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CONFLICT OF INTEREST DISCLOSURE

Dr. Takeuchi is a scientific advisor for the anti-ALK iAEP immunohistochemistry kit (ALK Detection Kit, Nichirei Bioscience, Tokyo, Japan). All remaining authors have made no disclosures.

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ALK fusion gene positive lung cancer and 3 cases treated with an inhibitor for ALK kinase activity

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ABSTRACT

Background: Anaplastic lymphoma kinase (ALK) fusion gene-positive lung cancer accounts for 4–5% of non-small cell lung carcinoma. A clinical trial of the specific inhibitor of ALK fusion-type tyrosine kinase is currently under way.

Methods: ALK fusion gene products were analyzed immunohistochemically with the materials obtained by surgery or by endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA). The echinoderm microtubule-associated protein-like 4(EML4)-ALK or kinesin family member 5B (KIF5B)-ALK translocation was confirmed by the reverse transcription polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH). After eligibility criteria were met and informed consent was obtained, 3 patients were enrolled for the Pfizer Study of Crizotinib (PF02341066), Clinical Trial A8081001, conducted at Seoul National University.

Results: Out of 404 cases, there were 14 of EML4-ALK non-small cell carcinoma (NSCLC) and one KIF5B-ALK NSCLC case (8 men, 7 women; mean age, 61.9 years, range 48–82). Except for 2 light smokers, all patients were non-smokers. All cases were of adenocarcinoma with papillary or acinar subtypes. Three were of stage IA, 5 of stage IIIA, 1 of stage IIIB and 6 of stage IV. Ten patients underwent thoracotomy, 3 received chemotherapy and 2 only best supportive care (BSC). One BSC and 2 chemotherapy cases were enrolled for the clinical trial. Patients with advanced stages who received chemotherapy or best supportive care were younger (54.0 ± 6.3) than those who were surgically treated (65.8 ± 10.1) ($p < 0.05$).

The powerful effect of ALK inhibitor on EML4-ALK NSCLC was observed. Soon after its administration, almost all the multiple bone and lymph node metastases quickly disappeared. Nausea, diarrhea and the persistence of a light image were the main side effects, but they diminished within a few months.

Conclusion: ALK-fusion gene was found in 3.7% (15/404) NSCLC cases and advanced disease with this fusion gene was correlated with younger generation. The ALK inhibitor presented in this study is effective in EML4-ALK NSCLC cases. A further study will be necessary to evaluate the clinical effectiveness of this drug.

1. Introduction

As the mechanisms of carcinogenesis become clearer, the target of cancer treatment is shifting from non-specific cytotoxic agents to specific agents that block key molecular events in the carcinogenesis of malignancy such as EGFR-TKI and anti-HER2 antibody (trastuzumab) [1–3]. Recently, Mano et al. [4–6] reported that a small inversion within chromosome 2p results in the formation of a fusion gene comprising portions of the

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echinoderm microtubule-associated protein-like 4 (EML4) gene and the anaplastic lymphoma kinase (ALK) gene in non-small-cell lung cancer. Transgenic mice that express EML4-ALK specifically in lung epithelial cells develop multiple foci of adenocarcinoma in the lung soon after birth, and the oral administration of a specific inhibitor of ALK tyrosine kinase activity eradicated completely the foci of adenocarcinoma. Clinical trials of specific inhibitors of EML4-ALK tumors are currently underway [7–11]. Kwak et al. [11] reported the effect of crizotinib in Clinical Trial A8081001 on the 82 patients with advanced ALK-positive disease. Over a mean treatment duration of 6.4 months, the overall response rate was 57% and the estimated probability of 6-month progression-free survival was 72%. We report 15 cases of ALK fusion gene-positive NSCLC cases and 3 cases in our experience with ALK inhibitor in the Pfizer Study of crizotinib (PF02341066), Clinical Trial A8081001, which was conducted at Seoul National University.

2. Materials and methods

Out of 404 patients who had undergone surgical resection (295 cases) or bronchoscopy (109 cases) in Chiba Cancer Center, Japan, from 2007 to 2009, 15 ALK fusion gene-positive NSCLC patients were initially screened by immunohistochemical procedures. Diagnoses were confirmed by RT-PCR and/or FISH for their molecular translocation.

2.1. ALK fusion protein detection by immunohistochemical methods

The intercalated antibody-enhanced polymer method of Takeuchi et al. [12,13] was used to detect ALK proteins. Formalin-fixed paraffin-embedded tissue was sliced at a thickness of 4 μm and the sections were placed on silane-coated slides. For antigen retrieval, the slides were heated for 40 min at 97 °C in target Retrieval Solution (pH 9.0; Dako). They were then incubated at room temperature, first with Protein Block Serum-free Ready-to-Use solution (Dako) for 10 min, and then with an anti-ALK antibody (5A4, Abcam) for 30 min. To increase the sensitivity of detection, we included an incubation step of 15 min at room temperature with rabbit polyclonal antibodies to mouse immunoglobulin (Dako). The immune complexes were then detected with the dextran polymer reagent and an AutoStainer instrument (Dako).

2.2. Confirmation of EML4-ALK fusion gene by RT-PCR and FISH

We confirmed the existence of ALK fusion gene expression by fluorescence in situ hybridization (FISH) and/or by the reverse transcription-polymerase chain reaction (RT-PCR).

2.3. Fluorescence in situ hybridization (FISH)

An EML4-ALK fusion assay was performed [10–12]. Unstained sections were processed with a Histology FISH Accessory Kit (Dako), subjected to hybridization with fluorescence-labeled bacterial artificial chromosome clone probes for EML4 and ALK (self-produced probes; EML4: RP11-996L7, ALK: RP11-984I21 and RP11-62B19), stained with 4,6-diamidino-2-phenylindole, and examined with a fluorescence microscope (BX51; Olympus). The FISH positivity criteria specified "over 50% cancer cells" for EBUS-TBNA samples.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

The multiplex PCR method proposed by the Japanese ALK lung cancer study group (ALCAS) was used to confirm the expression of ALK fusion gene [4–6].

Table 1
Characteristics of ALK fusion gene positive lung cancer patients.

Patient no	Sex	Age	SI	Histology	Variant	p Stage	Therapy	Recurrence	Distant meta	Survival (M)	Prognosis	ALK inhibitor case no
1	f	64	0	Ad: papillary	3	IIIA	Surgery	Positive	Bone, brain	21	Dead	
2	m	82	0	Ad: solid	2	IIIA	Surgery	Positive	Ascites	36	Alive	
3	f	68	0	Ad: papillary	3	IIIB	Surgery	Positive	Brain	34	Alive	
4	f	60	0	Ad: solid	3	IIIA	Surgery	Negative	None	29	Alive	
5	m	73	0	Ad: acinar	3	IA	Surgery	Negative	None	21	Alive	
6	m	66	0	Ad: papillary	KIF5B	IA	Surgery	Negative	None	15	Alive	
7	m	56	300	Ad: papillary	1	IA	Surgery	Negative	None	13	Alive	
8	m	46	0	Ad: acinar	5	IIIA	Surgery	Negative	None	22	Alive	
9	m	71	0	Ad: papillary	1	IIIA	Surgery	Negative		17	Alive	
10	f	73	0	Ad: acinar	1	IV	Surgery	Negative		14	Alive	
11	m	55	100	Ad: muc+	3	IV	BSC		Bone, brain	5	Dead	
12	m	48	0	Ad: muc+	1	IV	Chemo		Bone, brain	29	Dead	Case 1
13	f	49	0	Ad: muc+	3	IV	BSC		Bone, brain	15	Alive	Case 2
14	f	54	0	Ad: muc+	1	IV	Chemo		Bone, brain, pul	22	Alive	Case 3
15	f	64	0	Ad: acinar	3	IV	Chemo		Pul	2	Alive	

SI, smoking index; f, female; m, male; Ad, adenocarcinoma; muc+, mucin production; Distant meta, at the recurrence (surgery group) at the diagnosis (non-surgery group); pul, pulmonary metastasis; Case 1 was already reported by Nakajima et al. [16].

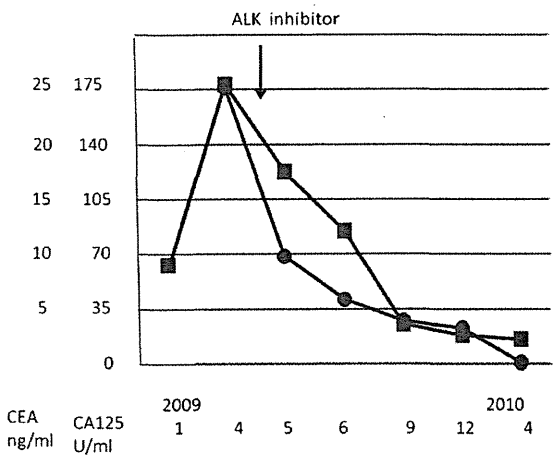

Fig. 1. Changes of tumor markers before and during the treatment with ALK inhibitor (Case 1) CEA (■), CA125 (●). Marked reduction of tumor markers was observed.

Total RNA was isolated from EBUS-TBNA or surgical samples using AllPrep DNA/RNA Mini Kit (Qiagen) and was reverse-transcribed into single strand cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). To detect a fusion cDNA derived from EML4 or KIF5B and ALK, PCR analysis was performed with the AmpliTaq Gold PCR Master Mix (Applied Biosystems), the forward primers derived from EML4, EA-F-cDNA-S (5′-GTGCAGTGTTTAGCATTCTTGGGG-3′), EA-F-2-g-S (5′-AGCTACATCACACACCTTGACTGG-3′), EA-F-cDNA-v3-S-2 (5′-TACCAGTGCTGTCTCAATTGCAGG-3″) and EA-W-cDNA-in-S (5′-GCTTTCCCCGCAAGATGGACGG-3′) and the forward primers derived from KIF5B, KA-F-cDNA-S-e24 (5′-CAGCTGAGAGAGTGAAAGCTTTGG-3′), KA-F-cDNA-S-e17 (5′-GACAGTTGGAGGAATCTGTCGATG-3′), KA-F-cDNA-S-e11 (5′-ATCCTGCGGAACACTATTCAGTGG-3′), and KA-cDNA-S-e2 (5′-TCAAGCACATCTCAAGAGCAAGTG-3′) and the reverse primer derived from ALK, EA-F-cDNA-A (5′-TCTTGCCAGCAAAG-CAGTAGTTGG-3′). PCR products were purified from gel bands using QIAquick Gel Extraction Kit (Qiagen) and confirmed by direct sequencing analysis.

2.5. Enrolment of patients for the Clinical Trial A8081001

Informed consent was obtained from each patient to be enrolled for the study [10]. Eligibility criteria for the enrolment of ALK translocation positive patients into the ALK TKI PI Trial were as required by the Committee of Clinical Trials A8081001.

