

## Prolongation of Overall Survival in Advanced Lung Adenocarcinoma Patients with the XAGE1 (GAGED2a) Antibody

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### Abstract

**Purpose:** The cancer/testis antigen XAGE1 (GAGED2a) is expressed in approximately 40% of advanced lung adenocarcinomas. We investigated the clinical relevance of the XAGE1 (GAGED2a) immune responses in patients with advanced lung adenocarcinoma.

**Experimental Design:** The XAGE1 (GAGED2a) antigen expression and EGFR mutation were determined with tumor tissues. The XAGE1 (GAGED2a) antibody and T-cell immune responses, as well as immune cell phenotypes, were analyzed with blood samples. Patients with EGFR wild-type (EGFRwt) tumors were treated with conventional platinum-based doublet chemotherapy and patients with EGFR-mutated (EGFRmt) tumors were treated with EGFR-TKI and conventional chemotherapy. The overall survival (OS) rates of the antibody-positive and -negative patients were investigated.

**Results:** The results showed that the OS of antibody-positive patients was prolonged significantly compared with that of antibody-negative patients with either XAGE1 (GAGED2a) antigen-positive EGFRwt (31.5 vs. 15.6 months,  $P = 0.05$ ) or EGFRmt (34.7 vs. 11.1 months,  $P = 0.001$ ) tumors. Multivariate analysis showed that the presence of the XAGE1 (GAGED2a) antibody was a strong predictor for prolonged OS in patients with XAGE1 (GAGED2a) antigen-positive tumors and in patients with either EGFRwt or EGFRmt tumors. On the other hand, XAGE1 (GAGED2a) antigen expression was a worse predictor in patients with EGFRmt tumors. Phenotypic and functional analyses of T cells indicated immune activation in the antibody-positive patients.

**Conclusions:** The findings suggest that production of the XAGE1 (GAGED2a) antibody predicts good prognosis for patients with lung adenocarcinoma as an immune biomarker and the protective effect of this naturally occurring immune response supports the concept of immunotherapy. *Clin Cancer Res*; 20(19); 5052–63. ©2014 AACR.

### Introduction

Cancer/testis (CT) antigen is a class of antigens that express predominantly in the testes in normal adult tissues and in various tumors (1–3). The CT database (4) lists 276 CT antigen genes, including 128 genes on the X chromosome (CT-X), nine genes on the Y chromosome, and 139 genes on various autosomes (non-X CT). Some CT antigens

have been shown to be highly immunogenic and are considered to be attractive targets for cancer vaccines (5–8).

XAGE1 was originally identified by the search for PAGE/GAGE-related genes using an expression sequence tag database (9) and was shown to exhibit CT antigen characteristics (10, 11). Five identical genes, XAGE1A to E, have been identified, being dispersed in a region of approximately 350 kilobases on chromosome Xp11.22 (12). The associated protein is designated as a G antigen family D member 2 (GAGED2), and GAGED2a and d isoforms have been identified (9, 12). Four transcript variants XAGE-1a, b, c, and d have been extensively studied and shown to be expressed in various tumors (13–16). The XAGE-1a and b transcripts code for 81 amino acid XAGE1 (GAGED2a) protein, whereas the XAGE-1d transcript codes for a 69 amino acid XAGE1 (GAGED2d) protein (17).

The XAGE1 (GAGED2a) antigen is expressed in approximately 40% of advanced lung adenocarcinomas (18–21). Approximately half of the patients with antigen-positive tumors naturally produced the XAGE1 (GAGED2a)

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

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### Translational Relevance

XAGE1 (GAGED2a) is a cancer/testis (CT) antigen expressed frequently in lung adenocarcinomas. The findings indicated that the XAGE1 (GAGED2a) immune response is relevant for better prognosis in patients with advanced lung adenocarcinomas and that the XAGE1 (GAGED2a) antibody response is a prognostic biomarker. On the other hand, XAGE1 (GAGED2a) antigen expression is predictive of a worse prognosis in patients with EGFR-mutated tumors.

antibody (19, 21). A CD4 T-cell response was detected in 14 of 16 and a CD8 T-cell response in 6 of 9 XAGE1 (GAGED2a) antibody-positive patients examined in our previous study (21). Frequent antibody and CD4 and CD8 T-cell responses indicate the strong immunogenicity of the XAGE1 (GAGED2a) antigen.

In this study, we investigated the clinical relevance of the XAGE1 (GAGED2a) immune responses in patients with advanced lung adenocarcinoma. A recent comprehensive analysis of human gene expression has identified the Ig  $\kappa$  constant (*IGKC*) gene as a strong prognostic marker in human solid tumors, including lung cancer (22). Identification of tumor-infiltrating plasma cells as the source of *IGKC* expression strongly suggests a role in immune responses and provides a compelling rationale for investigating the relation of humoral immune responses against lung cancer antigens and prognosis.

Patients with EGFR wild-type (EGFRwt) tumors were treated with conventional platinum-based doublet chemotherapy and patients with EGFR-mutated (EGFRmt) tumors were treated with EGFR-TKI as first line chemotherapy following conventional chemotherapy. Overall survival (OS) of the patients with EGFRmt tumors was prolonged compared with that of patients with EGFRwt tumors. The results in this study showed that the OS of the antibody-positive patients was prolonged significantly compared with that of antibody-negative patients with either XAGE1 (GAGED2a) antigen-positive EGFRwt or EGFRmt tumors. Phenotypic and functional analyses indicated immune activation in the antibody-positive patients. The findings suggest that production of the XAGE1 (GAGED2a) antibody predicts good prognosis of patients with lung adenocarcinoma as an immune biomarker and the protective effect of this naturally occurring immune response supports the concept of immunotherapy.

## Materials and Methods

### Patients and study design

The clinical relevance of XAGE1 (GAGED2a) immune responses was investigated in 145 patients with advanced (clinical stage IIIB and IV) lung adenocarcinoma. The patients were recruited into the study of the "Analysis of cancer antigen and host immune response," an ongoing

prospective observational cohort study initiated in April 2007 at the Kawasaki Medical School Hospital (Kurashiki, Japan) with approval of the local ethics committee (number: 603–6) and in accordance with the Declaration of Helsinki. The patients newly diagnosed with advanced lung adenocarcinoma were enrolled after obtaining written informed consent.

The diagnosis was done pathologically. Biopsy specimens from all 145 patients and additional pleural effusion from 22 patients were subjected to pathology and the results were obtained within a month after the first visit. Treatment started within a month after diagnosis. Survival was measured from the day of diagnosis. The peripheral blood samples were obtained during the period after diagnosis before starting treatment.

The XAGE1 (GAGED2a) antigen expression was determined by IHC and EGFR mutation by a PNA-LNA PCR clamp with tumor tissues. The XAGE1 (GAGED2a) antibody and T-cell immune responses, as well as immune cell phenotypes, were analyzed with blood samples obtained at diagnosis in most studies and with samples obtained later in kinetic studies. The patients with EGFRwt tumors were treated with conventional platinum-based doublet chemotherapy. Patients with EGFRmt tumors were treated with an EGFR tyrosine-kinase-inhibitor (EGFR-TKI) as first-line chemotherapy until progression or intolerable adverse effects following conventional platinum-based doublet chemotherapy. Patients were observed prospectively until death, loss of follow-up, or withdrawal of consent. Patient characteristics are shown in Supplementary Table S1A.

