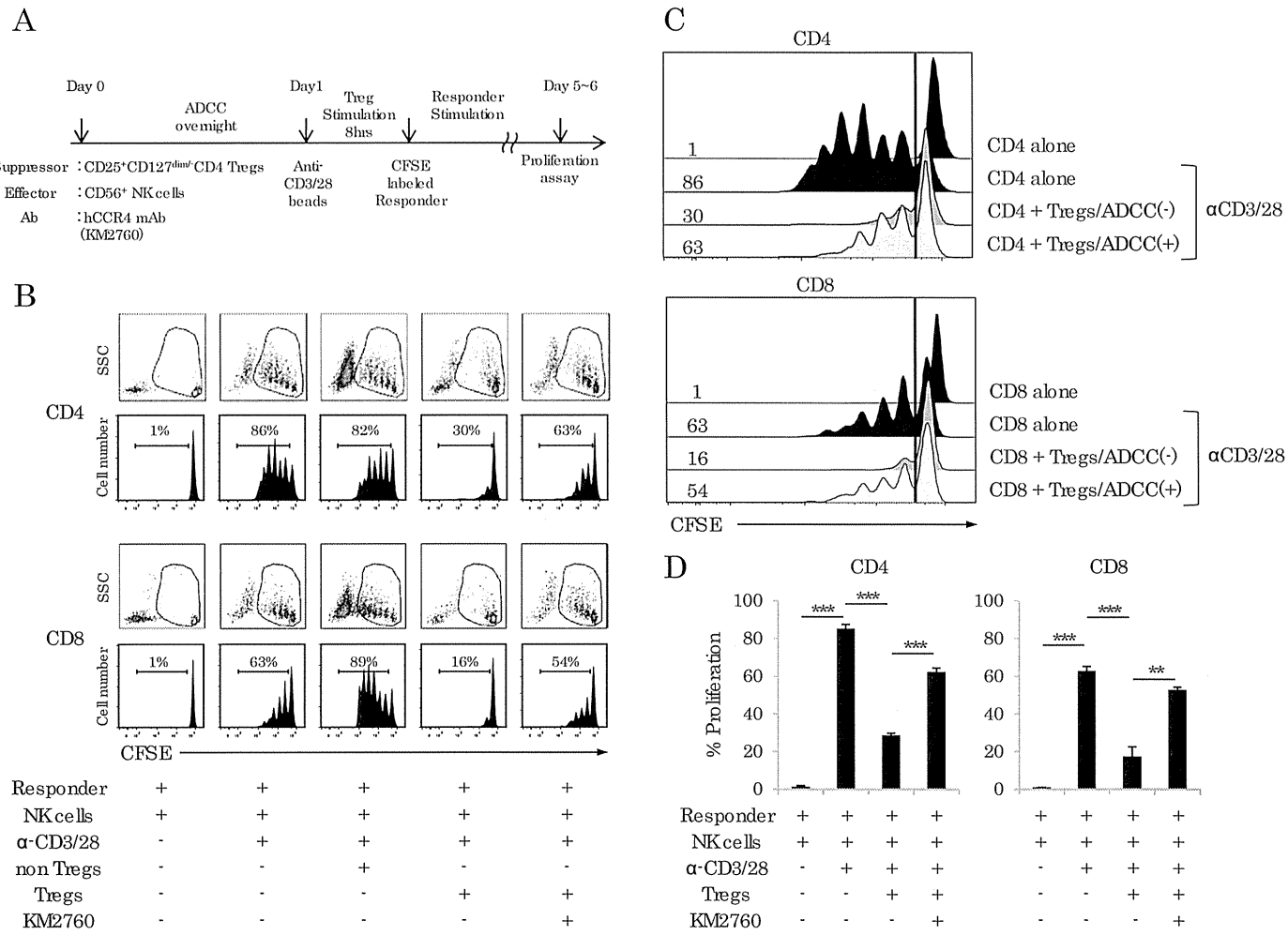

FIGURE 5. Inhibition of CD3/CD28-mediated proliferative response of CD4 and CD8 T cells by CD25+ Tregs and abrogation of inhibition by the treatment of Tregs with anti-hCCR4 mAb (KM2760). A, schema of the experimental protocol. CD127dim/- CD4 T cells were indirectly purified from the PBMCs of healthy donors using biotin-conjugated antibodies against CD8, CD19, CD123, and CD127 with antibiotin antibody-coated magnetic beads. CD25+ CD127dim/- CD4 Tregs were then purified and CD25- CD127dim/- CD4 T cells were used as control non-Tregs. CD56+ NK cells, and CD4 and CD8 T cells were purified from PBMCs also using antibody-coated magnetic beads. Tregs (1×10^4) and CD56+ NK cells (1×10^4) were incubated overnight with or without anti-hCCR4 mAb (KM2760) at a concentration of 10 μg/ml in 96-well culture plates. After washing the cells in the plates, anti-CD3/CD28 beads were added. The CFSE-labeled responder CD4 and CD8 T cells were then added and proliferation was determined after 5 to 6 days. In (B) representative results of three independent experiments are shown. Dot plots and histograms of CFSE-labeled CD4 and CD8 T cells after stimulation with anti-CD3/CD28, and inhibition of proliferation by Tregs and its abrogation by anti-hCCR4 mAb (KM2760) treatment are shown. C, layered presentation of the experiment shown in (B). D, the results in (B) are shown as the mean ± SD of triplicate experiments. Statistical analysis was done by Welch's t test (** $p < 0.01$, *** $p < 0.001$).