3. Results

There were 15 ALK fusion gene-positive cases which were screened immunohistochemically and confirmed by RT-PCR and FISH [14,15]. Eight patients were men and 7 women, of mean age

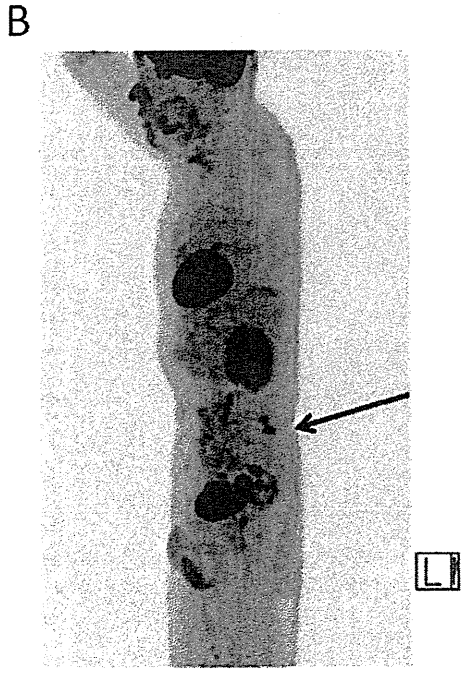

Fig. 2. FDG-PET scan of Case 1 performed at the same time (09/28/2009) as the previously reported Fig. 1D (Nakajima et al. [16]) shows bone metastasis of the left vertebral arch of L5 (arrow) in a sagittal view.

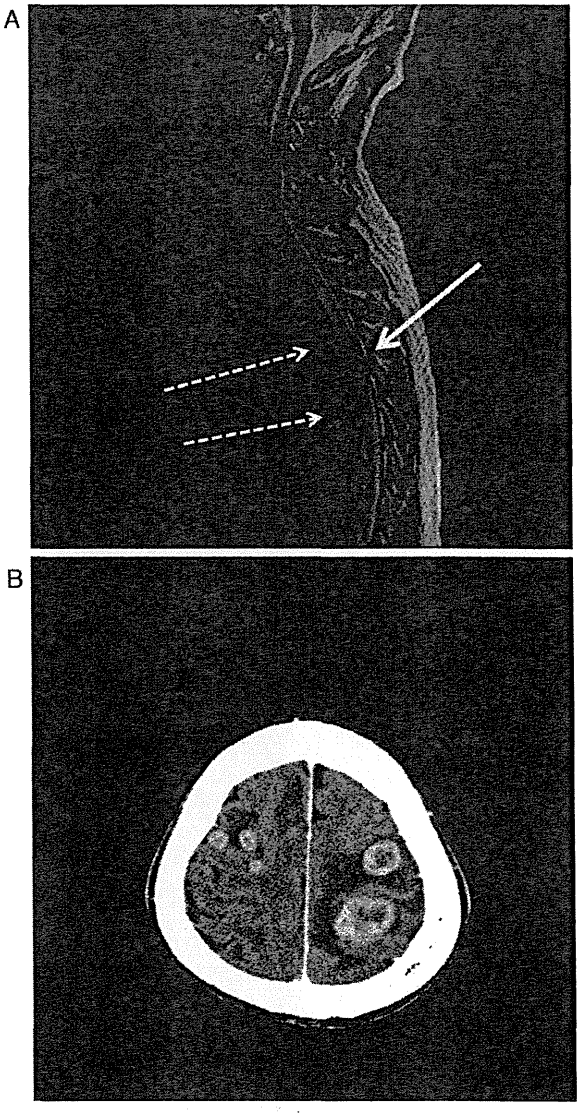

Fig. 3. MRI (Case 1) of the spinal cord on 04/05/2010 shows the metastases to the spinal cord (straight allow) and the spinal column (Th 4,6 dotted allow). B. CT scan (Case 1) of the brain on 04/05/2010 shows multiple brain metastases.

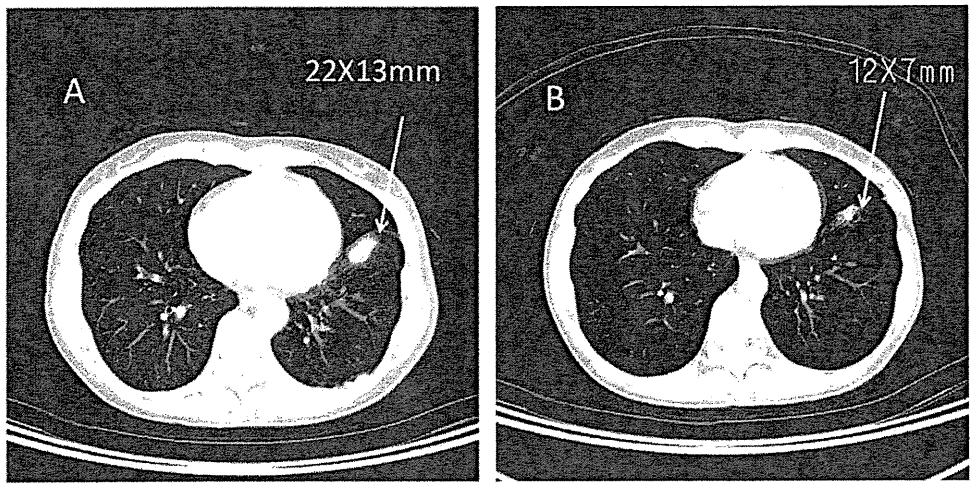

Fig. 4. CT scan (Case 2): A, 07/22/2009 (before ALK inhibitor) and B, 09/02/2009 (5 weeks after the initiation of the therapy). Left S8 tumor (arrow) decreased in size from 22X13 mm to 12X7 mm (PR).

61.9 years (range 48–82). Most were non-smokers, but 2 smoked lightly (Table 1). All tumors were adenocarcinomas with a papillary pattern predominant (5 cases), an acinar pattern predominant (3 cases), with mucin production (4 cases), etc. There were fourteen cases of fusion with EML4 and one KIF5B gene. There were 7 variant 3, 5 variant 1, and 1 each of variants 2 and 5. There were 3 stage IA, 5 stage IIIA, 1 stage IIIB and 6 stage IV cases. Ten cases were diagnosed after surgical resection, and 5, by tissue samples obtained with EBUS-TBNA. Ten cases underwent thoracotomy, 3 cases, chemotherapy, and 2 cases, only best supportive care. Of 5 cases diagnosed by EBUS-TBNA, 2 cases receiving chemotherapy and one receiving best supportive care were enrolled for the clinical trial. The mean age of the surgically treated group was 65.8 ± 10.1, and that of chemotherapy and BSC group was 54.0 ± 6.3. The difference was found by Student's t test to be statistically significant ($p < 0.05$), indicating that younger patients tend to have advanced cancer.

Out of 10 surgically treated cases, seven survived without a sign of recurrence, 3 had recurrence in both bone and brain tissue, and one died of bone and lymph node metastasis.

3.1. Case 1

Case 1 has already been reported in a case report (Nakajima et al.) [16] but without precise descriptions of the response to crizotinib, the adverse effects, the pattern of recurrence or the metastatic

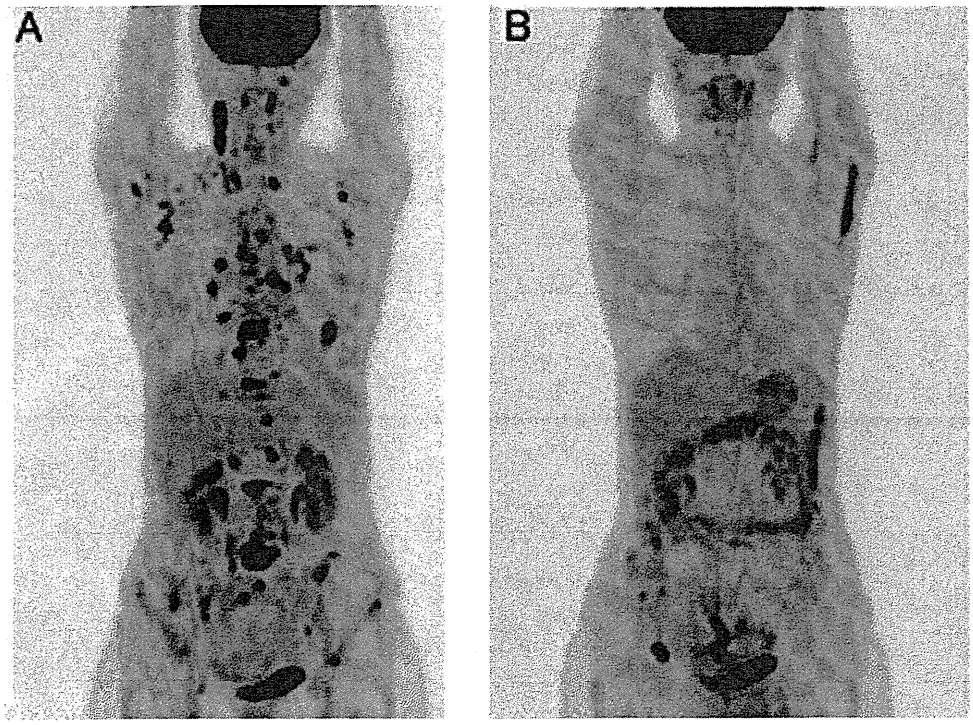

Fig. 5. FDG-PET scan (Case 2): A, 07/22/2009 (before ALK inhibitor) and B, 03/10/2010 FDG-PET scan shows marked reduction of accumulation in multiple bone and lymph node metastases 7 months after the initiation of the treatment.

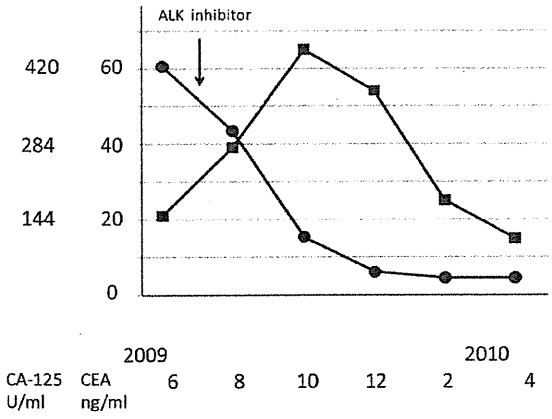

Fig. 6. Changes of tumor markers before and during the treatment with ALK inhibitor in case 2. CA125 (●) gradually decreased along with the treatment, but CEA (■) increased soon after the initiation of the therapy. The value of CEA then gradually decreased to 15.2 ng/ml in April 2010 (after 10 months).

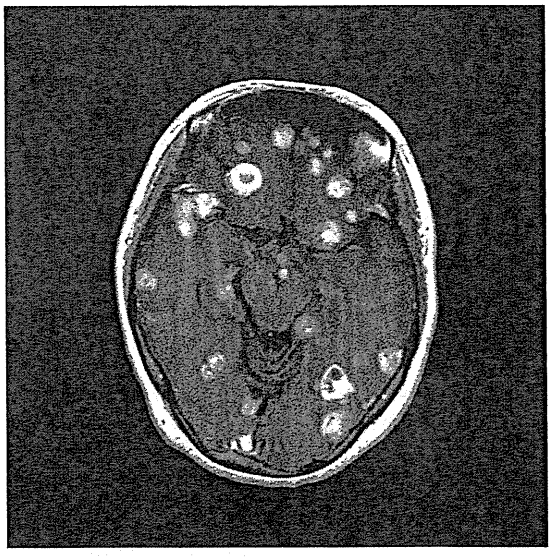

Fig. 7. Brain MRI of case 2 on 7/30/2010 showing multiple metastases.

tumor lesions. Such descriptions may contribute to a better understanding of the other cases, and so case 1 is described briefly below.

