な場ではないが，OK-432 と NY-ESO-1 タンパク質ワクチンと併用することで，Treg 存在下でも CTL が活性化可能[35]である。CpG-ODN は TLR-9 agonist であるが，CpG は樹状細胞の活性化を介して大量の type-1 インターフェロン産生を誘導し，自然免疫とともに獲得免疫を活性化することでペプチドワクチンの効果増強が得られる可能性が報告された[30]。さらに，LY6K および TTK 由来のペプチドワクチン療法に CpG-B を併用する第 I 相臨床試験において，CpG-ODN の癌ワクチンにおけるアジュバントとして有用であった[36]。

2010 年に免疫療法治療薬としては sipuleucel-T (Provenge) が，世界初の前立腺癌に有効な癌ワクチン療法として，2011 年には免疫チェックポイント阻害薬である抗 CTLA-4 抗体を FDA が認可した。PD-1 (Programmed Cell Death-1) といった腫瘍免疫において抑制的に働く補助刺激分子を標的とした薬剤も[37]免疫療法においては画期的なものとなるかもしれない。一方，膵癌に対するペプチドワクチンの分野では承認されたものはいまだないのが現状であり，ペプチドワクチンに代表される免疫療法薬が開発されるよう治験などの質の高い臨床試験の推進が必要である。

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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' *KpnI* 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