the population.[15] Thus, a small fraction of Th2, Th17, or IFNγ-producing cells was detected in the CD45RA- Foxp3 low positive, non-Treg fraction (Fr 3), although the majority of those cells were Foxp3-negative cells.

With regard to Th17 cells, it has recently been shown that the frequency of these cells secreting IL-17 was increased in patients with different types of tumors,[21] including lung cancer.[22] The density of intratumoral IL-17-positive cells in primary human NSCLC was inversely correlated with patient outcome and correlated with the smoking status of the patients.[23]

We also showed that CCR4 expression on activated/effector Tregs and also non-Tregs in TILs was down-regulated compared with that on those cells in PBMCs. It was noticed that chemokine receptors, including CCR4, were down-regulated quickly after interaction with the respective chemokines.[24] These findings suggested that CCR4 was functionally involved with chemotactic migration and accumulation of activated/effector Tregs and non-Tregs to the tumor sites.

We demonstrated that CCR4-expressing lymphocytes infiltrated in tumor tissue and some of them were likely Foxp3+ CD4 T cells as judged by IHC using TMA. CCR4-stained lymphocytes were detected in only 20% of the tumor tissues of 384 samples examined, whereas flow cytometric analysis showed that activated/effector Tregs were detected

in TILs from most of the 20 lung cancer patients we investigated. Detection of CCR4-expressing cells at a low frequency in TMA appeared to be due to the limited area of tumor tissue prepared for TMA and/or low sensitivity of IHC.[16]

Anti-hCCR4 mAb (KM2760) is a defucosylated chimeric mAb produced by Potelligent technology and has been shown to have more than 100 times stronger ADCC activity than the original antibody.[19] Leukemic cells in adult T-cell leukemia (ATL) express CCR4 on their surfaces and cytotoxicity of anti-hCCR4 mAb (KM2760) to those cells has been demonstrated.[25] Yamamoto et al.[26] reported that administration of even a small dose (0.1 mg/kg) of humanized anti-hCCR4 mAb (KW-0761) efficiently eliminated leukemia cells in the peripheral blood in ATL patients in clinical trials.[26] In this study, we showed that activated/effector Tregs also express CCR4 on their surface and that those cells could be efficiently eliminated in vitro by treatment with an anti-hCCR4 mAb (KM2760) by ADCC with NK cells. Migration of a $CD25^+$ CD4 Treg population sorted from PBMCs in healthy donors to a CCL22/MDC gradient was abrogated by the pretreatment of PBMCs with an anti-hCCR4 (KM2760) mAb. The inhibition of the proliferative response of CD4 and CD8 T cells stimulated with anti-CD3/CD28-coated beads by $CD25^+CD127^{dim/-}$ CD4 Tregs was abrogated by adding anti-hCCR4 mAb (KM2760) and $CD56^+$ NK cells to the culture. These in vitro findings of efficient elimination of Tregs in a migration assay and in a T-cell proliferation assay may give the basis for implementation of clinical trials focusing on depletion of Tregs by administration of anti-hCCR4 mAb to cancer patients with various solid tumors.

In this study, we showed that an anti-hCCR4 mAb (KM2760) had no direct blocking activity on the migration of purified CD4 T cells to the CCL22/MDC gradient by simply adding it to the culture during the assay. There is extensive redundancy in the binding for chemokines to chemokine receptors.[27] It is possible that chemokine receptors other than CCR4 are involved in the migration to the CCL22/MDC gradient under the CCR4 blockade.[28] Or it could simply be due to the lack of blocking activity for CCL22/MDC binding to CCR4.

Recently, Sugiyama et al.[29] showed depletion of activated/effector Tregs and augmentation of T-cell responses against the NY-ESO-1 antigen by magnetic bead depletion using a biotin anti-CCR4 mAb (1G1) and also by simply adding a mouse antihuman CCR4 mAb (KM2160) to the culture.[29] In our study, we showed that a defucosylated chimeric KM2760 derived from KM2160 efficiently depleted Tregs by ADCC with NK cells as above. However, with KM2760, no depletion of any Treg subpopulations and no effect on their migration to CCL22/MDC was observed without adding NK cells. The difference in the direct depletion effect between KM2160 and KM2760 by adding to the culture could be due to experimental systems, especially incubation time (7 days in their study and 4 hours in ours) or due to loss of depleting activity by chimerization and defucosylation of the antibody, although less likely. This point should be carefully addressed in future studies.