A 48-year-old non-smoking male patient had lung adenocarcinoma in the right lower lobe and multiple bone and lymph node metastases (T3N2M1 stage IV) at his first medical examination in November 2007. After several courses of chemotherapy, the patient was enrolled in a trial of crizotinib (PF02341066) from May 5th 2009 at Seoul National University, in which the drug was orally administered at 500 mg/day.

The effect of ALK inhibitor appeared rapidly. The patient's dyspnea improved within one week after drug administration. PS improved from 2 to 0 and a marked reduction in the tumor markers was observed (Fig. 1). Within 3 months after the start of therapy, almost all metastases disappeared except for those at the left vertebral arch of L5 (Fig. 2, arrow). The patient had severe adverse effects: diarrhea, nausea and persistence of light images started soon after the administration of the drug, but these gradually diminished over a 3-week period.

The control of the primary and metastatic tumors continued for 11 months until the patient visited Seoul University in April 2010, when he was hospitalized for paralysis of the lower extremities. MRI revealed spinal column (Th4-6) and spinal cord metastases (Fig. 3A). Soon after his hospitalization in our Cancer Center in April 2010, multiple brain metastases (Fig. 3B) were found, so the drug administration was stopped and he was transferred to a palliative care unit.

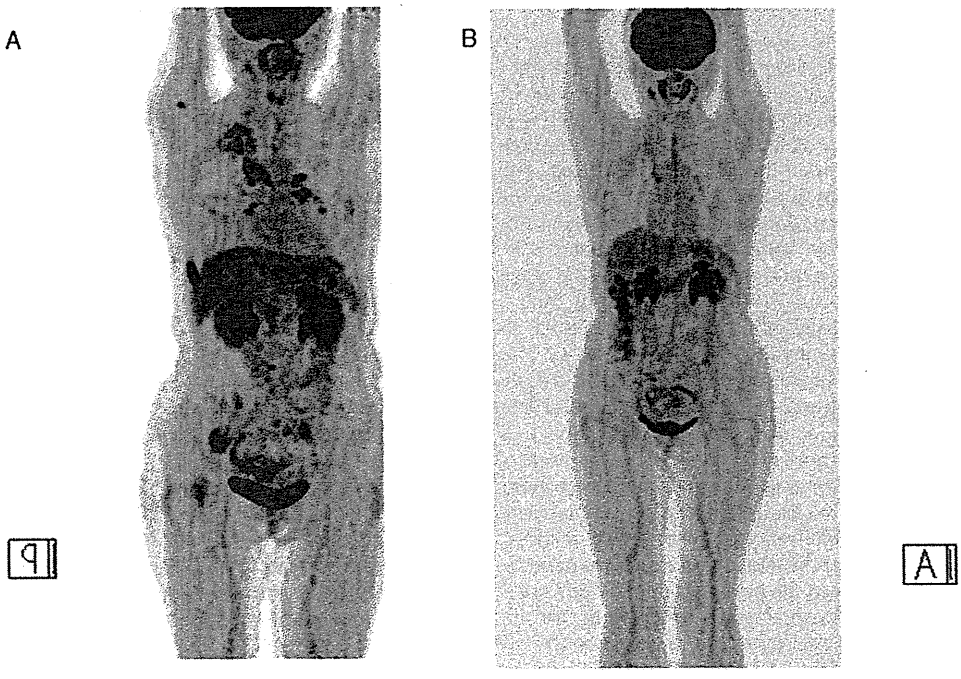

Fig. 8. FDG-PET scan: A, 09/08/2009 (before ALK inhibitor) and B, 07/05/2010 FDG-PET scan follow-up for 10 months indicated complete control of primary and distant metastases in case 3.

3.2. Case 2

A 49-year-old woman, a non-smoker with no history of illness, PS0, was introduced to the Orthopedics Department of our Center in April 2009 for back pain and multiple osteoplastic changes in the bones. Systematic examination revealed an abnormal shadow 22X13 mm in size in the left lower lobe (Fig. 4A). Bronchoscopy and a PET scan indicated left S8 adenocarcinoma with cervical, axial, mediastinal, hilar, pancreatic and retroperitoneal lymph node metastases, as well as cranial, thoracic (Th1-12), lumbar (L1-5), rib (1–12) pelvis, humerus, and femur metastases (Fig. 5A).

She refused any therapy except for best supportive care. One month after the examination, an additional immunohistochemical examination for EML4-ALK fusion protein was performed, and found to be positive. The presence of mRNA for EML4-ALK gene was also confirmed by RT-PCR and FISH from the mediastinal #4R lymph nodes obtained with EBUS-TBNA, which was performed 2 months later. EGFR mutation was negative, but the direct sequence of the EML4-ALK mRNA indicated that the translocation was variant 3 [9]. She decided to be enrolled to the crizotinib study (PF02341066) at a dosage of 500 mg/day at Seoul National University from July 2009.

She had nausea, diarrhea and light image persistence as in case 1, but her gastrointestinal symptoms were severer than those in case 1. Two weeks after the administration of ALK inhibitor, her back pain disappeared. A PET scan performed 5 weeks after the initiation of the therapy showed marked reduction of bone and lymph node metastases, and the primary tumor had decreased in size from 22X13 mm to 12X7 mm (Fig. 4A and B). Also, the SUV max dropped from 10.7 to 2.42. Changes of tumor markers were not parallel with the clinical course since the measured value of CA-125 dropped from 424 to 107 U/ml, but that of CEA increased from 21.5 to 65.4 ng/ml 4 months later. The value of CEA then gradually decreased to 15.2 ng/ml in April 2010 (10 months after that: Fig. 6). The PET scan conducted after 7 months indicated a partial response to multiple bone and lymph node metastases (Fig. 5B). The patient continued to take the drug until the end of July 2010, when brain metastases (Fig. 7) were found.

3.3. Case 3

A fifty-four-year-old woman, also a non-smoker, PS0, visited a doctor because of back pain in August 2008. Chest X-ray and CT scan showed an S3 59X22 mm tumor in the right upper lobe, combined with #4R, #2R mediastinal lymph nodes and intrapulmonary metastases. The tumor had invaded the SVC and the azygos vein. She had undergone bronchoscopy and EBUS-TBNA in October 2008. A diagnosis of lung adenocarcinoma was obtained with TBNA samples from #7 lymph nodes. Bone scans indicated cranial, costal, vertebral, scapular, pelvic and femoral metastases (T4N2M1 stage IV). She received 2 courses of CBDCA + GEM (1000 mg/m^2) and 7 courses of docetaxel (TXTL: 60 mg/m^2) from November 2008 to June 2009, but the effect was minimal.

EML4-ALK fusion gene was suggested immunohistochemically in August 2009 and confirmed by RT-PCR obtained by EBUS-TBNA samples from the primary tumor in September 2009. She was enrolled for the clinical trial from November 2009 with an oral administration of crizotinib 500 mg/day. Dyspnea and cough were alleviated within 2 weeks, and she complained of severe diarrhea, nausea, vomiting, light image persistence and perceived changes of taste. A PET scan one month after the start of the treatment demonstrated complete disappearance of the primary tumor as well as all the metastases except for a bone metastasis to the right 8th rib. A PET scan follow-up 8 months later indicated complete control of primary and metastatic tumors (Fig. 8A and B). CEA declined slowly from 1764 ng/ml to 79 ng/ml 6 months after the start of administration (Fig. 9). The patient had 12 brain metastases from 5 mm^3 to 309 mm^3 in volume and underwent gamma knife irradiation in August 2009, 2 months before the start of ALK inhibitor treatment. The irradiated field still showed little change for 5 months, but small new lesions appeared in the left occipital area 6 months after the start of the trial. Brain metastases grew very slowly, so we have maintained our observation until October 2010.

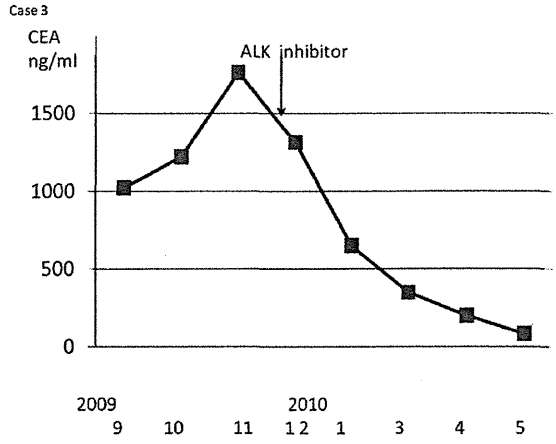

Fig. 9. CEA (■) declined slowly from 1764 ng/ml to 79 ng/ml 6 months after the start of the therapy in case 3.

4. Discussion

Above, we have reported the far-reaching effects of an ALK inhibitor on *EML4-ALK*-positive lung cancer patients. Soon after the administration of crizotinib, almost all metastases to bone and lymph nodes rapidly disappeared, followed by a marked reduction in the level of tumor markers in the sera. These observations clearly support the pivotal role of EML4-ALK oncokinase for the growth/survival of not only primary tumors but of the metastases. Such profound effects were rare among the patients when treated with conventional cytotoxic anticancer drugs.

The three cases which were enrolled for the study had surprisingly similar biological characteristics. They had multiple bone and lymph node metastases at the first medical examination, and were non-smokers at younger ages (48–54) who were resistant to chemotherapy. Adverse effects with crizotinib were also similar among them, including transient diarrhea, nausea, light image persistence, and subjective changes of taste. In addition, their response to ALK inhibitor was similar. Bone and lymph node metastases had disappeared within one month after the initiation of the therapy. The response of the primary tumor in case 2 was relatively slow compared with those of the metastases. The difference between the response of primary tumor and metastases to the ALK inhibitor in this case seems to indicate that the similar subclones of tumor cells in the primary tumors that were highly responsive to ALK inhibitor metastasized to distant organs and may give some explanation for the discrepancy in the time-course between CEA and CA125.

Molecular and immunohistochemical analyses in this cohort were conducted on the basis of the specimens obtained through EBUS-TBNA. Originally, EBUS-TBNA had been proposed useful for the pathological diagnosis of mediastinal involvement (N2 disease) of lung cancer [17–20]. However, we have already reported that EBUS-TBNA is also a versatile way of obtaining histological samples for the molecular analyses of cancer-related genes, such as *EGFR*, p53 et al. [21,22]. For those who have advanced NSCLC, it is often difficult to conduct surgery to obtain specimens from patients. Among such cases, however, EBUS-TBNA can usually be safely carried out to obtain specimens from enlarged mediastinal

lymph nodes or parathracheal tumors. We carried out EBUS-TBNA procedure for the reasons of its advantage in obtaining high quality core samples adequate for this purpose as well as its safety. We do not disregard the importance of TBB for the diagnosis of lung cancer; however, we needed histological samples to examine the immunohistochemistry and FISH for enrolment in a trial of crizotinib. Our experience with the three cases clearly demonstrates the importance and clinical relevance of obtaining such specimens for molecular analyses.

Although the initial effects of crizotinib are substantial in our cases, as well as in those reported by Bang et al. [10,11], such efficacy may not always last long. There was, for instance, development (case 1 and 2) and recurrence (case 3) of brain metastases while favorable control was maintained outside the brain. Given that the primary tumors and lymph node metastases were under control of crizotinib even at the appearance of brain metastases, the tumor cells outside the brain did not lose sensitivity to crizotinib. Relapses in the brain only may indicate either (i) subclones of the tumor acquired both the homing ability to the brain and resistance to crizotinib, or (ii) crizotinib may not penetrate the blood-brain barrier, leading to insufficient concentrations of crizotinib in the brain. It is thus highly important to examine in detail the molecular basis that would account for such acquired resistance to crizotinib, which may be secondary mutations within EML4-ALK itself or mutations/gene amplification of other genes, as demonstrated in the cases of acquired resistance of NSCLC to gefitinib/erlotinib [23–26].