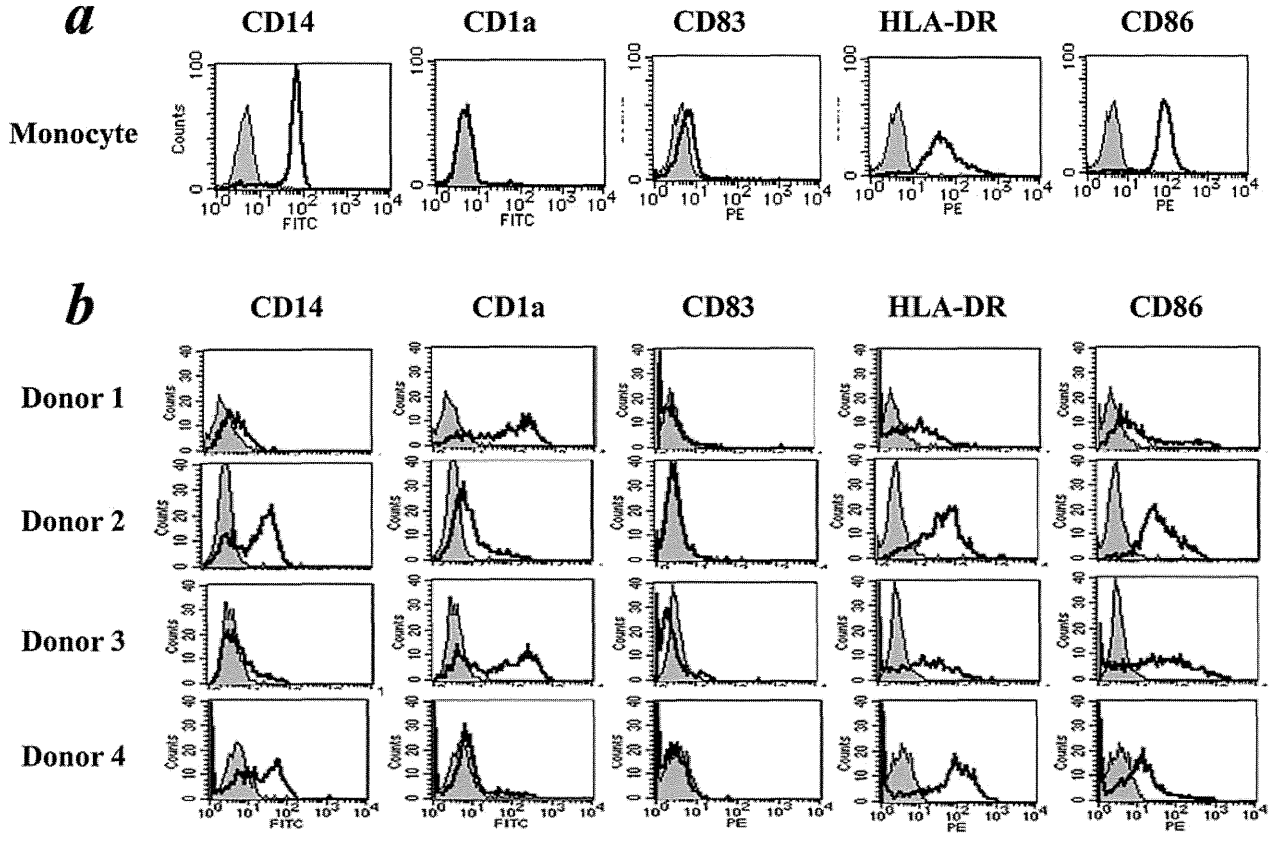

Figure 1. *a: Phenotype of monocytes isolated by magnetic beads from peripheral blood mononuclear cells. Cells were enriched and harvested immediately. b: Phenotype of monocyte-derived dendritic cells. Peripheral blood mononuclear cells were isolated from the peripheral blood of healthy individuals. 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).

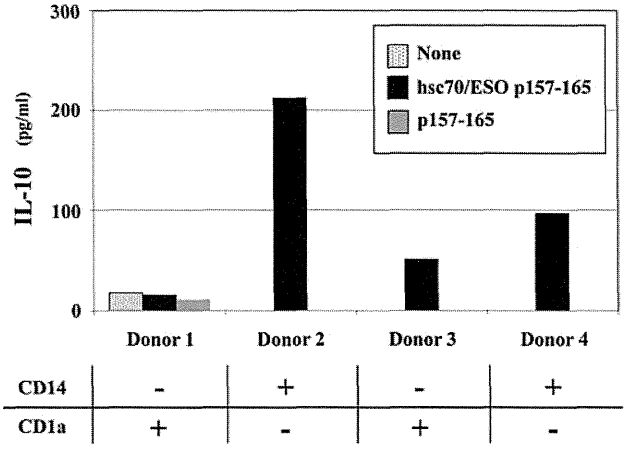

Figure 2. *Interleukin-10 (IL-10) production of dendritic cells (DCs). $CD14^-CD1a^+$ DCs and $CD14^+CD1a^-$ DCs were stimulated by hsc70/ESO p157-165 fusion protein or p157-165 in RPMI medium for 12 h. The IL-10 in the supernatants of pulsed DCs was measured by an enzyme-linked immunosorbent assay.*