Induction of immune responses by depleting Tregs has been reported previously.[30,31] In vitro depletion of $CD25^+$ cells induced activation of NY-ESO-1-specific naive CD4 T-cell precursors in stimulation with NY-ESO-1 peptides in PBMCs from healthy donors and from NY-ESO-1-expressing melanoma patients who had no NY-ESO-1 antibodies.[32] We previously showed that depletion of Tregs by in vivo administration of an anti-CD25 mAb (clone PC61) caused rejection of tumors that otherwise grew progressively in murine tumor models.[7] However, the effect of inducing tumor rejection by administration of the mAb was observed only up to day 2 after tumor inoculation. This is probably due to the depletion of the effector T cells, which were generated after recognition of the tumor cells and express CD25 on their cell surfaces.[33] There are some reports of clinical trials on the depletion of CD25 Tregs using anti-CD25 or diphtheria toxin-conjugated IL-2 (denileukin diftitox).[34,35] The results in those studies were controversial: successful depletion of $CD25^+$ cells and augmentation of the tumor immune response in one study,[36] but no effect in the others.

We are currently conducting a phase I clinical trial administering humanized anti-hCCR4 mAb (KW-0761) to patients with various solid tumors. Depletion of CCR4-expressing activated/effector Tregs in PBMCs will result in depletion of either CCR4-expressing or nonexpressing activated/effector Tregs in the tumor if they migrate from the peripheral blood. On the other hand, the findings that high and low frequencies of CCR4-expressing cells in activated/effector Tregs and resting/naive Tregs, respectively, in PBMCs may suggest that the CCR4 expression is correlated with Treg function, and only the CCR4-expressing population represents functional Tregs in TILs, although this remains to be clarified. Our preliminary results show efficient depletion of CCR4-expressing activated/effector Tregs in PBMCs, although those cells in the TILs were not analyzed.

Off-target effects could occur due to anti-CCR4 mAb therapy. CCR4 is expressed on Th2 and Th17 cells other than Tregs, but not on Th1 cells (data not shown). Depletion of these cells may cause impaired antibody and cellular responses against infection. CD8 and monocytes express no CCR4 (our unpublished observation).[29] Studies on ATL/ATLL patients and our preliminary study on solid tumor patients showed that eruption controllable by steroids probably caused by autoimmunity was commonly observed, whereas infection was rare.[26]

CCR4 expression on tumor cells is controversial. Frequent expression was reported with head and neck cancer[37] and moderate expression was reported with other cancers.[38,39] In lung cancer, however, IHC analysis of TMA in this study showed that CCR4 expression on tumor cells was observed in only 1 of 384 specimens. These findings suggest that the ADCC caused by anti-hCCR4 mAb (KW-0761) acts against CCR4-expressing lymphocytes, but not tumor cells, in lung cancer.

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Clinical Cancer Research

Personalized Medicine and Imaging

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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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 κ 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′-

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TTTCTCCGCTACTGAGACAC-3′ and X-2, 5′-CAGCTTGCGTTGTTTCAGCT-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_2O_2 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_2 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 (1×10^6/well) and CD8 (1×10^6/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_2 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).

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After incubation, responder CD4 or CD8 T cells (1×10^5) 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 μL) for 30 minutes on ice. After incubation, the cells were washed, fixed, and permeabilized with Cytofix/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-IFNγ-PE/Cy7 (clone 4S.B3; eBioscience), anti-TNFα-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-IFNγ-PerCP/Cy5.5 (clone 4S.B3; eBioscience), anti-TNFα-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 ≥ 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, IFNγ/TNFα, 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, IFNγ- and TNFα-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_{FH} 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