Conflict of interest

None declared.

Acknowledgements

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Ex Vivo Expansion of Human CD8$^+$ T Cells Using Autologous CD4$^+$ T Cell Help

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Abstract

Background: Using *in vivo* mouse models, the mechanisms of CD4$^+$ T cell help have been intensively investigated. However, a mechanistic analysis of human CD4$^+$ T cell help is largely lacking. Our goal was to elucidate the mechanisms of human CD4$^+$ T cell help of CD8$^+$ T cell proliferation using a novel *in vitro* model .

Methods/Principal Findings: We developed a genetically engineered novel human cell-based artificial APC, aAPC/mOKT3, which expresses a membranous form of the anti-CD3 monoclonal antibody OKT3 as well as other immune accessory molecules. Without requiring the addition of allogeneic feeder cells, aAPC/mOKT3 enabled the expansion of both peripheral and tumor-infiltrating T cells, regardless of HLA-restriction. Stimulation with aAPC/mOKT3 did not expand Foxp3$^+$ regulatory T cells, and expanded tumor infiltrating lymphocytes predominantly secreted Th1-type cytokines, interferon-γ and IL-2. In this aAPC-based system, the presence of autologous CD4$^+$ T cells was associated with significantly improved CD8$^+$ T cell expansion *in vitro*. The CD4$^+$ T cell derived cytokines IL-2 and IL-21 were necessary but not sufficient for this effect. However, CD4$^+$ T cell help of CD8$^+$ T cell proliferation was partially recapitulated by both adding IL-2/IL-21 and by upregulation of IL-21 receptor on CD8$^+$ T cells.

Conclusions: We have developed an *in vitro* model that advances our understanding of the immunobiology of human CD4$^+$ T cell help of CD8$^+$ T cells. Our data suggests that human CD4$^+$ T cell help can be leveraged to expand CD8$^+$ T cells *in vitro*.

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Competing Interests: MOB, LMN and NH have filed a patent application related to aAPC/A2. The patent application number is 10/850,294 and is entitled, "Modified Antigen-Presenting Cells." The authors confirm that this application does not alter their adherence to all PLoS ONE policies on the sharing of data and materials.

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Introduction

It is now well accepted that neoplastic cells are immunogenic and that tumors develop in the context of immune recognition by the host [1,2]. Tumor-associated antigens that serve as immune targets include cell lineage differentiation antigens, cancer-testes antigens, and neoantigens produced by mutations in the cancer cell's unstable genome. Mutational events can give rise to multiple immunogenic MHC class I and II restricted, non-self epitopes capable of inducing strong immune responses to the tumor [3,4]. In several maligancies, anti-tumor T cell responses, with infiltration of tumors by CD8$^+$ T lymphocytes and local production of interferon-γ and IL-2, have been associated with improved clinical prognosis [5–8].

Counter regulatory immune responses, however, also develop in the cancer-bearing host. Tumors subvert the immune response by secreting chemotactic factors that recruit immune suppressive elements, thereby inhibiting the function of anti-tumor effectors [9]. Tumor infiltration by T regulatory (Treg) cells has been correlated with inferior clinical outcomes in several tumors [10,11]. These findings have led to the proposal that immune recognition of cancer involves the balancing of opposing forces: anti-tumor effectors vs. pro-tumor regulatory elements [10,12,13]. In fact, a high ratio of Treg cells to CD8$^+$ T cells within the tumor microenvironment has been associated with poorer survival [14,15].

Adoptive T cell therapy is a promising treatment modality designed to amplify the anti-tumor immune response. Anti-tumor effectors are expanded *in vitro*, away from the pro-tumor milieu of the cancer bearing host, and then reinfused as a cellular therapy [16–21]. Successful approaches showing clinical activity include adoptive transfer of tumor antigen-specific T cell lines or clones

that have been derived from the peripheral blood. Specificity can be achieved by stimulating antigen-specific precursor T cells or through genetic modification of expanded bulk T cells to express cloned or chimeric T cell receptor (TCR) genes [22–26]. Alternatively, the nascent, endogenous immune effector response to the tumor can be amplified by expanding tumor-infiltrating lymphocytes (TIL) *in vitro*. Adoptive cell transfer of *in vitro* activated TIL has achieved major clinical responses when patients first undergo lymphodepletion and are then given high dose IL-2 after adoptive transfer [17,27]. Lymphodepletion augments the persistence and function of transferred TIL not only by reducing or temporarily eliminating Treg cells, but also by reducing cytokine sinks that results in the accumulation of homeostatic cytokines such as IL-7 and IL-15 [28,29].

The optimal method for generating clinically effective T cell grafts *in vitro* has yet to be established [21,30]. In order to achieve massive numerical expansion of T cells, current methods necessitate the use of soluble monoclonal antibodies (mAb), allogeneic feeder PBMC, EBV transformed lymphoblastoid cell lines, and/or undefined culture supernatants. Consequently, these requirements present formidable challenges and costs that prevent the widespread clinical application of this therapy. While adoptive transfer of anti-tumor $CD4^+$ T cells can be efficacious, expansion of anti-tumor $CD8^+$ T cells is also an important goal, particularly in light of the association between their persistence and clinical responses [18,31–33].

Insights into requirements for augmenting the expansion of both $CD4^+$ and $CD8^+$ T cells will help further improve methods to generate T cell grafts for adoptive therapy. $CD4^+$ T cells help generate effective immune responses by sustaining $CD8^+$ T cell proliferation, preventing exhaustion, and establishing long-lived functional memory [34]. In mouse models, common γ-chain receptor cytokine and CD40 signaling can mediate $CD4^+$ T cell help [34–44]. In clinical studies, $CD4^+$ T cells have also been implicated in promoting the persistence and anti-tumor activity of antigen-specific $CD8^+$ T cells in patients [45,46]. However, the mechanisms of human $CD4^+$ T cell help are less well understood. To conduct a mechanistic analysis of human $CD4^+$ T cell help, we developed a novel, human cell-based aAPC, aAPC/mOKT3, which induces both $CD4^+$ and $CD8^+$ T cell expansion without allogeneic feeder cells. The removal of allogeneic feeder cells from our T cell culture system enabled us to precisely isolate molecules mediating help of $CD8^+$ T cell expansion that are expressed or secreted by human $CD4^+$ T cells.

Results

K562-based aAPC expressing membranous OKT3 induces $CD3^+$ T cell expansion

We and others have previously reported the generation of aAPC derived from the human erythroleukemia cell line K562 [47–51]. K562 serves as an excellent platform for generating aAPC since it expresses no HLA class I or II molecules, but highly expresses adhesion molecules such as CD54 and CD58. Using K562, we developed a novel aAPC, aAPC/mOKT3, capable of expanding $CD3^+$ T cells regardless of HLA subtype (Figure 1A, Figure S1). This aAPC was engineered to express a membranous form of the anti-CD3 mAb, OKT3, on its cell surface, thus obviating the need for adding soluble mAb to T cell cultures or loading it onto aAPC as described elsewhere [51,52]. aAPC/mOKT3 also ectopically expresses immunostimulatory molecules CD80 and CD83. We and others have shown that CD83 delivers a CD80 dependent signal that promotes lymphocyte longevity [47,53,54].

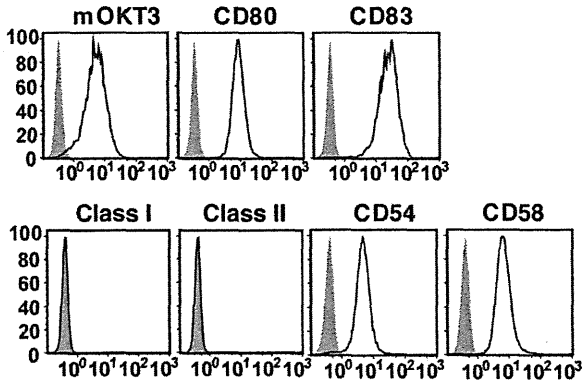

Figure 1. Generation of aAPC/mOKT3. Surface expression of a transduced membranous form of anti-CD3 mAb, and transduced CD80, CD83, and endogenous HLA class I, class II, CD54, and CD58 on aAPC/mOKT3 is shown. A membranous form of anti-CD3 mAb on aAPC/mOKT3 (open) and wild type K562 (shaded) was stained using goat anti-mouse IgG (H+L). Other surface molecules were stained with each specific mAb (open) and isotype control (shaded) and analyzed by flow cytometry. Note the lack of endogenous expression of HLA class I and II on aAPC/mOKT3.
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Stimulation of $CD3^+$ T cells with aAPC/mOKT3 induces robust $CD8^+$ T cell expansion

Peripheral $CD3^+$ T cells expanded with aAPC/mOKT3 were phenotypically characterized after 28 days in culture (Figure 2). While the number of both $CD4^+$ and $CD8^+$ T cells increased, $CD8^+$ T cells expanded substantially better than $CD4^+$ T cells, and therefore dominated cultures from every donor tested (Figure 2A). This is in contrast to other pan T cell expansion systems such as anti-CD3/CD28 mAb-coated beads, which invariably favor the expansion $CD4^+$ T cells over $CD8^+$ T cells [55] (Figure 2B). Similar fold expansion of $CD3^+$ T cells was obtained with the aAPC/mOKT3-based and antibody-coated bead-based expansion systems. T cells expanded using aAPC/mOKT3 displayed a central memory~effector memory phenotype ($CD45RA^-$ $CD54RO^+$ $CD62L^{+/-}$) and retained expression of receptors for IL-2, IL-7, and IL-21 (Figure 2C). CD40 ligand was highly expressed by $CD4^+$ T cells but not $CD8^+$ T cells. Importantly, expanded $CD4^+$ $CD25^+$ T cells did not express Foxp3, indicating that immunoinhibitory Treg cells did not proliferate well (Figure 2D).

aAPC/mOKT3 induces unbiased $CD3^+$ T cell expansion, preserving the repertoire for viral and tumor-associated antigens

In order to evaluate whether stimulation with aAPC/mOKT3 induced broad expansion of $CD3^+$ T cells, TCR Vβ repertoire analysis was performed. No obvious skewing in the TCR Vβ usage of both $CD4^+$ and $CD8^+$ T cell populations was revealed, supporting "unbiased" T cell expansion by aAPC/mOKT3 (Figure 3A). Moreover, HLA-restricted antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTL) against viral and tumor antigens could be generated from $CD3^+$ T cells initially expanded for four weeks using aAPC/mOKT3 (Figure 3B and 3C). The functional avidity of these tumor antigen-specific T cells was sufficient to recognize tumor targets endogenously expressing antigen, confirming that the T cell repertoire for tumor antigen recognition was preserved (Figure 3C). We also confirmed that stimulation with aAPC/mOKT3 induced the expansion of tumor-antigen specific T cells. After 28 days in culture, MART1 peptide specific $CD8^+$ T cell expansion was 420–1,150 fold (Figure S1D).