### Overlapping peptides

Overlapping XAGE1 (GAGED2a) peptides spanning the entire protein were synthesized using Fmoc chemistry on a Multiple Peptide Synthesizer (AMS422, ABIMED) at Okayama University (Okayama, Japan). The following series of 35 12-mer peptides: 1–12, 3–14, 5–16, 7–18, 9–20, 11–22, 13–24, 15–26, 17–28, 19–30, 21–32, 23–34, 25–36, 27–38, 29–40, 31–42, 33–44, 35–46, 37–48, 39–50, 41–52, 43–54, 45–56, 47–58, 49–60, 51–62, 53–64, 55–66, 57–68, 59–70, 61–72, 63–74, 65–76, 67–78 and 69–81, and the following series of 17 16-mer peptides: 1–16, 5–20, 9–24, 13–28, 17–32, 21–36, 25–40, 29–44, 33–48, 37–52, 41–56, 45–60, 49–64, 53–68, 57–72, 61–76, and 65–81 were used.

### Synthetic XAGE1 (GAGED2a) protein

XAGE1 (GAGED2a) protein (81 amino acids) was synthesized using a peptide synthesizer by GL Biochemistry.

### Reverse transcription PCR

Total RNA was obtained from cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Two micrograms of each sample were subjected to cDNA synthesis using a Ready-To-Go first strand beads kit (GE Healthcare). Sequences of primer pairs for XAGE1 (transcript variant b) were X-1, 5'-

TTTCTCCGCTACTGAGACAC-3' and X-2, 5'-CAGCTTGC-GTTGTTTCAGCT-3', and sequences for G3PDH were G3PDH-S, 5'-ACCACAGTCCATGC CATCAC-3', G3PDH-AS, 5'-TCCACCACCCTGTTGCTG TA-3'. The amplification was performed using 30 cycles as described (19).

Thirteen of 145 specimens were examined by both IHC and reverse transcription PCR (RT-PCR). The numbers of RT-PCR-positive and -negative specimens were 3 and 10, respectively. Two of three positive specimens were also positive for IHC, but one was negative. All 10 negative specimens were negative for IHC.

### EGFR mutation

EGFR mutations were examined by a PNA-LNA PCR clamp using paraffin-embedded tissue samples in Mitsubishi Chemical Medicine.

### IHC

IHC for XAGE1 (GAGED2a) antigen expression was done with transbronchial or CT-guided lung biopsy specimens from all 145 patients and for additional pleural effusion cells from 22 patients. Tumor biopsy specimens or cells in pleural effusion were fixed with buffered formalin and embedded in paraffin. Five-micrometer sections were deparaffinized with xylene and ethanol. Antigen retrieval and inactivation of endogenous peroxidase were done as described previously (17, 19, 20). After washing, the USO 9-13 mAb was added at a concentration of 2 µg/mL and incubated overnight at room temperature. After washing, the sample slides were stained by a streptavidin-biotin complex (SimpleStain MAX-PO kit; Nichirei), followed by reaction with 3, 3'-diaminobenzidine in H<sub>2</sub>O<sub>2</sub> and counterstained with hematoxylin solution. More than 5% stained cells was considered positive as previously reported (19, 20).

### ELISA

Synthetic XAGE1 (GAGED2a) protein (1 µg/mL) in a coating buffer was adsorbed onto a 96-well ELISA plate (Nunc) and incubated overnight at 4°C. Plates were washed with PBS and blocked with 5% FCS/PBS (200 µL/well) for 1 hour at 37°C. After washing, 100 µL of serially diluted serum was added to each well and incubated for 2 hours at 4°C. After washing, each horseradish peroxidase-conjugated goat anti-human IgG (MBL), IgG1 (Southern Biotechnology Associates), IgG2 (Southern Biotechnology Associates), IgG3 (Southern Biotechnology Associates), and IgG4 (Southern Biotechnology Associates) were added to the wells, and the plates were incubated for 1 hour at 37°C. After washing and development, absorbance was read at 490 nm.

### Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation using a Histo-paque 1077 (Sigma-Aldrich). CD4 and CD8 cells were purified by magnetic cell sorting (Miltenyi Biotec). The residual cells were kept for use as antigen-

presenting cells (APC). The cells were stored in liquid N<sub>2</sub> until use. After thawing, PBMCs were incubated with the monoclonal antibodies for 20 minutes at 4°C. Anti-CD3-V450 (clone UCHT1; BD Horizon, BD Bioscience), anti-CD4-V500 (clone RPA-T4; BD Horizon), anti-CD8-APC/Cy7 (clone SK1; BD Pharmingen), anti-CD183 (CXCR3)-PerCP/Cy5.5 (clone G025H7; BioLegend), anti-CD196 (CCR6)-PE/Cy7 (clone 11A9; BD Pharmingen), anti-CD185 (CXCR5)-Alexa Fluor 488 (clone RF8B2; BD Pharmingen), anti-CD294 (CRTH2)-PE (clone BM16; BioLegend) were used for phenotypic analysis of CD4. Anti-CD3-V450 (clone UCHT1; BD Horizon), anti-CD45-APC (clone HI30; BD Pharmingen), anti-CD14-PE/Cy7 (clone M5E2; BD Pharmingen), anti-HLA-DR-APC/Cy7 (clone L243; BioLegend), anti-CD11b-PE (clone ICRF44; BioLegend), anti-CD15-V500 (clone HI98; BD Horizon), anti-CD33-PerCP/Cy5.5 (clone P67.6; BD Bioscience), and anti-Lineage cocktail 1 (lin 1)-FITC (BD Bioscience) were used for phenotypic analysis of myeloid-derived suppressor cells. Anti-CD3-V450 (BD Horizon), anti-CD4-V500 (BD Horizon), anti-CD8-APC/Cy7 (BD Pharmingen), anti-CD278 (ICOS)-PE (clone DX29; BD Pharmingen), anti-CD134 (OX40)-PerCP/Cy5.5 (clone Ber-ACT35; BioLegend), anti-CD357(GITR)-Alexa Fluor 488 (clone eBioAITR; eBioscience), anti-CD137(4-1BB)-APC (clone 4B4-1; BioLegend), anti-CD279-PE/Cy7 (clone EH12.2H7; BioLegend), anti-CD272(BTLA)-PE (clone MH26; BioLegend), anti-Tim-3-APC (clone F38-2E2; eBioscience), and anti-CD244(2B4)-FITC (clone eBioDM244; eBioscience) were used for analysis of activation and inhibitory molecules on T cells. After incubation, the cells were washed and analyzed by FACS Canto II (BD Bioscience).

### Foxp3 staining

Intracellular Foxp3 staining was performed using a Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions. Anti-CD4-V500 (BD Horizon), anti-CD45RA-APC/H7 (clone HI100; BD Pharmingen), and anti-Foxp3-FITC (clone 259D/C7; BD Pharmingen) were used for phenotypic analysis of regulatory T cells.

### ***In vitro* stimulation of CD4 and CD8 T cells with the XAGE1 (GAGED2a) antigen and detection of cytokine production**

CD4 ( $1 \times 10^6$ /well) and CD8 ( $1 \times 10^6$ /well) T cells were cultured with an equal number of irradiated (40 Gy), autologous CD4- and CD8-depleted cells as APC in the presence of a mixture of 17 16-mer overlapping peptides ( $10^{-6}$  mol/L) for CD4 T cells and in the presence of synthetic XAGE1 (GAGED2a) protein ( $10^{-6}$  mol/L) for CD8 T cells on a 48-well culture plate (BD Bioscience) for 12 days at 37°C in a 5% CO<sub>2</sub> atmosphere. The medium was AIM-V (Invitrogen) supplemented with 5% heat-inactivated pooled human serum, 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 10 U/mL recombinant IL2 (Takeda Chemical Industries), and 10 ng/mL recombinant IL7 (Peprotech).