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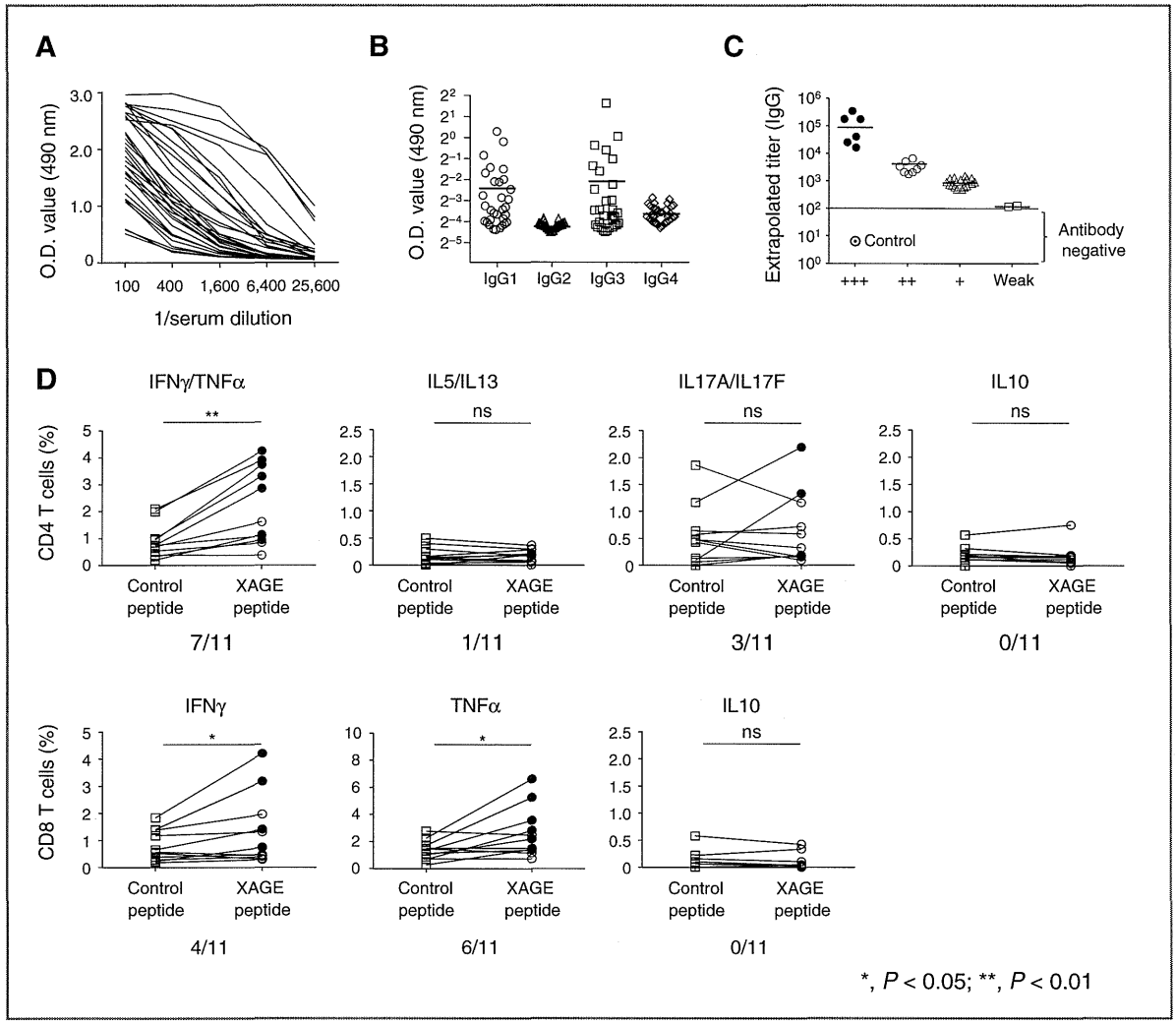

Figure 1. Antibody and T-cell responses against XAGE1 (GAGED2a) in patients with advanced lung adenocarcinoma. A, titration curves of positive sera from 33 of 145 patients investigated. ELISA was done using synthetic XAGE1 (GAGED2a) protein. The IgG response was defined as positive for sera with an extrapolated 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 ≥ 100. D, CD4 and CD8 T cells (1×10^6) 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_{FH} 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_{FH}/total MDSC, but not the T_{FH}/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