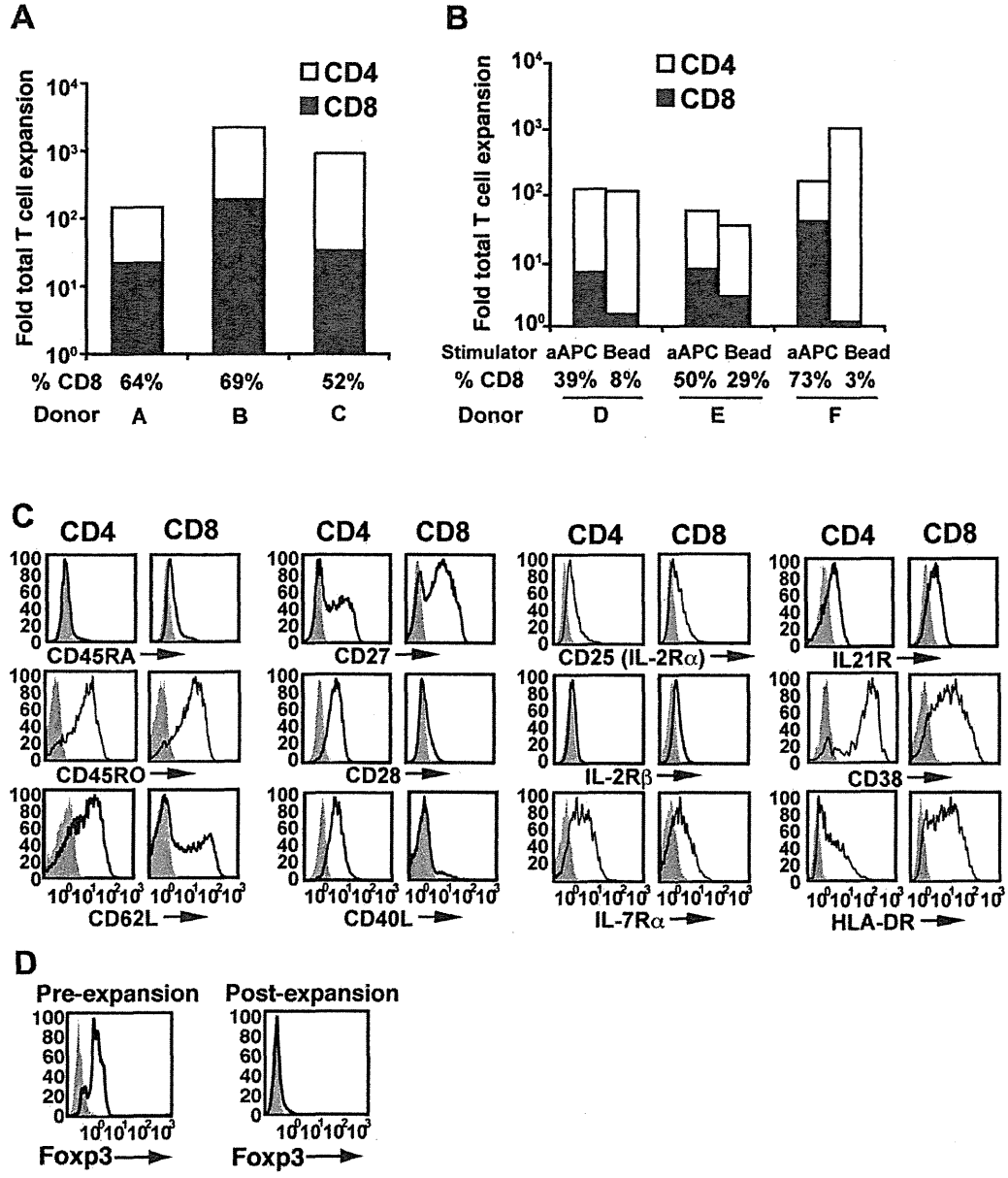

Figure 2. aAPC/mOKT3 expands both CD4+ and CD8+ T cells without using allogeneic feeder PBMC. (**A**) $CD3^+$ T cells were stimulated twice with aAPC/mOKT3 and supplemented with IL-2 between stimulations. Fold expansion of $CD3^+$ T cells over one month is shown for three donors. Shading shows the proportion of expanded $CD4^+$ (white) and $CD8^+$ (black) T cells, and percent $CD8^+$ T cells is indicated. (**B**) $CD3^+$ T cells were stimulated twice with aAPC/mOKT3 or beads (Dynabeads CD3/CD28) and supplemented with IL-2 between stimulations. Fold expansion of $CD3^+$ T cells over one month is shown for three donors. Shading shows the proportion of expanded $CD4^+$ (white) and $CD8^+$ (black) T cells, and percent $CD8^+$ T cells is indicated. (**C**) $CD3^+$ T cells were expanded as described in Figure 2A. Expression of surface molecules on gated $CD4^+$ and $CD8^+$ T cells is shown (open). Isotype mAb staining was used as a control (shaded). (**D**) $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells, present pre-expansion, were absent in expanded cultures. $CD4^+$ $CD25^+$ cells, pre- and post-expansion, were stained intracellularly with anti-Foxp3 mAb (open) and isotype control (shaded).
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aAPC/mOKT3 expands functional TIL but not contaminating Treg cells

Using aAPC/mOKT3, lymphocytes derived from malignant ascites (breast and ovarian cancer) and melanoma metastases were successfully expanded without adding any allogeneic feeder cells (Figure 4A). As observed with peripheral $CD3^+$ T cells in Figure 2A, $CD8^+$ T cells predominantly expanded in all cultures, including those that initially contained a minimal percentage of $CD8^+$ T cells. Importantly, $Foxp3^+$ cells did not proliferate well (Figure 4B). As with peripheral $CD3^+$ T cells, expanded TIL had a central memory~effector memory phenotype ($CD45RA^-$ $CD62L^{+/-}$) consistent with a lack of terminal differentiation (Figure S2). Furthermore, expanded T cells highly expressed CD27 and CD28 which are associated

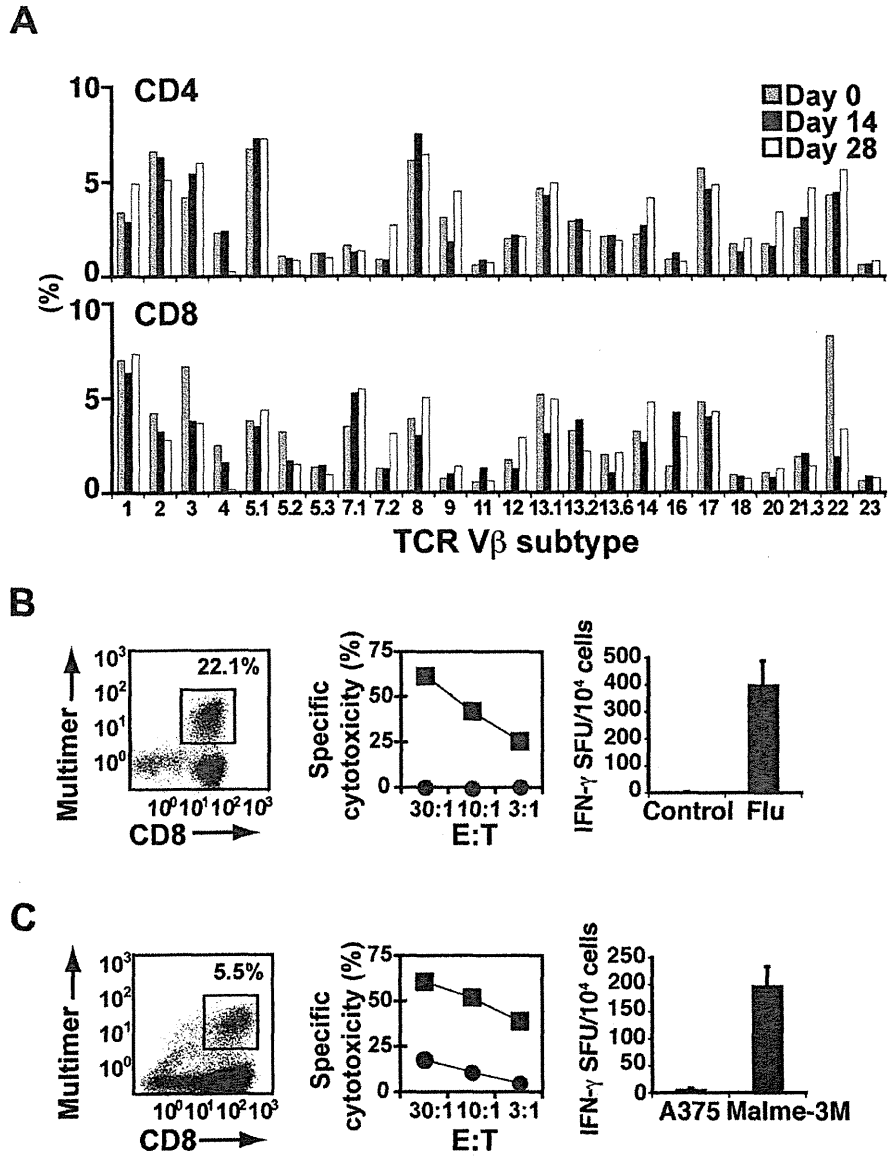

Figure 3. Expansion with aAPC/mOKT3 does not induce skewing of the TCR Vβ repertoire. (**A**) TCR Vβ subfamily analysis before and after stimulation with aAPC/mOKT3 is shown. $CD3^+$ T cells were stimulated with aAPC/mOKT3 on days 0 and 14 and were treated with IL-2 at 300 IU/ml between stimulations. TCR Vβ usage analysis was performed on days 0, 14, 28. Data shown is on gated $CD4^+$ and $CD8^+$ T cells. (**B, C**) $A2^+$ $CD3^+$ T cells were stimulated twice with aAPC/mOKT3 for one month. Subsequently, $CD8^+$ T cells were purified from expanded $CD3^+$ T cells and further stimulated with aAPC/A2 pulsed with Flu or MART1 peptide. (**B**) Flu specificity was demonstrated by multimer staining (left). Functional competence was demonstrated by antigen-specific cytotoxicity (middle) and IFN-γ secretion (right). T2 cells pulsed with Flu peptide (■) or control peptide (●) were used as targets. (**C**) MART1 specificity was similarly demonstrated by multimer staining (left). The HLA-$A2^+$/$MART1^+$ melanoma line, Malme-3M (■), and the HLA-$A2^+$/$MART1^-$ melanoma line, A375 (●), were used as targets in cytotoxicity (middle) and IFN-γ ELISPOT assays (right).
doi:10.1371/journal.pone.0030229.g003

with T cell survival and persistence *in vivo* [56-59]. They also secreted high quantities of IFN-γ and IL-2, while IL-4 secretion was lower and no IL-10 was produced (Figure 4C). These results demonstrate that the aAPC/mOKT3-based system can expand tumor-infiltrating $CD8^+$ T cells in the presence of autologous $CD4^+$ T cells, and that they display phenotypic and functional characteristics consistent with central memory~effector memory T cells.