After incubation, responder CD4 or CD8 T cells ( $1 \times 10^5$ ) harvested from the stimulation culture were washed and then pulsed with 17 16-mer OLPs for CD4 T cells or 35 12-mer OLPs for CD8 T cells with GolgiStop monensin (Sigma Chemical Co.) for 3 hours. After incubation, cytokine production by CD4 and CD8 T cells was detected by intracellular cytokine staining (ICS).

#### Intracellular cytokine staining

The cells harvested from culture were washed and stained with anti-CD3-PerCP/Cy5.5 (clone SK7; eBioscience) and anti-CD4-V500 (BD Horizon), or anti-CD3-APC/Cy7 (clone HIT3a; BioLegend), anti-CD8-V500 (clone RPA-T8; BD Horizon), and anti-CD107a-FITC (clone H4A3; BD Pharmingen; 2  $\mu$ L) for 30 minutes on ice. After incubation, the cells were washed, fixed, and permeabilized with Cytotfix/Cytoperm solution (Pharmingen, Becton Dickinson) for 20 minutes at 4°C. Then, the cells were washed in Perm/Wash solution (Pharmingen), and pelleted cells were stained for intracellular cytokines using anti-IFN $\gamma$ -PE/Cy7 (clone 4S.B3; eBioscience), anti-TNF $\alpha$ -PE/Cy7 (clone MAb11; eBioscience), anti-IL-5-PE (clone JES1-39D10; BD Horizon), anti-IL-13-PE (clone JES10-5A2; BD Horizon), anti-IL-17A-Brilliant Violet 421 (clone BL168; BioLegend), anti-IL-17F-V450 (clone O33-782; BD Pharmingen), and anti-IL-10-APC (clone JES3-19F1; BioLegend) for detection of CD4 cytokines or anti-IFN $\gamma$ -PerCP/Cy5.5 (clone 4S.B3; eBioscience), anti-TNF $\alpha$ -Brilliant Violet 421 (clone MAb11; BioLegend), and anti-IL-10-APC (clone JES3-19F1; BioLegend) for detection of CD8 cytokines for 30 minutes on ice. After incubation, the cells were washed and analyzed by FACS Canto II (BD Bioscience). The data were analyzed using FlowJo software (version 7.6.5; Tree Star). If the number of cytokine-staining cells stimulated with XAGE1 (GAGED2a) OLPs was more than 2-fold the number of staining cells stimulated with control peptides, it was defined as positive (23).

#### Overall survival

The diagnosis of lung cancer was done pathologically within a month after the first visit. OS was measured from the day of diagnosis and analyzed by the Kaplan–Meier method. Differences in survival between patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses using Cox proportional hazards regression model were performed to assess the association of each factor with OS. *P* values less than 0.05 were considered significant.

#### Statistical analysis

Statistical analysis was performed with the Student *t* test for two groups and with ANOVA for multiple groups using IBM SPSS Statistics 19 for Windows (IBM). Quantitative data without a normal distribution were analyzed with nonparametric tests, and data with a normal distribution were analyzed with parametric tests. For a two-sample comparison of continuous variables, Wilcoxon rank-sum test was performed. For analysis of the correlation of the extrapolated titer and each parameter, Pearson rank test

was performed. Results are expressed as the mean or 95% confidence interval (95% CI).

## Results

### XAGE1 (GAGED2a) antibody response in patients with advanced lung adenocarcinoma

Characteristics of 145 patients with advanced (clinical stage IIIB and IV) lung adenocarcinoma investigated in this study are shown in Supplementary Table S1A. We evaluated the serum IgG response against XAGE1 (GAGED2a) in the patients by ELISA using a synthetic protein. An extrapolated titer was calculated for each serially diluted serum sample as described (24). The IgG response was defined as positive for sera with extrapolated titers exceeding or equal to 100. Thirty-three patients were antibody positive and titration curves of sera are shown in Fig. 1A. The dominant IgG subtypes were IgG1 and IgG3, and no IgG2 or IgG4 response was observed (Fig. 1B). The positive response was further classified by extrapolated titers as +++  $\geq 6,400$ , 6,400 > ++  $\geq 1,600$ , 1,600 > +  $\geq 400$ , and 400 > weak  $\geq 100$  (Fig. 1C).

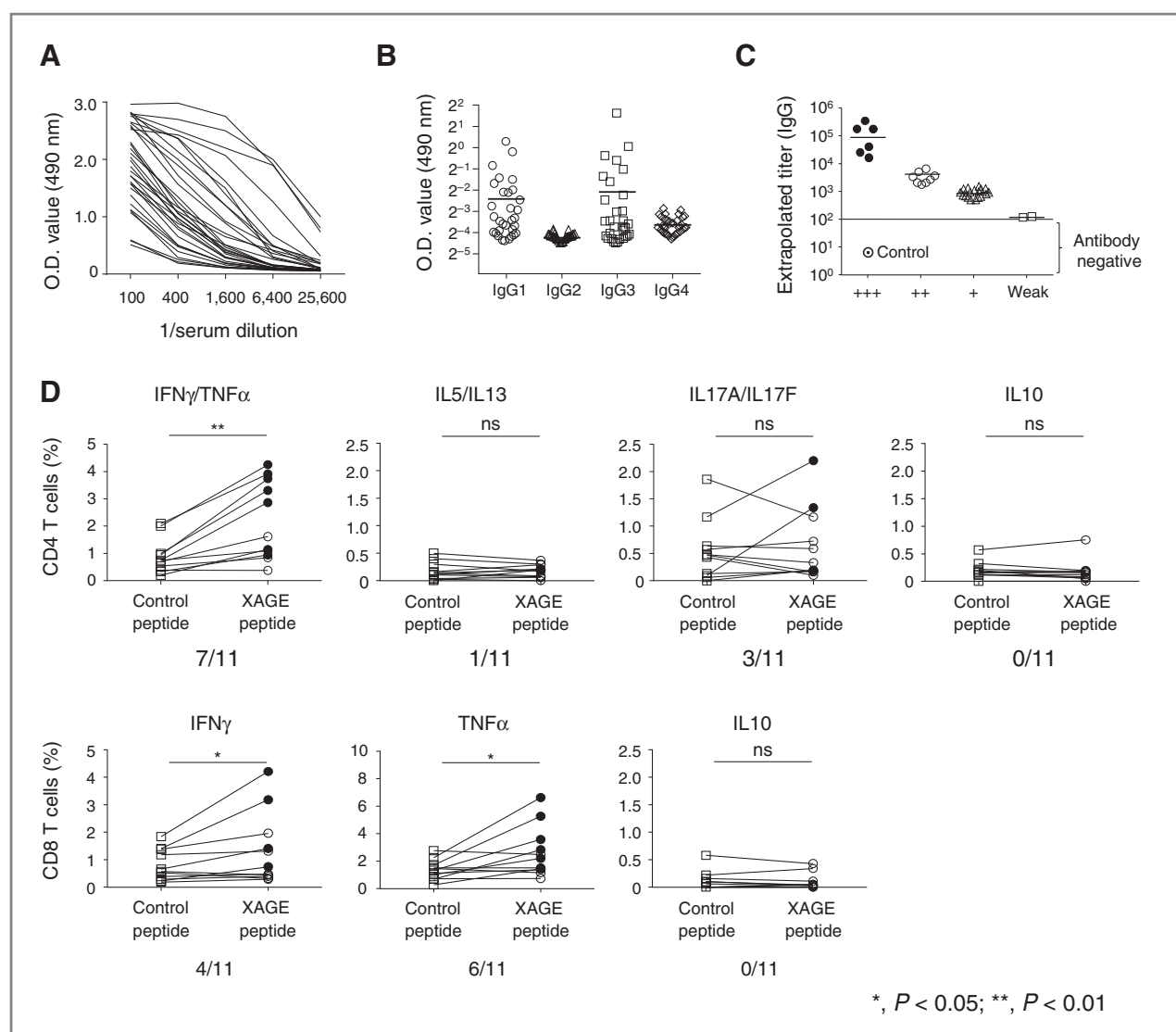
A higher antibody response frequency was observed in patients with EGFRmt tumors than in patients with EGFRwt tumors of the 145 patients (Supplementary Table S1A). However, no significant difference was observed for the antibody response in any characteristics in 58 patients with XAGE1 (GAGED2a) antigen-positive tumors (data not shown).