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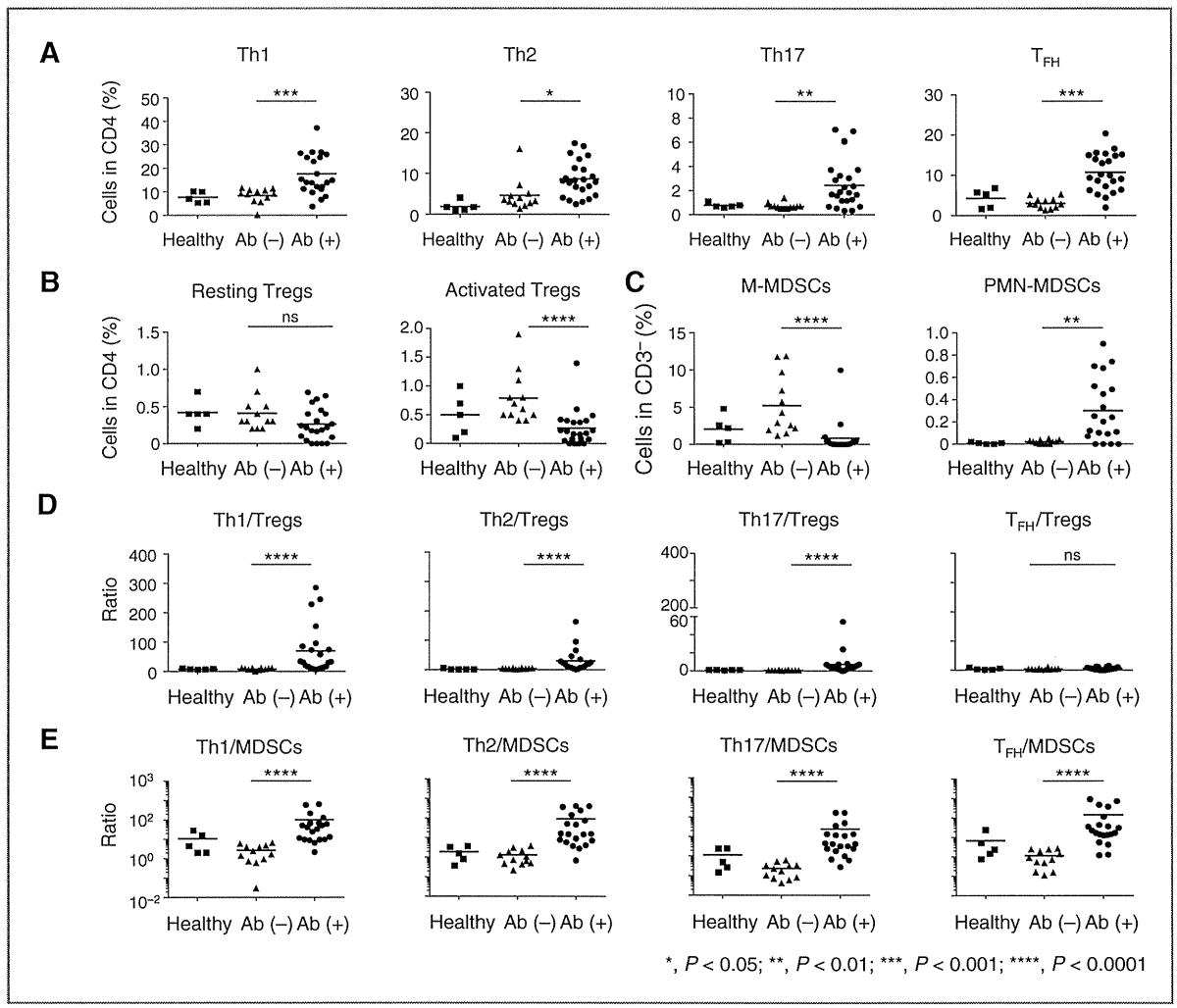

Figure 2. Phenotypic analyses of CD4 T cells (A and B) and MDSCs (C) in PBMCs from 23 XAGE1 (GAGED2a) antibody-positive and 12 negative patients, and 5 healthy donors by FACS. D and E show the ratio of each phenotype of T cells to Tregs and MDSCs, respectively. Statistical analysis was done by the Student *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