IL-2 and IL-21 are necessary, but not sufficient, for $CD4^+$ T cell-mediated help of $CD8^+$ T cell expansion

Using the aAPC/mOKT3-based expansion system, we compared the expansion of $CD8^+$ T cells in the presence or absence of $CD4^+$ T cells. $CD8^+$ T cells expanded much better in the presence of $CD4^+$ T cells (Figure 5A), suggesting the presence of $CD4^+$ T cell help for $CD8^+$ T cells in these aAPC/mOKT3-based cultures. We tested whether this "help" was mediated by soluble factors or

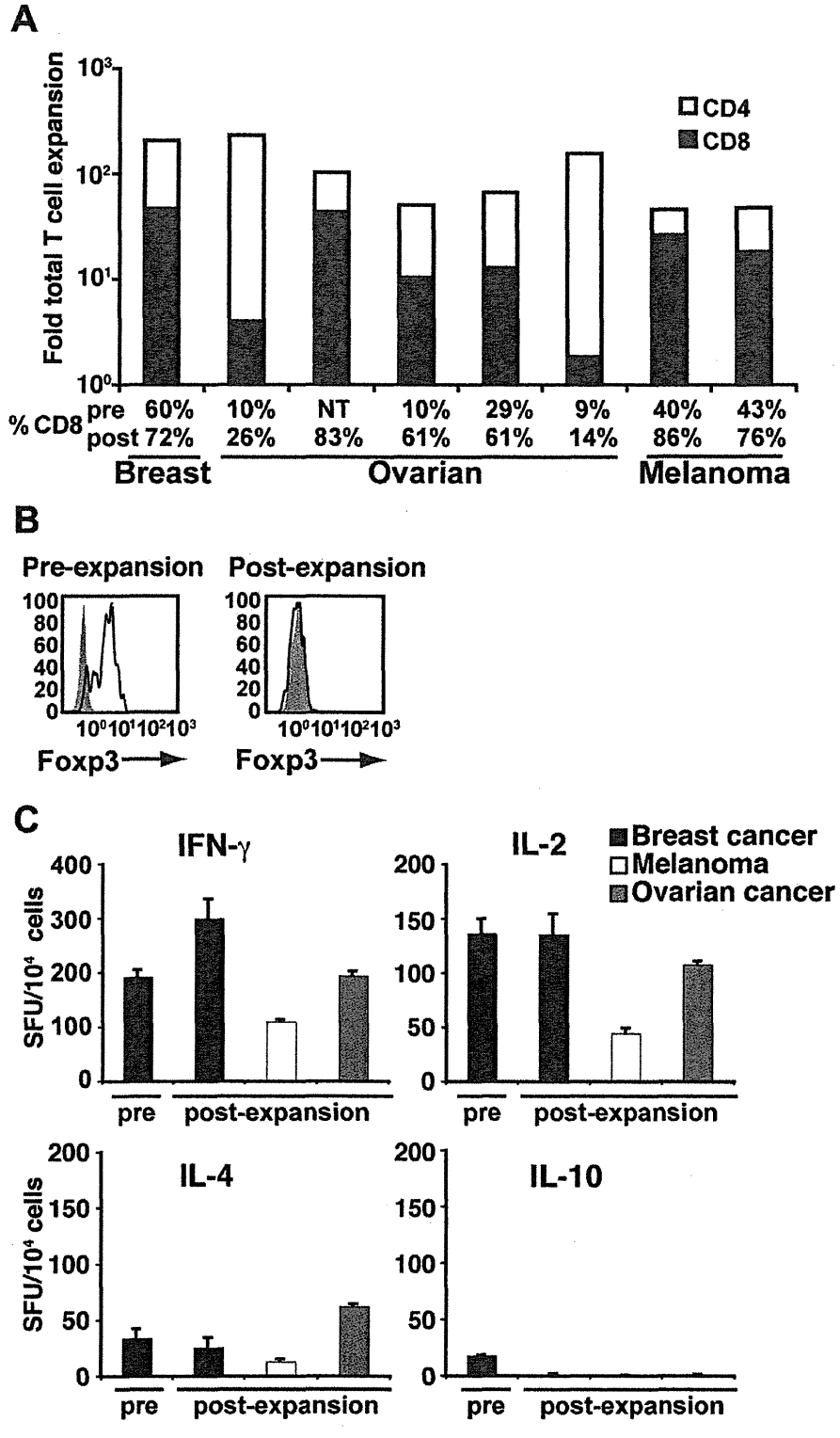

Figure 4. aAPC/mOKT3 expanded TIL are Foxp3 negative and secrete predominantly Th1 cytokines. (**A**) Expansion of TIL obtained from breast and ovarian cancer ascites and melanoma metastases is shown. Shading indicates the proportion of $CD4^+$ (white) and $CD8^+$ (black) T cells in expanded cultures. The percentage of $CD8^+$ T cells in pre- and post-expansion cultures is shown. Note that in all samples tested, the percentage of $CD8^+$ T cells increased even in those that initially contained a minimal percentage of $CD8^+$ T cells. NT denotes not tested. (**B**) $CD4^+$ $CD25^+$ $Foxp3^+$ Treg

cells, present pre-expansion, were not detectable after one month of culture. $CD4^+$ $CD25^+$ cells were intracellularly stained with anti-Foxp3 mAb (open) and isotype control (shaded). (**C**) IFN-γ, IL-2, IL-4, and IL-10 secretion of expanded TIL was determined by ELISPOT assays. Cytokine secretion by TIL from the breast cancer ascites specimen prior to expansion is shown as a control. Pre-expansion samples from melanoma and ovarian cancer specimens were not studied because of low initial cell numbers.
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cell-cell contact using the transwell assay (Figure 5B). A single stimulation, without any exogenously added cytokines, expanded $CD8^+$ T cells by an average of 40.5% better when $CD4^+$ T cells were present but separated from $CD8^+$ T cells by the transwell membrane ($P<0.005$). In co-cultures where $CD4^+$ and $CD8^+$ T cells were mixed, allowing for direct cell-cell contact, $CD8^+$ T cells expanded more than in cultures where they were separated from $CD4^+$ T cells by the transwell membrane ($P<0.05$). These results suggest that observed $CD4^+$ T cell help involves both soluble factors and cell-cell contact.

To identify molecules mediating the observed $CD4^+$ T cell help, culture supernatants of $CD4^+/CD8^+$ T cell mixed and separate cultures were tested for a panel of soluble factors (Figure 5C and Table S1). Greater quantities of MIP-1α, MIP-1β, and RANTES were detected in $CD4^+/CD8^+$ T cell mixed cultures compared to separate cultures, suggesting increased production in mixed cultures. In contrast, IL-2 and IL-21, as well as IL-10, IL-17, TNF-α, and TNF-β, were detected at lower levels in mixed cultures, consistent with more consumption or less production of these cytokines.

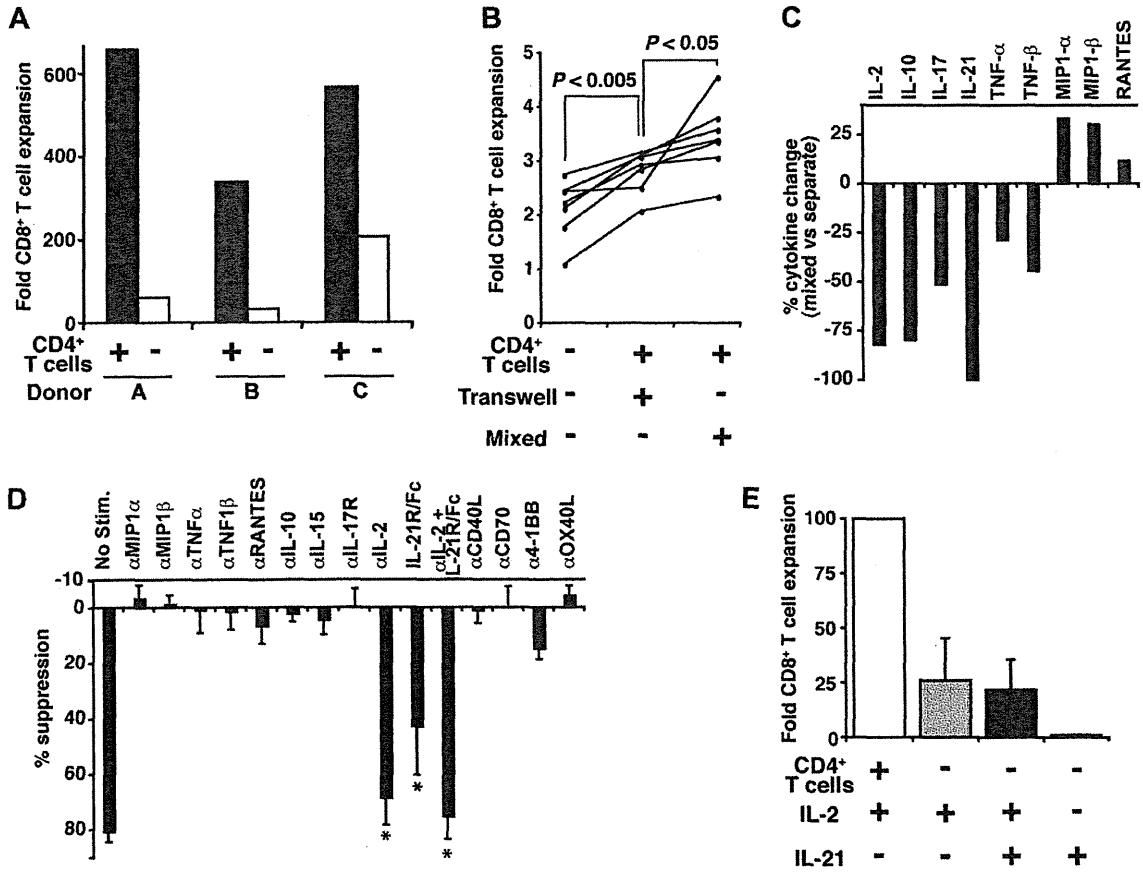

Figure 5. Autologous $CD4^+$ T cell secretion of IL-2/IL-21 is necessary but not sufficient to help $CD8^+$ T cells proliferate. (**A**) $CD8^+$ T cells were stimulated twice by aAPC/mOKT3 with or without $CD4^+$ T cells and treated with IL-2 between stimulations. Fold expansion of $CD8^+$ T cells over 28 days is shown for 3 donors. (**B**) $CD8^+$ T cells were stimulated only once by aAPC/mOKT3 with or without $CD4^+$ T cells in transwell plates. No IL-2 or other cytokines were given. Fold expansion of $CD8^+$ T cells over 6 days is shown for 7 donors. (**C**) Culture supernatants were tested for a panel of soluble factors to identify mediators of $CD4^+$ T cell help. Relative changes in cytokines, comparing mixed vs. separate cultures, are shown. Data is representative of two donors. Absolute values for two donors are shown in Table S1. (**D**) Suppression of $CD8^+$ T cell expansion in the presence of $CD4^+$ T cells by blocking reagents is presented as percent suppression relative to control. Values indicate mean of four independent experiments; error bars show s.d. *$P<0.005$. (**E**) $CD8^+$ T cells were stimulated twice with aAPC/mOKT3 in the presence or absence of $CD4^+$ T cells. IL-2, IL-21, or both were added in each condition. Fold expansion of $CD8^+$ T cells over 28 days is shown. Percent expansion was calculated by dividing the number of expanded $CD8^+$ T cells by the number of $CD8^+$ T cells expanded in the presence of $CD4^+$ T cells. Values indicate mean of six independent experiments; error bars show s.d.
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To differentiate between "more consumption" and "less production," CD4+/CD8+ T cell mixed cultures were stimulated in the presence of blocking reagents, and suppression of CD8+ T cell expansion was assessed (Figure 5D). Blockade of IL-2 and IL-21 resulted in a reduction of expansion by 68.8% ($P<0.005$) and 42.9% ($P<0.005$), respectively. These results indicate that the decreased levels of IL-2 and IL-21 in CD4+/CD8+ T cell mixed cultures were due to more consumption rather than less production and that these cytokines may be necessary mediators of CD4+ T cell help in this human-based in vitro system. To test whether IL-2/IL-21 could substitute for the observed CD4+ T cell help, CD8+ T cells stimulated with aAPC/mOKT3 were supplemented with IL-2, IL-21, or both (Figure 5E). CD8+ T cells did not expand without IL-2. The addition of IL-2 with or without IL-21 did not improve CD8+ T cell expansion to the level observed when cocultured with CD4+ T cells, demonstrating that IL-2 plus IL-21 are not sufficient to replace CD4+ T cell help.