### Detection of CD4 and CD8 T-cell responses in PBMCs from XAGE1 (GAGED2a) antibody-positive advanced lung adenocarcinoma patients

Purified CD4 and CD8 T cells in PBMCs from XAGE1 (GAGED2a) antibody-positive advanced lung adenocarcinoma patients were stimulated for 12 days with CD4- and CD8-depleted PBMCs treated with XAGE1 (GAGED2a) 17 16-mer OLPs or a synthetic protein, respectively. After culture, the cells were collected and cytokine production was examined for CD4 T cells after 3-hour stimulation with XAGE1 (GAGED2a) 17 16-mer OLPs and for CD8 T cells after 3-hour stimulation with XAGE1 (GAGED2a) 35 12-mer OLPs by ICS. As shown in Fig. 1D and Supplementary Fig. S1, IFN $\gamma$ /TNF $\alpha$ , IL5/IL13, and IL17A/IL17F-producing CD4 T cells were detected in PBMCs from 7, 1, and 3 of 11 patients examined, respectively. IL10-producing CD4 T cells were not detected in any of the patients. On the other hand, IFN $\gamma$ - and TNF $\alpha$ -producing CD8 T cells were detected in PBMCs from 4 and 6 of 11 patients, respectively. IL10-producing CD8 T cells were not detected in any. No XAGE1 (GAGED2a) antibody responses or CD4 or CD8 T-cell responses were detected in healthy individuals as reported previously (21).

### Phenotypic analyses of CD4 T cells and MDSCs in PBMCs from XAGE1 (GAGED2a) antibody-positive patients

Th1, Th2, Th17, and T<sub>FH</sub> CD4 T cells, resting and activated CD4 Tregs (25), and M- and PMN-MDSCs in PBMCs from XAGE1 (GAGED2a) antibody-positive patients were analyzed by flow cytometry (Supplementary Fig. S2). As



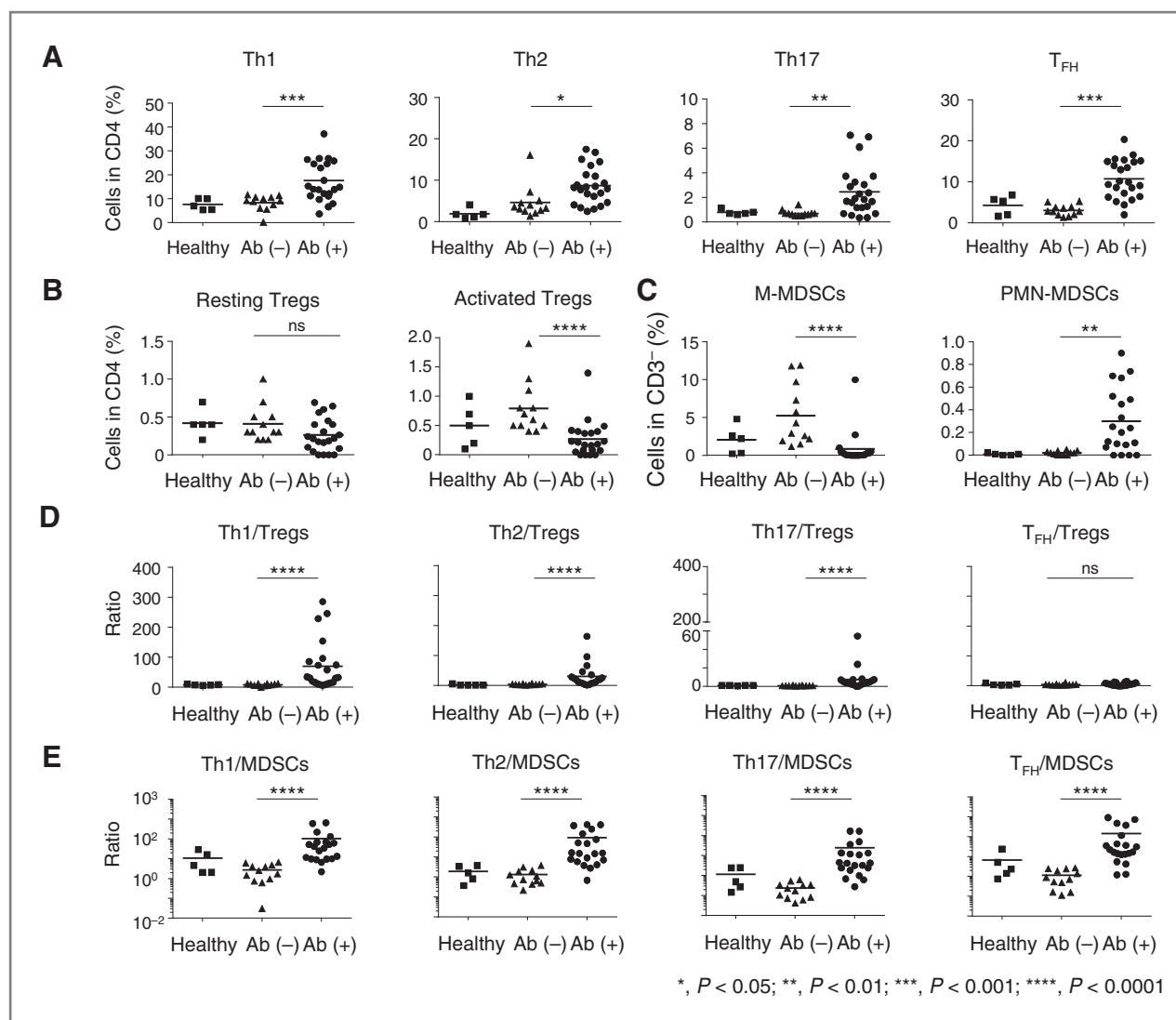
**Figure 1.** Antibody and T-cell responses against XAGE1 (GAGED2a) in patients with advanced lung adenocarcinoma. **A**, titration curves of positive sera from 33 of 145 patients investigated. ELISA was done using synthetic XAGE1 (GAGED2a) protein. The IgG response was defined as positive for sera with an extrapolated titer exceeding or equal to 100. **B**, serum IgG subtypes reactive to synthetic XAGE1 (GAGED2a) protein determined by ELISA with diluted sera (1:100) using a subtype-specific antibody. **C**, classification of positive responses against XAGE1 (GAGED2a) by an extrapolated titer as +++  $\geq 6,400$ , 6,400 > ++  $\geq 1,600$ , 1,600 > +  $\geq 400$ , and 400 > weak  $\geq 100$ . **D**, CD4 and CD8 T cells ( $1 \times 10^6$ ) purified from PBMCs of XAGE1 (GAGED2a) antibody-positive patients were cultured with an equal number of irradiated (40 Gy), autologous CD4 $^-$ , and CD8-depleted PBMCs as APCs in the presence of XAGE1 (GAGED2a) 17 16-mer OLPs ( $10^{-6}$  mol/L) or the synthetic protein ( $10^{-6}$  mol/L), respectively, using 48-well culture plates for 12 days. The cells were then collected and cytokine production was examined for CD4 T cells after 3-hour stimulation with XAGE1 (GAGED2a) 17 16-mer OLPs and for CD8 T cells after 3-hour stimulation with XAGE1 (GAGED2a) 35 12-mer OLPs by intracellular cytokine staining (ICS). Control peptides (a mixture of MGARASVLSGGELDR and ASVLSGGELDRWEKI) were from Con B gag motifs of the human immunodeficiency virus. Cytokine-staining cells stimulated with XAGE1 (GAGED2a) OLPs at more than 2-fold the number of staining cells stimulated with control peptides was defined as positive (filled circles). The number of positive patients out of 11 patients investigated is shown in each panel. Statistical analysis was done by the Wilcoxon rank test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). Each line indicates a single patient.