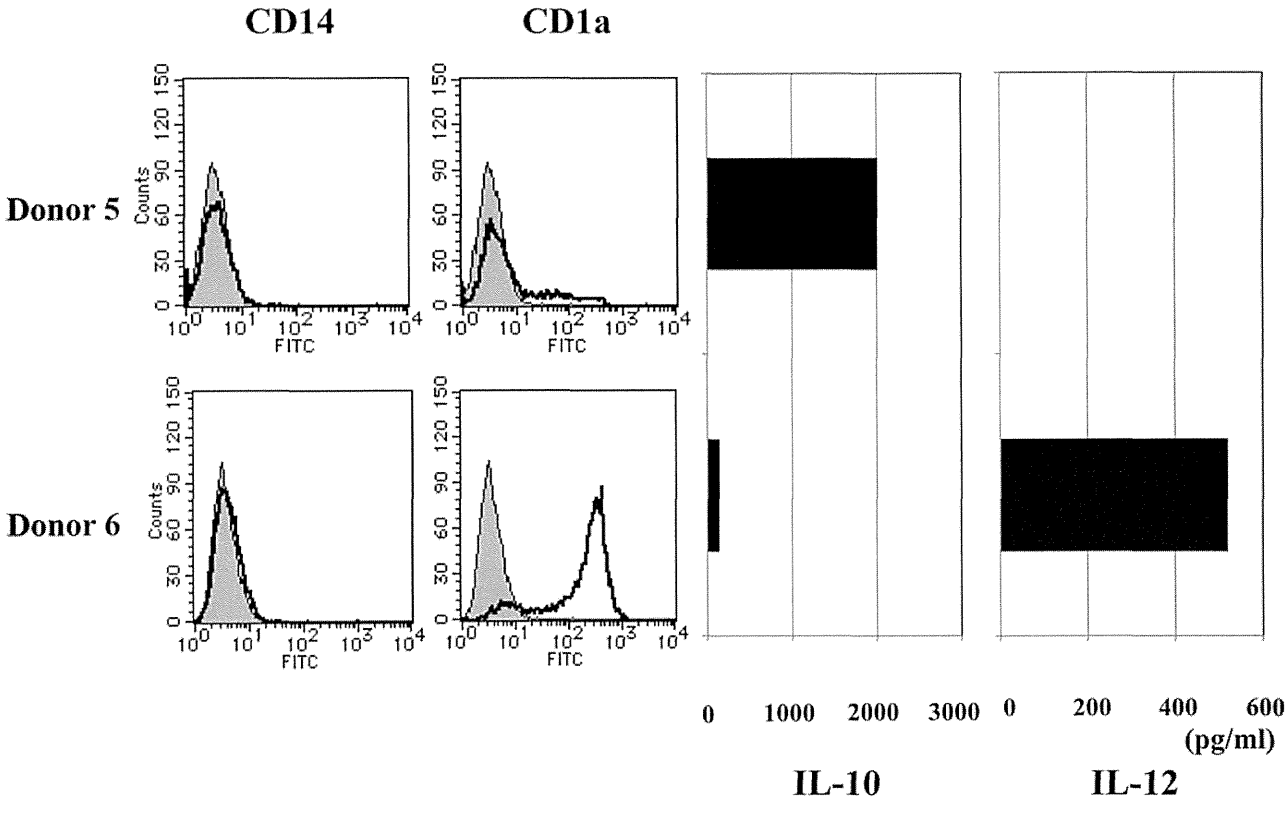

Figure 3. *Interleukin-10 (IL-10) and interleukin-12 (IL-12) production of dendritic cells (DCs). $CD14^{-}CD1a^{+}$ DCs and $CD14^{+}CD1a^{-}$ DCs were activated, as described in Materials and Methods by the hsc70/ESO p157-165 fusion protein. IL-10 and IL-12 in the supernatants of cells were measured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