Exogenous IL-2/IL-21 and upregulation of IL-21 receptor can partially recapitulate CD4+ T cell help of CD8+ T cell expansion *in vitro*

Interestingly, we observed that higher expression of the IL-21 receptor (IL-21R) on CD8+ T cells occurred when CD4+ T cells were present during stimulation by aAPC/mOKT3 (Figure 6A). Higher IL-21R expression on CD8+ T cells was not induced by supplementing cultures with IL-2 and IL-21 (data not shown). This prompted us to hypothesize that increased upregulation of IL-21R on CD8+ T cells is critical for the full effect of IL-21 secreted by CD4+ T cells. We constitutively expressed IL-21R on CD8+ T cells (Figure 6B, left) and stimulated them with aAPC/mOKT3 in the presence of IL-2/IL-21. In accordance with the transduction efficiency of IL-21R to 75.9%, CD8+ T cell proliferation partially increased to levels seen in the presence of CD4+ T cells (Figure 6B, right). This indicates that elevated expression of IL-21R is necessary and can partially recapitulate CD4+ T cell help for CD8+ T cell proliferation.

Discussion

A novel human cell-based aAPC expanded CD3+ T cells *in vitro* without the addition of allogeneic feeder PBMC. Phenotypic analysis of expanded healthy donor T cells and TIL showed, that while both CD4+ and CD8+ T cells expanded, CD8+ T cells predominated. In this model system, we demonstrated that CD8+ T cell expansion depended on the presence of CD4+ T cells, suggesting that CD4+ T cells provided help to proliferating CD8+ T cells. The CD4+ T cell secreted cytokines, IL-2 and IL-21, and the CD4+ T cell-dependent upregulation of IL-21R on CD8+ T cells were necessary for the observed CD4+ T cell help.

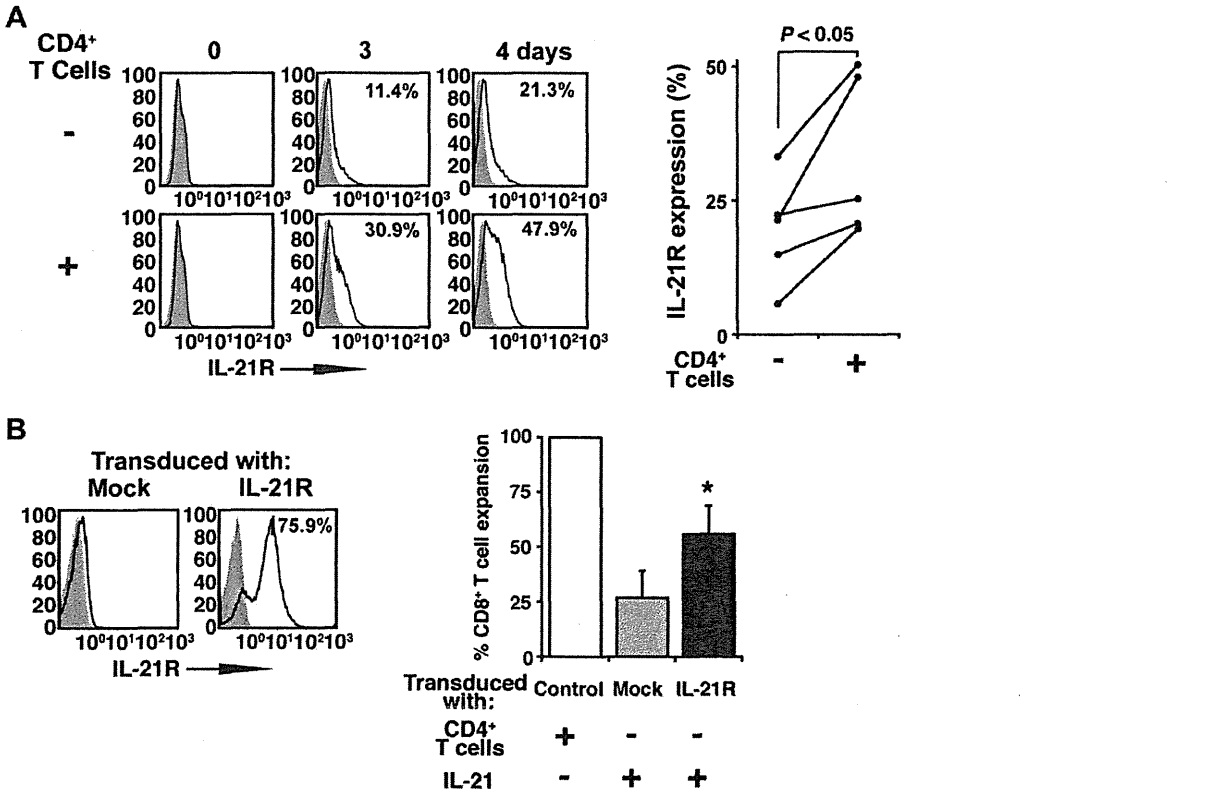

Figure 6. IL-2/IL-21 and upregulation of IL-21R expression replace CD4+ T cell help of CD8+ T cell expansion *in vitro*. (**A**) IL-21R expression on CD8+ T cells stimulated with aAPC/mOKT3 in the presence or absence of CD4+ T cells was studied by flow cytometry. On the left, histogram plots for 1 donor is shown and, on the right, IL-21R expression on day 4 is displayed for 5 donors. (**B**) IL-21R expression on CD8+ T cells ectopically transduced with mock or IL-21R is shown (left). Expansion of transduced CD8+ T cells stimulated twice by aAPC/mOKT3 with or without IL-21 is compared (right). Percent expansion was calculated by dividing the number of expanded transduced CD8+ T cells by that of CD8+ T cells stimulated in the presence of CD4+ T cells. Values indicate mean of four independent experiments; error bars show s.d. *$P<0.005$.
doi:10.1371/journal.pone.0030229.g006

IL-2 and IL-21 have previously been shown to mediate $CD4^+$ T cell help in murine *in vivo* studies. IL-2, one of the few effector cytokines made by naïve $CD4^+$ T cells, expands activated T cells and is essential in the development of $CD8^+$ T cell memory responses to pathogens [60]. While $CD8^+$ T cell responses during acute viral infections were relatively independent of IL-2, the development of protective $CD8^+$ T cell memory responses required IL-2 exposure during priming [35–37]. In vivo models also indicate that IL-21 is critical for containing chronic viral infections and preventing the deletion of high affinity antiviral $CD8^+$ T cells. IL-21 secretion by $CD4^+$ T cells enables the generation, sustained proliferation, and maintenance of polyfunctional $CD8^+$ T cells during chronic infection [39–41].

Our results confirmed a role for IL-2 and IL-21 in human $CD4^+$ T cell help. By using a standardized aAPC, we were able to single out and examine the effects of cocultured $CD4^+$ T cells, unhindered by immunostimulatory and inhibitory factors produced by allogeneic feeder cells. Stimulation of T cells with aAPC/mOKT3 induced the secretion of cytokines and chemokines, including high levels of interferon-γ, MIP-1α, and MIP-1β. Among all the cytokines and chemokines studied, blocking experiments identified IL-2 and IL-21 as necessary for $CD4^+$ T cell help of $CD8^+$ T cell expansion. These cytokines alone, however, were not sufficient to replace $CD4^+$ T cells. We showed that $CD4^+$ T cells help by enhancing IL-21R expression on $CD8^+$ T cells, rendering them more responsive to secreted IL-21. Taken together, the secretion of IL-2/IL-21 and the induction of IL-21R are necessary and sufficient to partially recapitulate human $CD4^+$ T cell help of $CD8^+$ T cell expansion *in vitro*.

Transwell assays showed that the $CD4^+$ T cell dependent expansion of $CD8^+$ T cells was also mediated by cell-cell contact factors. CD40-CD40 ligand interactions have been shown to mediate $CD4^+$ T cell help through CD40-mediated activation of dendritic cells, which are then "licensed" to stimulate $CD8^+$ T cells [43,44,61]. CD40 ligation was also shown to increase IL-21R expression on B lymphocytes suggesting a mechanism for IL-21R upregulation on $CD8^+$ T cells [62]. However, we did not observe any suppression of $CD8^+$ T cell expansion following blockade of CD40 ligand (Figure 5D) even though expanded $CD4^+$ T cells strongly expressed CD40 ligand (Figure 2C). Furthermore, stimulation with aAPC/mOKT3 in the presence of CD40 ligation and the addition of IL-21 did not consistently enhance $CD8^+$ T cell expansion (data not shown). Therefore, these results are in agreement with others who have shown that $CD4^+$ T cells do not provide direct help to $CD8^+$ T cells through CD40 ligation [63,64]. It should be noted that blocking of CD70, 4-1BB, or OX40 signaling also did not suppress the expansion of $CD8^+$ T cells in the presence of $CD4^+$ T cells (Figure 5D).

aAPC induced polyclonal expansion of both $CD4^+$ and $CD8^+$ T cells as shown by the absence of clonal skewing of the TCR Vβ repertoire. The ability to further expand antigen-specific T cells capable of killing tumor targets indicated that the TCR repertoire for highly avid T cells was preserved. Also, expanded TIL secreted higher amounts of Th1 cytokines, IFN-γ and IL-2, which are associated with anti-tumor immunity. While aAPC/mOKT3 induced substantial expansion of $CD8^+$ T cells in the presence of $CD4^+$ T cell help, terminal effector T cell differentiation did not occur, as demonstrated by the central memory~effector memory phenotype ($CD45RA^-$ $CD45RO^+$ $CD62L^{+/-}$). Retention of CD62L expression would enable homing to lymph nodes, where encounter with antigen presented by professional APC could augment immune responses [65]. CD27, which is down-regulated in late stage effector T cells, was also highly expressed. CD27 expression by *in vitro* expanded TIL and T cell clones has been associated with persistence and clinical responses after adoptive transfer [56,57,59,66].

We also found that expanded T cells were not contaminated by cells with the $CD4^+$ $CD25^+$ $Foxp3^+$ Treg phenotype even when $CD4^+$ $CD25^+$ $Foxp3^+$ T cells were present prior to stimulation. We previously found that K562-based aAPC expressing HLA-DR molecules did not expand $Foxp3^+$ cells even though aAPC itself produces modest amounts of the Treg cell growth factor TGF-β [48]. We previously reported that aAPC also secretes IL-6 [47]. It is possible that IL-6, secreted by aAPC, might interfere with $Foxp3^+$ Treg cell expansion [67,68].