shown in Fig. 2A, all Th1, Th2, Th17, and T<sub>FH</sub> CD4 T-cell levels were elevated in XAGE1 (GAGED2a) antibody-positive patients compared with those in antibody-negative patients. A decrease in activated, but not resting, Treg levels was also observed (Fig. 2B). Furthermore, a decrease in the M-MDSC level and an increase in the PMN-MDSC level were observed (Fig. 2C). Th1, Th2, and Th17/total Treg and Th1, Th2, and Th17/total MDSC levels were increased (Fig.

2D and E). An increase in T<sub>FH</sub>/total MDSC, but not the T<sub>FH</sub>/Treg level, was observed (Fig. 2D and E).

#### Analysis of CD4 and CD8 T cells expressing T-cell activation and inhibitory molecules in PBMCs from XAGE1 (GAGED2a) antibody-positive patients

CD4 and CD8 T cells expressing T-cell activation molecules ICOS, OX40, 4-1BB, and GITR, and T-cell inhibitory



**Figure 2.** Phenotypic analyses of CD4 T cells (A and B) and MDSCs (C) in PBMCs from 23 XAGE1 (GAGED2a) antibody-positive and 12 negative patients, and 5 healthy donors by FACS. D and E show the ratio of each phenotype of T cells to Tregs and MDSCs, respectively. Statistical analysis was done by the Student *t* test for two groups and by ANOVA for multiple groups (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ). Each dot indicates a single patient.

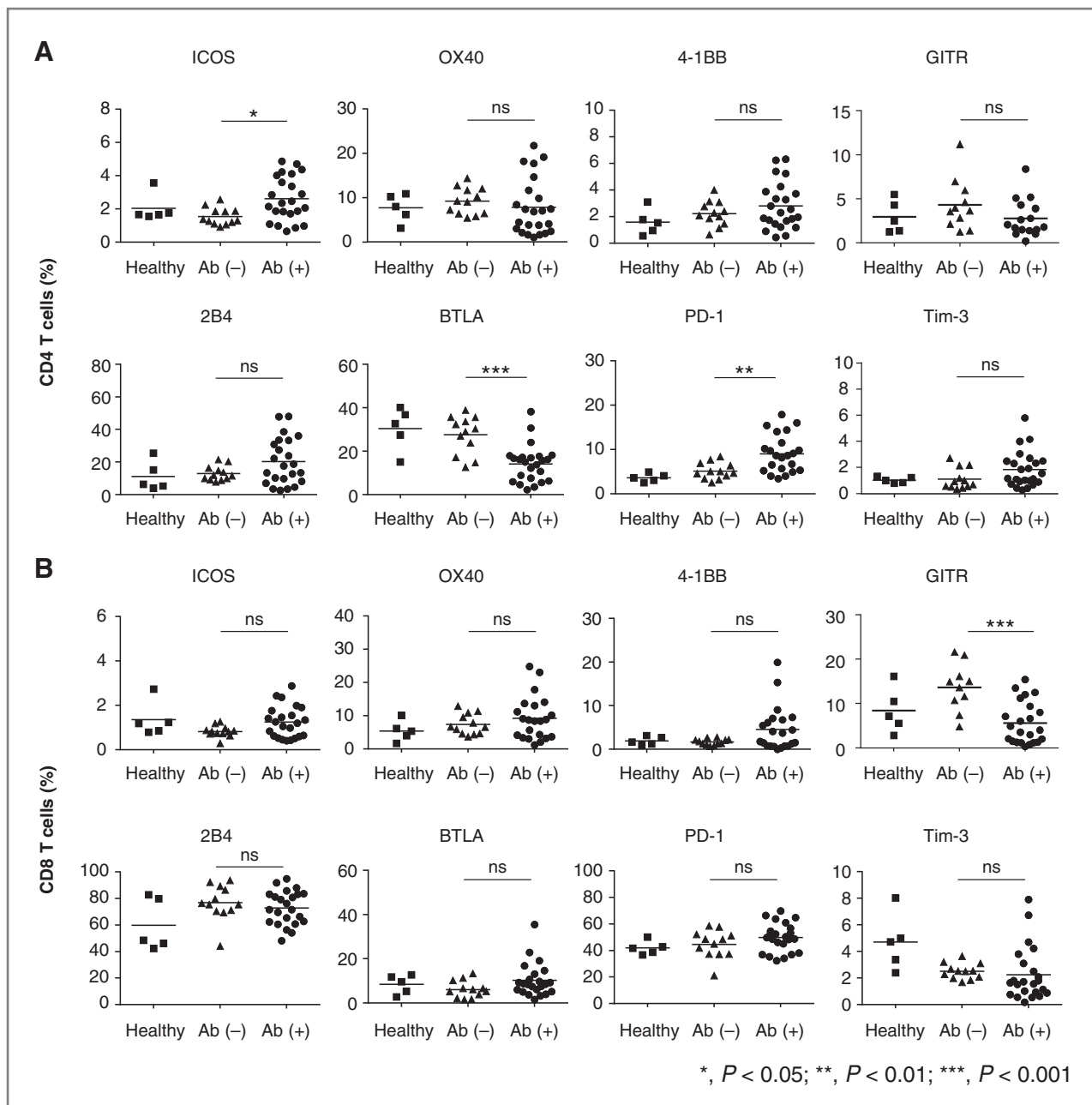
molecules 2B4, BTLA, PD-1, and Tim-3 in PBMCs from XAGE1 (GAGED2a) antibody-positive patients were investigated. As shown in Fig. 3 and Supplementary Fig. S3, an increase in ICOS and PD-1-positive cell levels and a decrease in BTLA-positive cell levels were observed in CD4 T cells from antibody-positive patients compared with those in CD4 T cells from antibody-negative patients. On the other hand, a decrease in GITR-positive cell levels was observed in CD8 T cells from antibody-positive patients.

#### Overall survival of XAGE1 (GAGED2a) antibody-positive and -negative patients

The OS of XAGE1 (GAGED2a) antibody-positive and -negative patients was analyzed for 145 patients with

advanced lung adenocarcinoma. Patient characteristics are shown in Supplementary Table S1A, as described above.