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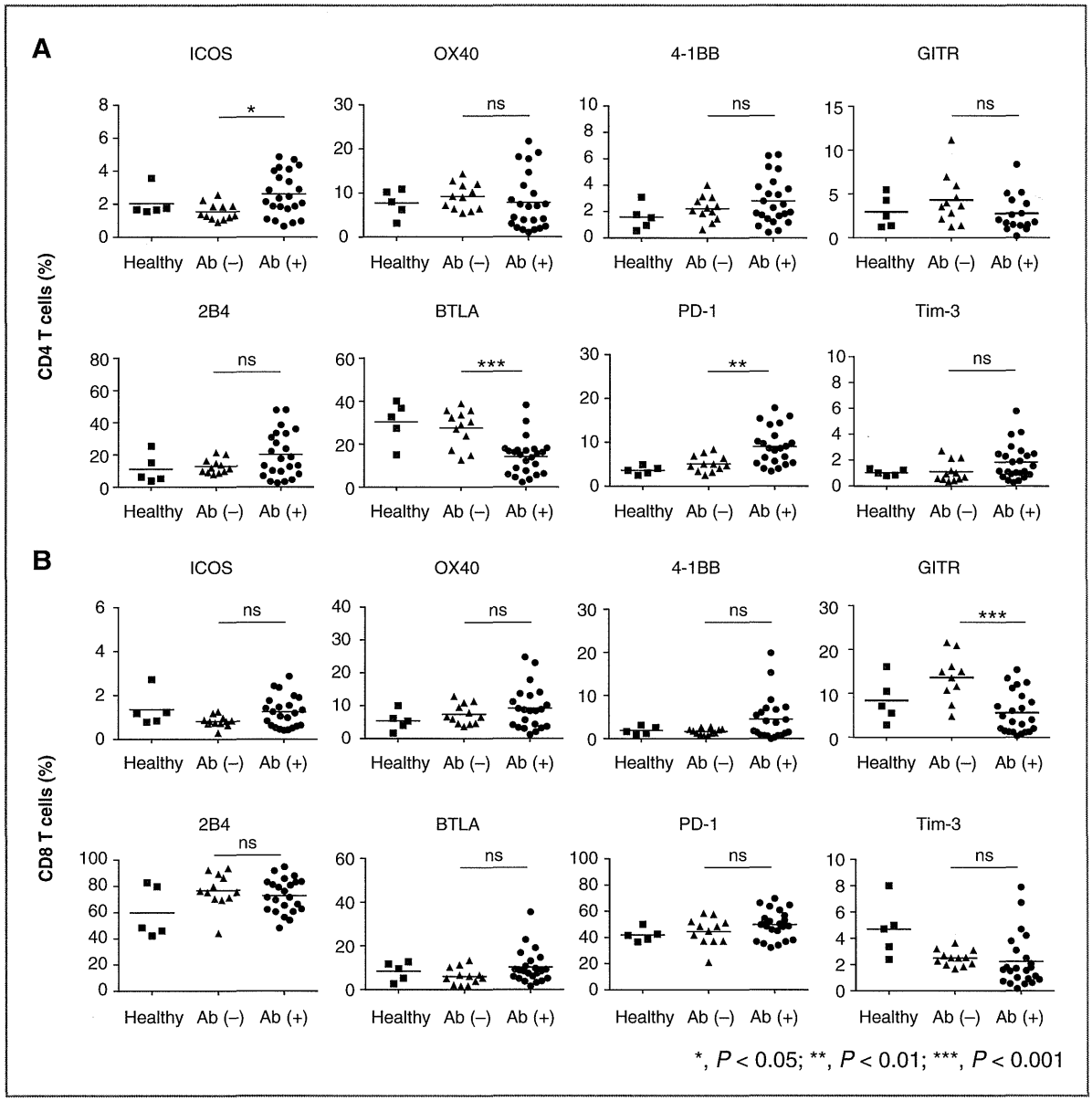

Figure 3. Analysis of CD4 (A) and CD8 (B) T cells expressing T-cell activation (ICOS, OX40, 4-1BB, and GITR) and inhibitory (2B4, BTLA, PD-1, and Tim-3) molecules in PBMCs from XAGE1 (GAGED2a) antibody-positive and negative patients, and healthy donors by FACS. Statistical analysis was done by the Student *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

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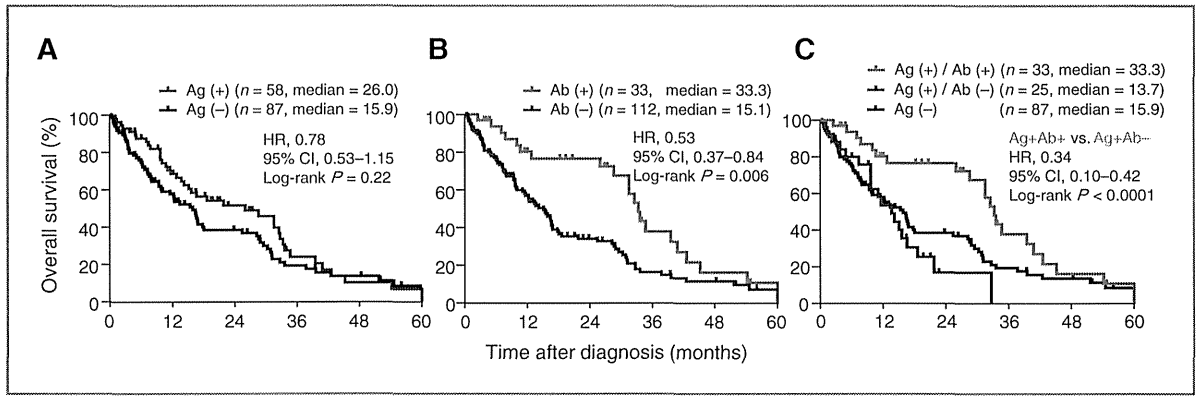

Figure 4. OS of patients with advanced lung adenocarcinoma with XAGE1 (GAGED2a) antigen-expressing or nonexpressing tumors (A), with and without the XAGE1 (GAGED2a) antibody (B), and with the antigen expressing or nonexpressing tumors and with or without the antibody (C).