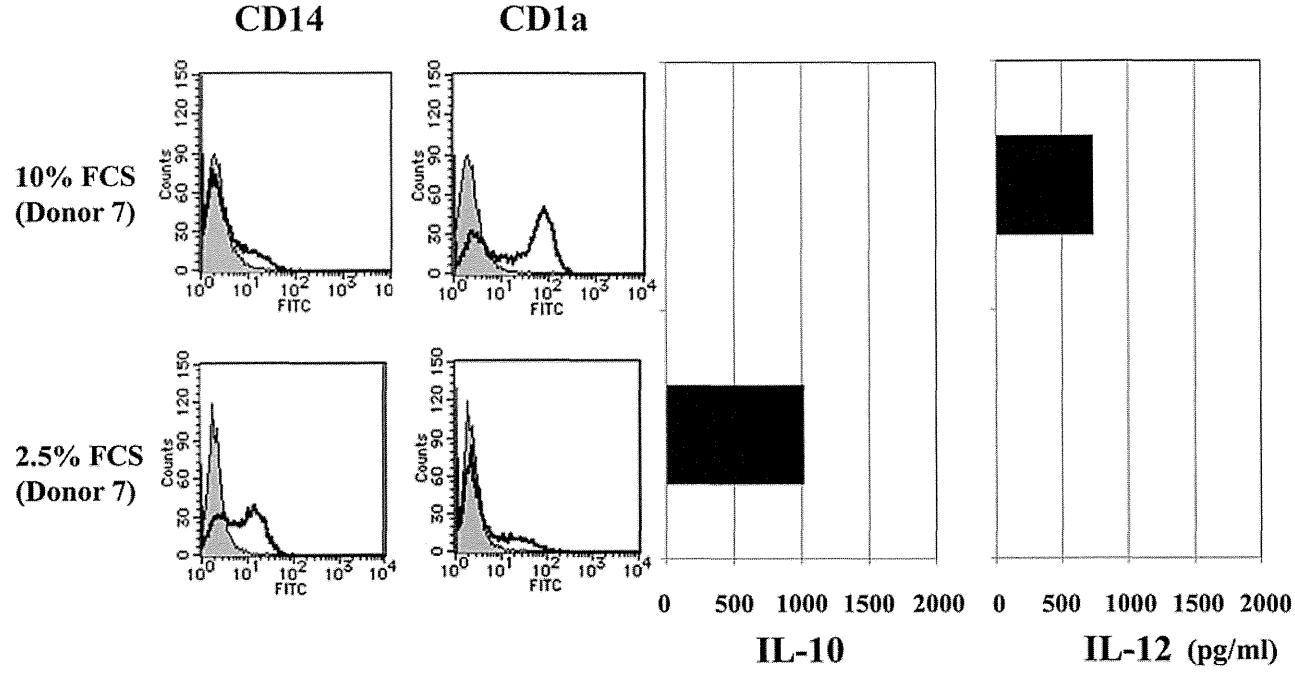

Figure 4. *Cytokine production of dendritic cells (DCs) in different culture medium. The percentage of fetal calf serum (FCS) in RPMI medium for generation of monocyte-derived DCs was changed from 10% to 2.5%.*

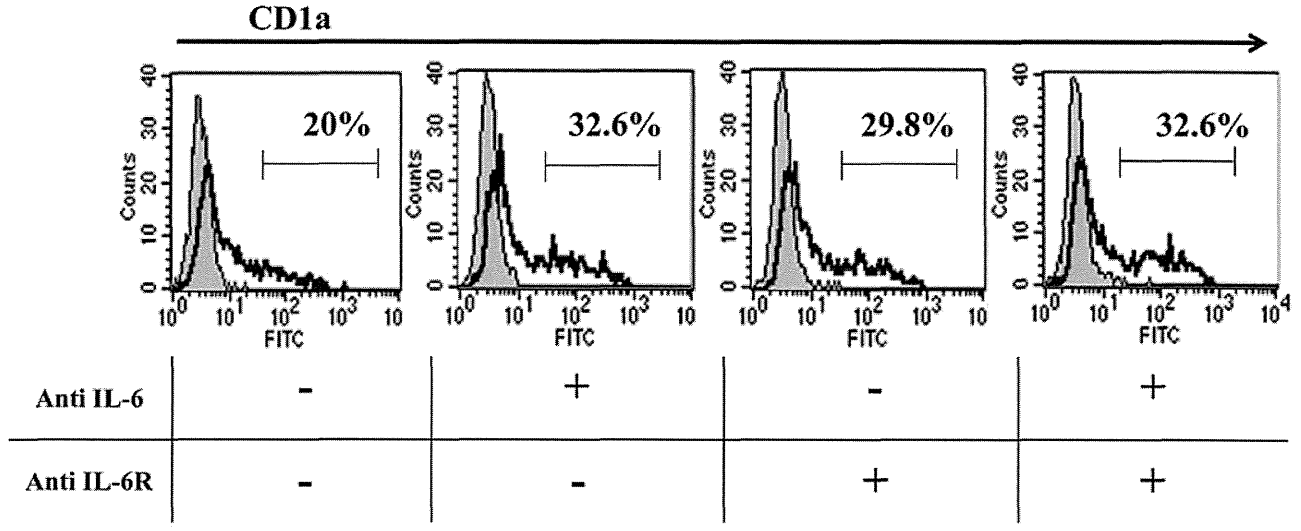

Figure 5. *The population of CD1a$^+$ dendritic cells (DCs) after neutralizing Interleukin-6 (IL-6) and/or IL-6R. Antibodies to human IL-6 and IL-6R were added to the culture on day 0 and 3 of the generation of DCs from monocytes, in a medium with 2.5% fatal calf serum (FCS). The CD1a expression of harvested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