OS was first analyzed for the patients with XAGE1 (GAGED2a) antigen-positive and -negative tumors. As shown in Fig. 4A, no significant difference was found in OS between them ( $P = 0.22$ , HR, 0.78). However, prolongation of OS was observed in XAGE1 (GAGED2a) antibody-positive patients compared with antibody-negative patients ( $P = 0.006$ , HR, 0.53; Fig. 4B). The median OS times in the antibody-positive and -negative patients were 33.3 months and 15.1 months, respectively. Antibody-negative patients were then stratified by the XAGE1 (GAGED2a) antigen expression in the tumor. As shown in Fig. 4C, the antibody-negative patients with antigen-positive tumors showed shortened survival. The median



**Figure 3.** Analysis of CD4 (A) and CD8 (B) T cells expressing T-cell activation (ICOS, OX40, 4-1BB, and GITR) and inhibitory (2B4, BTLA, PD-1, and Tim-3) molecules in PBMCs from XAGE1 (GAGED2a) antibody-positive and negative patients, and healthy donors by FACS. Statistical analysis was done by the Student *t* test for two groups and by ANOVA for multiple groups (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). Each dot indicates a single patient.

OS in the patients with antigen-positive tumors was 33.3 months when antibody-positive, but only 13.7 months when antibody-negative ( $P < 0.0001$ , HR, 0.34). Furthermore, prolongation of OS was dependent on the IgG titer (Supplementary Fig. S4).

The patients were further stratified by the absence or presence of the EGFR mutation in the tumor. The patients with EGFRmt tumors treated with EGFR-TKI and conventional platinum-based doublet chemotherapy showed prolonged OS compared with the patients with EGFRwt tumors

treated with conventional chemotherapy alone ( $P = 0.017$ ; Fig. 5A and C and Supplementary Fig. S5A). OS was prolonged by the presence of antibodies in patients with XAGE1 (GAGED2a) antigen-positive EGFRwt or EGFRmt tumors (Fig. 5B and D). The median OS in the patients with XAGE1 (GAGED2a) antigen-positive EGFRwt tumors was 31.5 months when antibody-positive, but only 15.6 months when antibody-negative ( $P = 0.05$ , HR, 0.46; Fig. 5B). On the other hand, in the patients with XAGE1 (GAGED2a) antigen-positive EGFRmt tumors, the median OS was 34.7

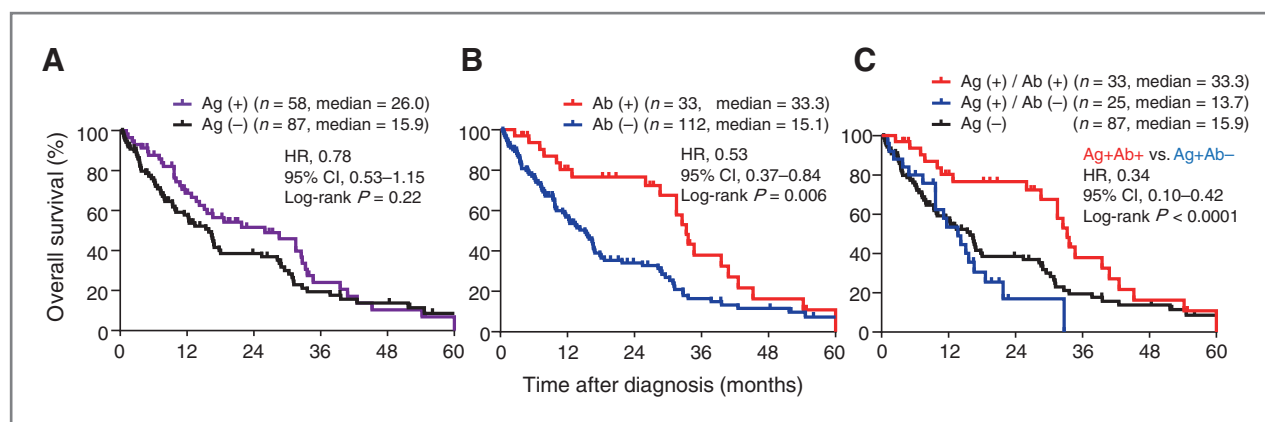


Figure 4. OS of patients with advanced lung adenocarcinoma with XAGE1 (GAGED2a) antigen-expressing or nonexpressing tumors (A), with and without the XAGE1 (GAGED2a) antibody (B), and with the antigen expressing or nonexpressing tumors and with or without the antibody (C).

months when antibody-positive, but only 11.1 months when antibody-negative ( $P = 0.001$ , HR, 0.28; Fig. 5D). No significant difference was found in OS in the antibody-positive patients with EGFRwt and EGFRmt tumors, or the patients with antigen-negative EGFRmt tumors ( $P = 0.36$ ; Fig. 5B and D and Supplementary Fig. S5B).

#### OS of patients with different EGFR mutation tumors

EGFR mutation types were defined in 44 tumors (Supplementary Table S1B). Mutations in exon 18 (G719A/C/S), exon 19 (E746-A750 deletion: type 1, E746-A750 deletion: type 2 and L747-P753 deletion and S insertion), and exon 21 (L858R and L861Q) were detected. As shown in Supplementary Fig. S6, no significant difference in OS was observed in patients with different EGFR mutation tumors ( $P = 0.44$ ).

#### Univariate and multivariate analyses

We performed univariate and multivariate analyses on OS in 145 patients with advanced lung adenocarcinoma. Univariate analysis showed that the presence of the XAGE1 (GAGED2a) antibody, EGFR mutation, good Eastern Cooperative Oncology Group performance status, or never or light smoking habit were significant predictors for prolonged OS (Supplementary Table S2A). On the other hand, multivariate analysis showed that the presence of the XAGE1 (GAGED2a) antibody was a strong predictor for prolonged OS in patients with XAGE1 (GAGED2a) antigen-positive tumors ( $P < 0.0001$ , HR, 0.18) and in patients with either EGFRwt ( $P = 0.04$ , HR, 0.50) or EGFRmt tumors ( $P = 0.002$ , HR, 0.17; Supplementary Table S2B). XAGE1 (GAGED2a) antigen expression was a worse predictor in patients with EGFRmt tumors ( $P = 0.004$ , HR, 5.23).

#### Augmented or sustained antibody response, but increased immune inhibition, during the late phase of disease progression

The XAGE1 (GAGED2a) antibody response was examined for a prolonged period until death by disease progression in 13 XAGE1 (GAGED2a) antigen-positive advanced

lung adenocarcinoma patients. The numbers of antibody-positive and -negative patients were 10 and 3, respectively. As shown in Fig. 6A, antibody response was augmented in 5 of 10 antibody-positive patients and sustained in 5 other patients. No positive conversion was observed in 3 antibody-negative patients. With 5 antibody response-augmented patients, various immune parameters were compared at diagnosis and at the late phase of disease progression. As shown in Fig. 6B, an increase in CXCR5<sup>+</sup> (T<sub>FH</sub>) CD4 T-cell level, but not CXCR3<sup>+</sup> (Th1) CD4 T-cell level, was observed. No change in resting or activated Treg levels was observed (Fig. 6C). M- and PMN-MDSC levels were increased at the late phase (Fig. 6C). An increase in Tim-3 expression level was observed in CD8 T cells (Fig. 6D). Functional analysis showed that impaired XAGE1 (GAGED2a)-specific CD4 (Fig. 6E) and CD8 (Fig. 6F) T-cell responses for cytokine production were observed frequently at the late phase.

#### Discussion

In this study, we demonstrated that XAGE1 (GAGED2a) antibody-positive advanced lung adenocarcinoma patients showed prolonged OS when compared with the OS of antibody-negative patients. In patients with XAGE1 (GAGED2a) antigen-positive tumors, no significant difference was found in OS in patients with EGFRwt and EGFRmt tumors when they were XAGE1 (GAGED2a) antibody-positive (Fig. 5 and Supplementary Fig. S5). It should be noted that the patients with EGFRmt tumors treated with EGFR-TKI and conventional platinum-based doublet chemotherapy showed prolonged OS compared with those with EGFRwt tumors treated with conventional chemotherapy alone (Supplementary Fig. S5). The presence of the antibody greatly prolonged OS in patients with XAGE1 (GAGED2a) antigen-positive EGFRwt tumors, resulting in OS close to that of antibody-positive patients with EGFRmt tumors.