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 $CXCR5^+$ (T_{FH}) CD4 T-cell level, but not $CXCR3^+$ (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

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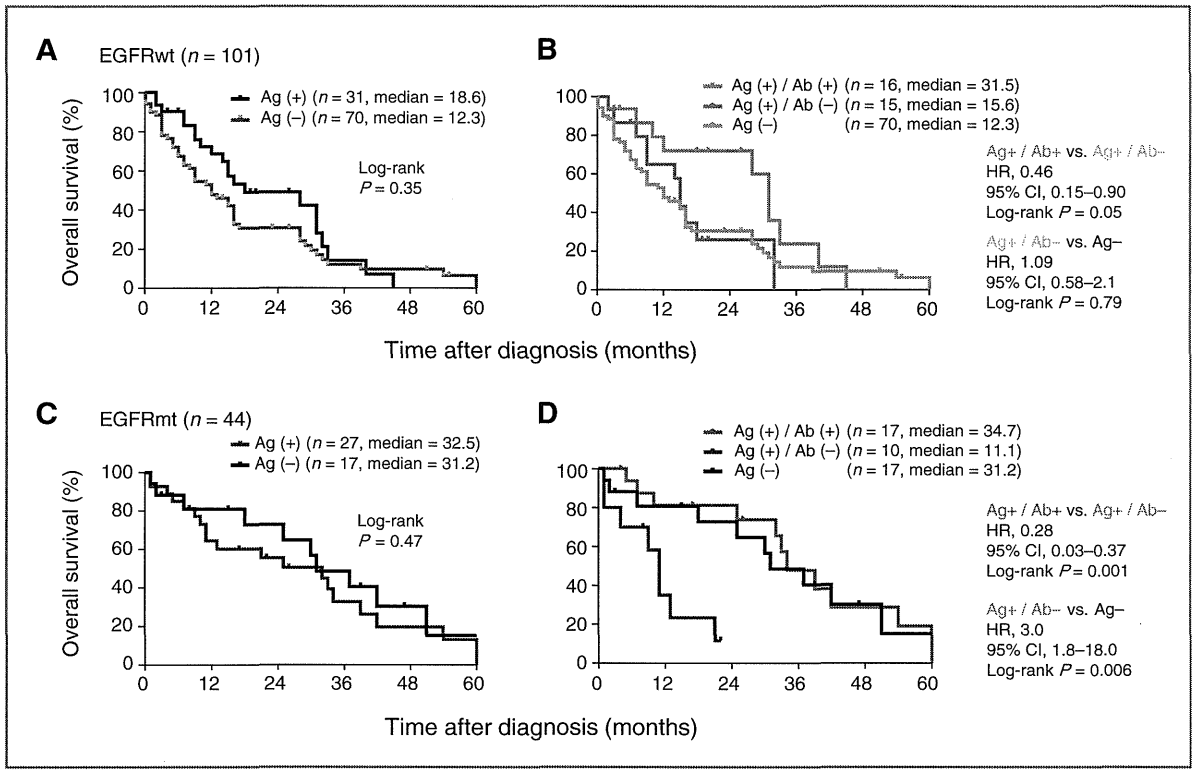

Figure 5. OS of the patients with advanced lung adenocarcinoma with XAGE1 (GAGED2a) antigen-expressing or nonexpressing EGFRwt (A and B) and EGFRmt (C and D) tumors and with or without the antibody (B and D).