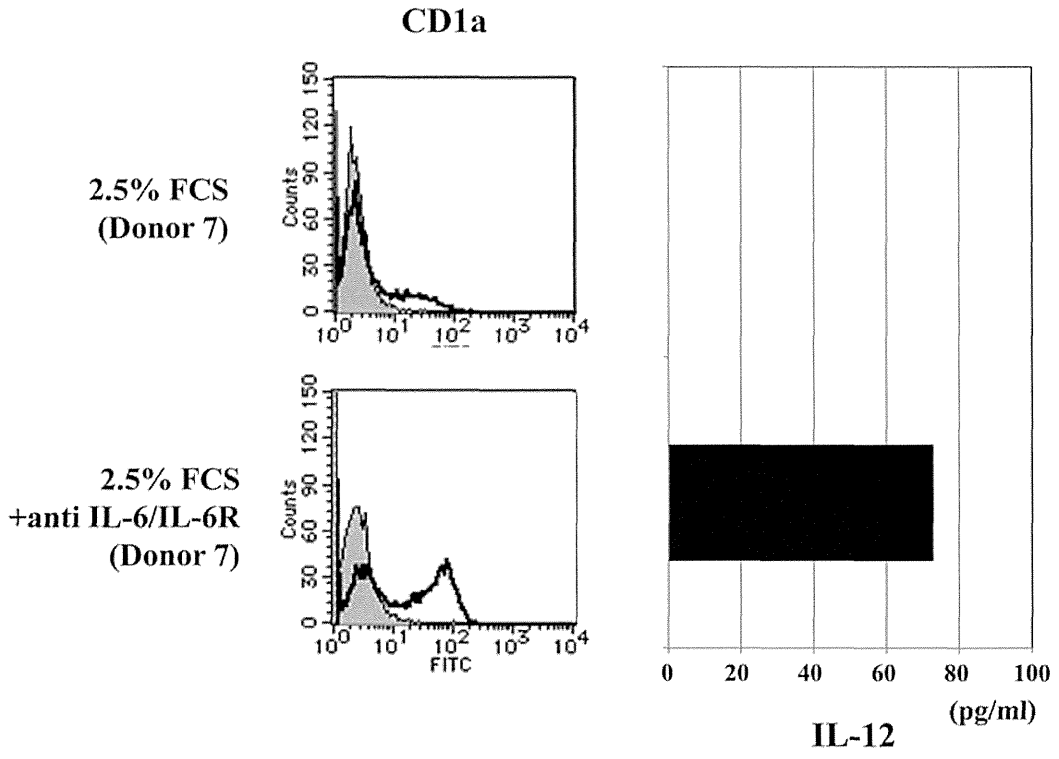

Figure 6. *Interleukin-12 (IL-12) production of monocyte-derived dendritic cells (mo-DCs) following culture with antibodies to interleukin-6 (IL-6) and IL-6R. The DCs generated in RPMI plus 2.5% fetal calf serum (FCS) and IL6/IL-6R antibodies up-regulated their expression of CD1a. The IL-12 production 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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ORIGINAL ARTICLE

Immunological milieu in the peritoneal cavity at laparotomy for gastric cancer

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Abstract

AIM: To investigate the immunological repertoire in the peritoneal cavity of gastric cancer patients.

METHODS: The peritoneal cavity is a compartment in which immunological host-tumor interactions can occur. However, the role of lymphocytes in the peritoneal cavity of gastric cancer patients is unclear. We observed 64 patients who underwent gastrectomy for gastric cancer and 11 patients who underwent laparoscopic cholecystectomy for gallstones and acted as controls. Lymphocytes isolated from both peripheral blood and peritoneal lavage were analyzed for surface markers of lymphocytes and their cytokine production by flow cytometry. $CD4^+CD25^{high}$ T cells isolated from the patient's peripheral blood were co-cultivated for 4 d with the intra-peritoneal lymphocytes, and a cytokine assay was performed.

RESULTS: At gastrectomy, $CCR7^-$ $CD45RA^-$ $CD8^+$ effector memory T cells were observed in the peritoneal cavity. The frequency of $CD4^+$ $CD25^{high}$ T cells in both the peripheral blood and peritoneal cavity was elevated in patients at advanced stage [control *vs* stage Ⅳ in the peripheral blood: 6.89 (3.39-10.4) *vs* 15.34 (11.37-19.31), $P < 0.05$, control *vs* stage Ⅳ in the peritoneal cavity: 8.65 (5.28-12.0) *vs* 19.56 (14.81-24.32), $P < 0.05$]. On the other hand, the suppression was restored with $CD4^+$ $CD25^{high}$ T cells from their own peripheral blood. This study is the first to analyze lymphocyte and cytokine production in the peritoneal cavity in patients with gastric cancer. Immune regulation at advanced stage is reversible at the point of gastrectomy.

CONCLUSION: The immunological milieu in the peritoneal cavity of patients with advanced gastric cancer elicited a Th2 response even at gastrectomy, but this response was reversible.

Key words: Cytokines; Gastric cancer; Lymphocytes; Peritoneal cavity; Regulatory T cell

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