The patients with XAGE1 (GAGED2a) antigen-positive EGFRmt tumors showed shortened OS when the patients were antibody-negative compared with that of those with



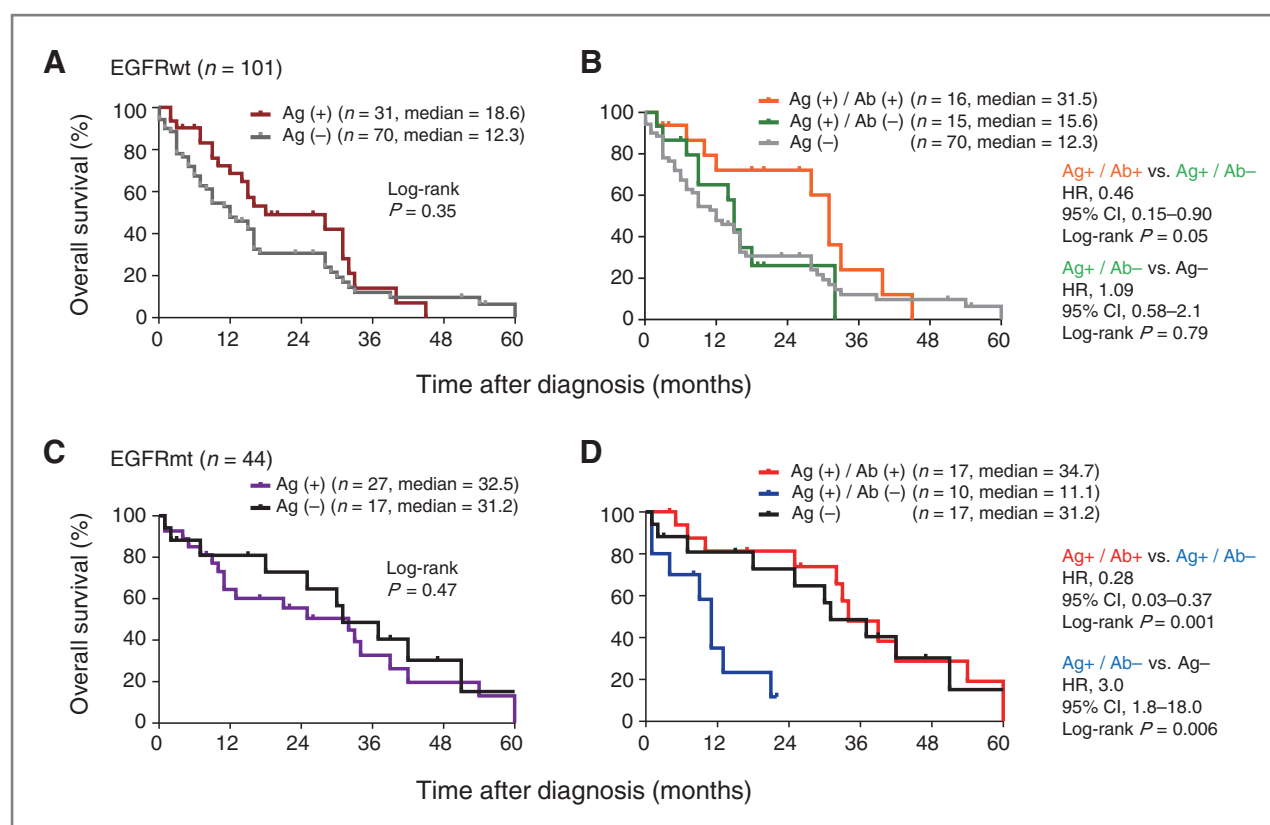


Figure 5. OS of the patients with advanced lung adenocarcinoma with XAGE1 (GAGED2a) antigen-expressing or nonexpressing EGFRwt (A and B) and EGFRmt (C and D) tumors and with or without the antibody (B and D).

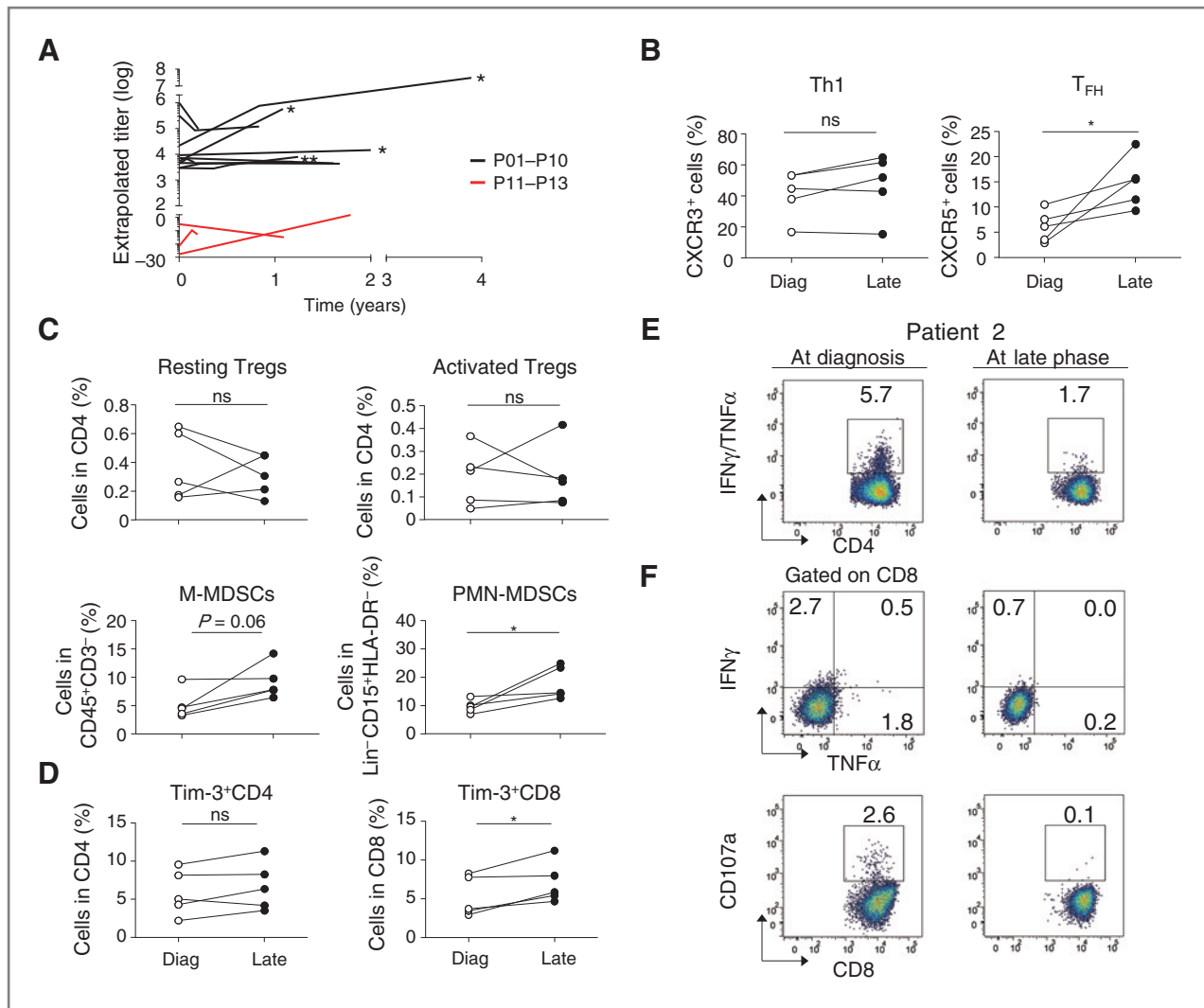
antigen-negative EGFRmt tumors. It should be noted that the survival shortening effect of XAGE1 (GAGED2a) antigen expression was observed only in patients with EGFRmt tumors, but not in those with EGFRwt tumors. These findings suggest specific involvement of the XAGE1 (GAGED2a) antigen in EGFRmt tumors or with EGFR-TKI treatment. The presence of the XAGE1 (GAGED2a) antigen in a tumor may facilitate EGFR-mediated tumorigenesis and/or hamper the effect of EGFR-TKI, and the presence of the XAGE1 (GAGED2a) antibody may inhibit this effect. EGFR signaling was delivered via the PI3K, AKT, and mTOR, or Ras, Raf, MEK, and MAPK pathways to activate many tumorigenic genes (26–30). These involve cell cycle, cell proliferation, antiapoptosis, invasion, or metastasis (31–33). Although the XAGE1 (GAGED2a) antigen has been shown to locate in the nucleus, the possibility of direct molecular interaction between XAGE1 (GAGED2a) and mutated EGFR, its downstream molecules, or EGFR-TKI itself remains to be addressed.