Adoptive transfer of *in vitro* expanded T cells has led to clinically significant anti-tumor responses in patients [30]. By leveraging autologous $CD4^+$ T cell help, aAPC/mOKT3 eliminates the use allogeneic feeder cells for T cell expansion, potentially increasing the availability of adoptive therapy as a cancer treatment. We previously reported the development of K562-based aAPCs dedicated to the expansion of HLA-restricted antigen-specific $CD4^+$ and $CD8^+$ T cells [47,48]. Antigen-specific $CD4^+$ and $CD8^+$ T cells expanded *in vitro* with these aAPC had a central memory~effector memory phenotype ($CD45RA^-$ $CD62L^{+/-}$) and possessed surprisingly prolonged *in vitro* longevity without feeder cells or cloning. In a recent clinical trial, HLA-A2-restricted MART1 peptide-specific $CD8^+$ T cells generated *in vitro* with aAPC were infused to advanced melanoma patients [69]. Without lymphodepletion or IL-2 administration, transferred T cells could persist for >16 months, established anti-tumor immunological memory *in vivo*, trafficked to tumor, and induced clinical responses. aAPC/mOKT3 extends the K562 platform to the stimulation of T cells regardless of HLA subtype. The aAPC/mOKT3-based T cell expansion system facilitates the understanding of mechanisms for human $CD4^+$ T cell help and provides a novel strategy to expand T cells for *in vitro* and *in vivo* uses.

Materials and Methods

Ethics Statement

All specimens and clinical data were collected under protocols approved by the Institutional Review Board at the Dana-Farber Cancer Institute (DFCI). All patients provided written informed consent for the collection of samples and subsequent analysis.

cDNAs and cell lines

cDNAs encoding the heavy and light chains for a membranous form of anti-CD3 mAb (OKT3, mIgG2a) were cloned from hybridoma cells (ATCC, VA). HLA null K562 transduced with CD80 and CD83 has been described previously [47,53]. $CD80^+$ $CD83^+$ K562 cells were retrovirally transduced with the heavy and light chains of a membranous form of anti-CD3 mAb. After drug selection, anti-CD3 mAb expressing cells were isolated by magnetic bead guided sorting (Miltenyi Biotec, CA). High expression of a membranous form of anti-CD3 mAb on the cell surface was confirmed by flow cytometry. The parental cell line K562 lacks the endogenous expression of any HLA molecule, but does endogenously express the adhesion molecules CD54 and CD58.

Retrovirus supernatants expressing IL-21R was harvested from PG13 cells. Fresh $CD8^+$ T cells purified from healthy donors were first activated with anti-CD3 (0.75 μg/ml) and anti-CD28 (1 μg/ml) mAbs (Fitzgerald Industries International, MA) for two days. Pre-activated T cells were infected with IL-21R or mock retrovirus supernatants every 24 hr at an MOI of 10 for 10 days and treated with 50 IU/ml IL-2 between infections. Following the assessment

of IL-21R expression by flow cytometry analysis, infected T cells were stimulated with aAPC/mOKT3.

T2, A375, and Malme-3M cell lines were obtained from ATCC as described elsewhere [47].

T cell expansion

Healthy donor PBMC were obtained by leukapheresis performed at the DFCI Kraft Family Blood Donor Center. Cells were isolated by Ficoll-Hypaque density gradient centrifugation and $CD3^+$, $CD4^+$, or $CD8^+$ T cells were purified by negative selection via MACS sorting according to the manufacturer's protocol (Miltenyi Biotec, CA). TIL samples were processed by centrifugation of malignant ascites or mechanical and enzymatic digestion of melanoma metastases with collagenase as previously described [70]. $CD3^+$ TIL were obtained by positive or negative selection via MACS sorting (Miltenyi Biotec, CA). aAPC/mOKT3 cells were irradiated (200 Gy) and added to purified T cells at a T cell to aAPC ratio of 20:1 unless otherwise noted. Dynabeads CD3/CD28 (Invitrogen, CA) were used as stimulators according to the manufacturer's instruction at a T cell to bead ratio of 1:3. Expanding T cells were cultured in RPMI 1640 containing 10% human AB sera and gentamycin (Invitrogen, CA), and between stimulations, unless otherwise noted, 300 IU/ml IL-2 (Prometheus, CA) was added every 3-4 days. In the absence of $CD4^+$ T cells, $CD8^+$ T cells expanded only in the presence of IL-2. Where indicated, 50 ng/ml IL-21 (Peprotech, NJ) was added every 3-4 days. Unless otherwise noted, T cells were restimulated every two weeks. Expanded cells were characterized two weeks after the second stimulation. Cell viability was >90% by trypan blue exclusion.

To test whether antigen-specific cultures can be generated from $CD3^+$ T cells polyclonally expanded with aAPC/mOKT3, $CD3^+$ T cells derived from HLA-A*0201 $(A2)^+$ donors were initially stimulated and expanded with aAPC/mOKT3 for one month. Subsequently, $CD8^+$ T cells were purified and further stimulated with Flu or MART1 peptide-pulsed aAPC/A2 as previously described [47,53].

Analysis of cultured T cells

Flow cytometry analysis was performed using mAbs for the following antigens: CD4, CD8, CD25, CD28, CD56, CD62L, and IL-2Rβ (Coulter, CA); CD40 ligand, CD80, IL-7Rα, OX40, OX40 ligand, and 4-1BB (BD Biosciences, CA); CD27, CD45RA, CD45RO and CD83 (Invitrogen, CA); CCR4 and CCR7 (R&D Systems, MN); ICOS, NKG2D, and PD-1 (eBioscience, CA); CD38, Foxp3, HLA-DR, and 4-1BB ligand (Biolegend, CA); CD40 and CD70 (Ancell, MN); IL-21R (R&D Systems, MN; or BD Biosciences, CA). Goat anti-mouse IgG (H+L) Fab (Jackson ImmunoResearch, PA) was used to detect surface expression of murine Ig. Assessment of TCR Vβ subfamily usage was performed using TCR Vβ mAbs (Beta Mark, Coulter, CA).

To assess the production/consumption of soluble factors in T cell cultures, purified $CD4^+$, $CD8^+$, or a 1:1 mixture of $CD4^+$ and $CD8^+$ T cells were stimulated with irradiated aAPC/mOKT3 for 72 hours and supernatants were measured for: GM-CSF, IFN-γ, IL-2, IL-4, IL-10, IL-12, IL-15, IL-17, MIP-1α, MIP-1β, RANTES, TNF-α, TNF-β, and TRAIL (R&D Systems, MN); IL-7 (Diaclone/Cell Sciences, MA); IL-18 (Medical & Biological Laboratories, Japan); and IFN-α (PBL Biomedical Laboratories, NJ). IL-21 (eBiosciences, CA) was measured at 48-hours. Relative changes in cytokines resulting from mixed cultures of $CD4^+$ and $CD8^+$ T cells vs. separate $CD4^+$ and $CD8^+$ T cell cultures were determined by the following formula: (x-y)/y, where x = cytokine secreted by $CD4^+$ and $CD8^+$ T cell mixed co-cultures and y is the average of cytokine produced in separately stimulated $CD4^+$ and $CD8^+$ T cell cultures.

IFN-γ ELISPOT and standard chromium release assays were performed as described elsewhere [47,53]. IL-2, IL-4 and IL-10 ELISPOT assays were performed according to the manufacturer's protocol (R&D Systems, MN).

Transwell and blocking assays

Transwell assays were performed by placing purified $CD4^+$, $CD8^+$, or a mixture of $CD4^+$ and $CD8^+$ T cells into Millicell-24 plate chambers (Millipore) which were separated by a 0.4 μm filter allowing free movement of soluble factors but not cells. T cells were stimulated once with aAPC/mOKT3 in the absence of exogenous cytokines. Six days later, expansion of $CD8^+$ T cells was determined.

Blocking assays were performed in 96-well round bottomed plates where $CD4^+$ and $CD8^+$ T cells were combined 1:1 and then stimulated with irradiated mOKT3/aAPC in the presence of blocking reagents. Blocking mAbs used recognized IL-2, IL-10, IL-15, IL-17R, MIP-1α, MIP-1β, OX40 ligand, RANTES, TNFα, and TNFβ (R&D Systems, MN); 4-1BB (Neomarkers, CA); CD40 ligand (Biolegend, CA); and CD70 (Ancell, MN). IL-21 was blocked using recombinant human IL-21R subunit/Fc chimeric protein (R&D Systems, MN) as previously described [71]. Six days later, $CD8^+$ T cell expansion was determined.

Statistical analysis

Data analysis was performed using the paired, one-sided Student's t-test where $P<0.05$ was considered to be statistically significant.

Supporting Information

Figure S1 K562-based aAPC/mOKT3, expressing a membranous form of anti-CD3 mAb, stimulates $CD3^+$ T cell expansion. **(A)** $CD3^+$ T cells were stimulated twice with aAPC/mOKT3 and supplemented with IL-2 at the following concentrations: 10 IU/ml (gray), 300 IU/ml (white) and 6,000 IU/ml (black). Fold expansion over 28 days is demonstrated. Without IL-2 addition, T cell expansion over the 28-day culture period was minimal. Data for three separate donors is shown. **(B)** $CD3^+$ T cells were stimulated twice with aAPC/mOKT3 at the indicated aAPC: T cell ratios. Cultures were supplemented with IL-2 (300 IU/ml) between stimulations. Fold expansion of $CD3^+$ T cells over one month is shown for two donors. **(C)** Phenotype of fresh healthy donor $CD3^+$ T cells prior to stimulation is depicted to compare with the T cells shown in Figure 2C which were expanded with aAPC/mOKT3. Expression of surface molecules on gated $CD4^+$ and $CD8^+$ T cells is shown (open). Isotype mAb staining was used as a control (shaded). **(D)** HLA-$A2^+$ healthy donor $CD8^+$ T cells were stimulated with MART1 peptide-pulsed aAPC/A2 as previously described [47,53]. MART1 specific T cells were then stimulated twice with aAPC/mOKT3 in the presence of autologous $CD4^+$ T cells. Fold expansion of MART1 T cells over one month is shown for three donors.
(TIF)

Figure S2 TIL expanded with aAPC/mOKT3 express CD27 and CD28 and have a central memory~effector memory phenotype. $CD3^+$ T cells from malignant ovarian ascites were stimulated twice with aAPC/mOKT3, and cultures were supplemented with IL-2 at 300 IU/ml. **(A)** Fresh, unstimulated TIL and **(B)** aAPC/mOKT3 expanded TIL were stained with indicated mAb (open) and isotype control (shaded).

TIL were analyzed after a one month expansion. Data depicted is on gated $CD4^+$ and $CD8^+$ T cells.
(TIF)

Table S1 Soluble factors in T cell cultures stimulated with aAPC/mOKT3. Concentrations of soluble factors (pg/ml) in supernatants of $CD4^+$ separate, or $CD8^+$ separate, and $CD4^+$ and $CD8^+$ mixed T cell cultures stimulated by aAPC/mOKT3 were measured by ELISA. [a]Percent change was calculated as detailed in Methods. [b]not applicable. Data from two different donors is depicted.
(DOC)

Author Contributions

Conceived and designed the experiments: MOB LMN NH. Performed the experiments: MOB OI MT SA AB GM MIM MMM APM NH. Analyzed the data: MOB LMN NH. Contributed reagents/materials/analysis tools: MOB OI YY MT SA HM LMN NH. Wrote the paper: MOB LMN NH.

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