The function of the XAGE1 (GAGED2a) molecule is largely unknown. However, Caballero and colleagues (34) recently showed that XAGE1 depletion in an SK-MEL-37 melanoma cell line by siRNAs reduced proliferation, clonogenic survival, migration, and invasion of the cells. The tumorigenic effect of cancer/testis antigen on the X chromosome (CT-X) was also shown in *SSX4* (34) and

*MAGE* (35, 36). For XAGE-related GAGE genes, the proteins bind to the metazoan transcriptional regulator, germ cell-less (GCL), at the nuclear envelope and cause tumorigenesis (37). These findings suggest that the tumorigenic effect is a common characteristic of CT-X antigens.

CT-X expression has been shown to be a marker of poor outcome in non-small cell lung cancer (NSCLC) (38). The expressions of NY-ESO-1, MAGE-A1, MAGE-A3, and SSX2 were associated with shorter survival in lung adenocarcinoma. Especially, high-level expression of NY-ESO-1 or MAGE was a strong predictor for worse outcome independent of confounding factors such as stage, histology, and therapy. With XAGE1 (GAGED2a), no significant difference was observed in OS with the antigen-positive and negative patients. Moreover, we previously reported that no correlation was found between the expression pattern (diffuse, intermediate, or focal) and OS (19). It is possible that the higher frequency of antibody response causing prolonged survival may obscure the difference.

The prolongation of OS by the presence of the XAGE1 (GAGED2a) antibody counteracting the survival shortening effect of XAGE1 (GAGED2a) antigen expression may not result from direct interaction of the antibody and antigen. The XAGE1 (GAGED2a) antigen resides in the cells, usually in the nucleus as mentioned above. It is unlikely that the antibody enters the cell and interacts with XAGE1



**Figure 6.** Augmented or sustained antibody response, but increased immune inhibition, during the late phase of disease progression in XAGE1 (GAGED2a) antigen-positive, advanced lung adenocarcinoma patients. A, kinetic XAGE1 (GAGED2a) antibody response by ELISA during a prolonged period until death by disease progression in 10 XAGE1 (GAGED2a) antibody-positive (P01–P10, black lines) and 3 antibody-negative (P11–P13, red lines) patients. Five patients showing an increase in antibody titer are denoted by asterisks and were analyzed for CXCR3 and CXCR5-positive CD4 T cells (B), resting and activated Tregs, and M- and PMN-MDSCs (C), and Tim-3 expression in CD4 and CD8 T cells (D) at diagnosis or at the late phase by FACS. Statistical analysis was done by the Wilcoxon rank test (\*,  $P < 0.05$ ). Each line indicates a single patient. E and F show the responses of CD4 and CD8 T cells, respectively, against the XAGE1 (GAGED2a) antigen determined for cytokine production by ICS as described in the Fig. 1D legend. In F, the expression of the CD107a molecule on CD8 T cells was also analyzed by FACS.

(GAGED2a). Rather, it is likely that T-cell responses elicited concomitantly with the antibody response contribute to the antitumor effect. Our previous (21) and present results showed frequent occurrence of CD4 and CD8 T-cell responses in XAGE1 (GAGED2a) antibody-positive patients and no such T-cell responses in antibody-negative patients. Thus, CD4 and CD8 T-cell responses seemed to be associated with the antibody response in patients with XAGE1 (GAGED2a)-positive tumors. In patients with NY-ESO-1-positive tumors, such a naturally occurring integrated immune response was frequently observed (39, 40). NY-ESO-1 is a prototype of the CT antigen and has been shown to be strongly antigenic (41). XAGE1 (GAGED2a) seemed

to be less immunogenic than NY-ESO-1 (21), but still capable of eliciting an integrated immune response. In our previous study, we demonstrated that XAGE1 (GAGED2a) expression resulted in shorter survival in patients with NSCLC when the MHC class I expression was downregulated in the tumor (20). However, when the tumor coexpressed XAGE1 (GAGED2a) and MHC class I, survival was clearly prolonged. These findings suggest the involvement of CD8 T-cell activation in recognizing the XAGE1 (GAGED2a) antigen on HLA class I may contribute to prolonged survival.

On the other hand, recent exome analysis to determine mutations in the tumor has revealed the relevance of

immune responses to multiple mutated gene products in the tumor (42–45). Because XAGE1 (GAGED2a) expression is mostly heterogeneous, the immune response to XAGE1 (GAGED2a) could be a surrogate for such immune responses to mutated antigens.

In this study, we characterized various immune parameters in XAGE1 (GAGED2a) antibody-positive patients and showed elevated immune responsiveness. XAGE1 (GAGED2a)-reactive CD4 and CD8 T cells were detected in the antibody-positive patients. Increases in Th1, 2, 17, T<sub>FH</sub> levels and decreases in activated Treg and M-MDSC levels were observed in the antibody-positive patients. An increase in ICOS and PD-1–expressing CD4 T-cell levels and a decrease in BTLA-expressing CD4 T-cell levels were observed in the antibody-positive patients. These findings suggested that in XAGE1 (GAGED2a) antibody-positive patients, immune activation involving CD4 and CD8 T cells occurred in response to the XAGE1 (GAGED2a) antigen, supporting elicitation of an integrated immune response.

At the late phase of disease progression long after finishing treatment, the XAGE1 (GAGED2a) antibody response was still augmented or sustained. CXCR3<sup>+</sup> (Th1) and CXCR5<sup>+</sup> (T<sub>FH</sub>) CD4 T-cell levels were retained or increased. However, increases in M- and PMN-MDSC, and Tim-3–expressing CD8 T-cell levels were observed. A reduction in XAGE1 (GAGED2a)-reactive CD4 and CD8 T-cell responses was frequently observed at the late phase. These findings suggest that immune regulation is one of the causes leading to disease progression resulting in death, even in patients with prolonged survival.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Writing, review, and/or revision of the manuscript:** Y. Ohue, M. Oka, E. Nakayama

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** Y. Ohue, H. Matsumoto, Y. Nishio, M. Isobe, M. Fukuda, A. Uenaka, M. Oka

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