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

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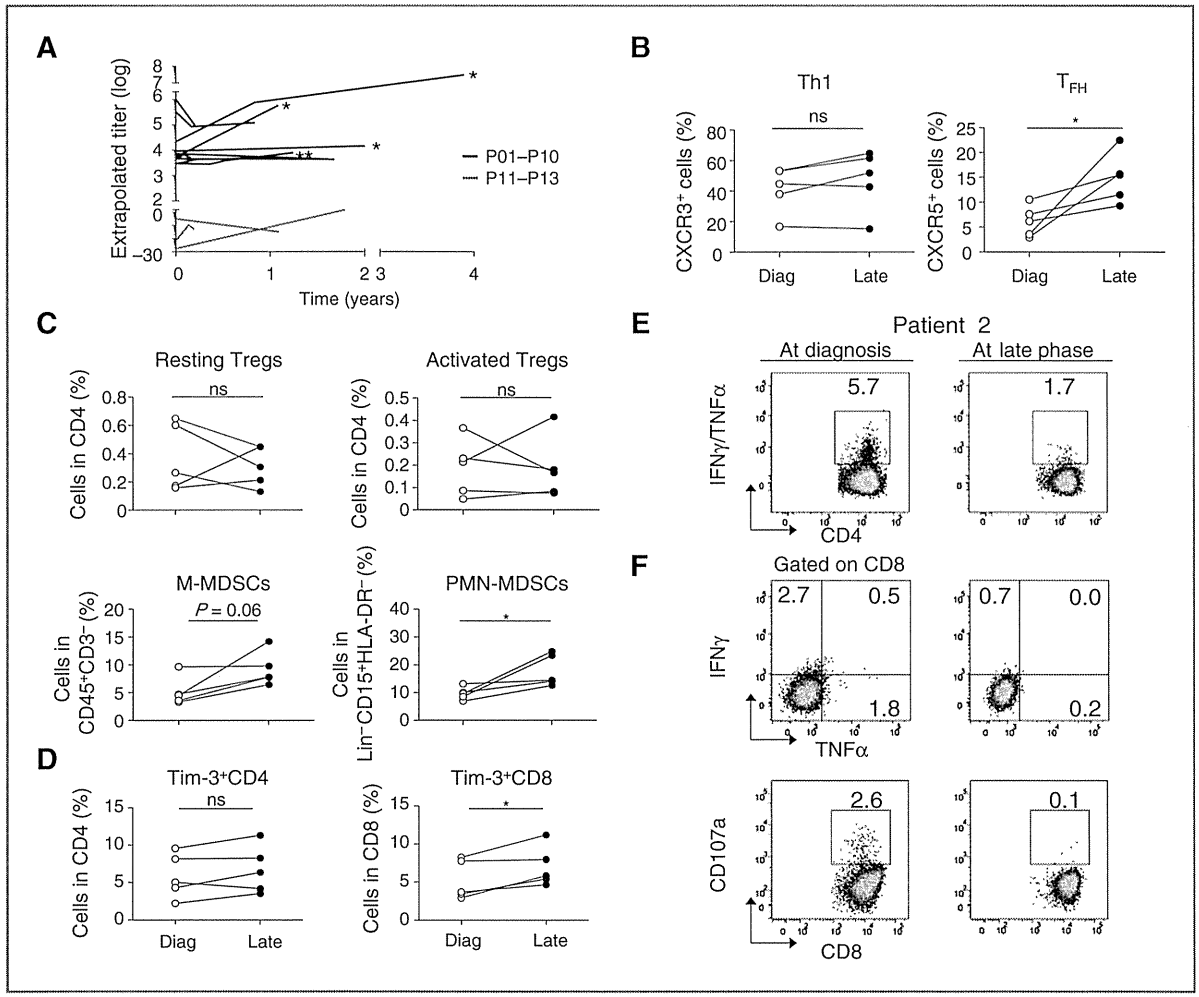

Figure 6. Augmented or sustained antibody response, but increased immune inhibition, during the late phase of disease progression in XAGE1 (GAGED2a) antigen-positive, advanced lung adenocarcinoma patients. A, kinetic XAGE1 (GAGED2a) antibody response by ELISA during a prolonged period until death by 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

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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_{FH} 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. $CXCR3^+$ (Th1) and $CXCR5^+$ (T_{FH}) 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: Y. Ohue, Y. Mizote
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Ohue, K. Kurose, M. Fukuda, M. Oka
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Ohue, Y. Mizote, M. Oka, E. Nakayama
Writing, review, and/or revision of the manuscript: Y. Ohue, M. Oka, E. Nakayama
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Prolongation of Overall Survival in Advanced Lung Adenocarcinoma Patients with the XAGE1 (GAGED2a) Antibody

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Genetic variants of immunoglobulin γ and κ chains influence humoral immunity to the cancer-testis antigen XAGE-1b (GAGED2a) in patients with non-small cell lung cancer

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Summary

GM (γ marker) allotypes, genetic variants of immunoglobulin γ chains, have been reported to be associated strongly with susceptibility to lung cancer, but the mechanism(s) underlying this association is not known. One mechanism could involve their contribution to humoral immunity to lung tumour-associated antigens. In this study, we aimed to determine whether particular GM and KM (κ marker) allotypes were associated with antibody responsiveness to XAGE-1b, a highly immunogenic lung tumour-associated cancer-testis antigen. Sera from 89 patients with non-small cell lung cancer (NSCLC) were allotyped for eight GM and two KM determinants and characterized for antibodies to a synthetic XAGE-1b protein. The distribution of various GM phenotypes was significantly different between XAGE-1b antibody-positive and -negative patients ($P = 0{\cdot}023$), as well as in the subgroup of XAGE-1b antigen-positive advanced NSCLC ($P = 0{\cdot}007$). None of the patients with the GM 1,17 21 phenotype was positive for the XAGE-1b antibody. In patients with antigen-positive advanced disease, the prevalence of GM 1,2,17 21 was significantly higher in the antibody-positive group than in those who lacked the XAGE-1b antibody ($P = 0{\cdot}026$). This phenotype also interacted with a particular KM phenotype: subjects with GM 1,2,17 21 and KM 3,3 phenotypes were almost four times (odds ratio = 3·8) as likely to be positive for the XAGE-1b antibody as the subjects who lacked these phenotypes. This is the first report presenting evidence for the involvement of immunoglobulin allotypes in immunity to a cancer-testis antigen, which has important implications for XAGE-1b-based immunotherapeutic interventions in lung adenocarcinoma.

Keywords: cancer-testis antigen, GM/KM allotypes, humoral immunity, non-small cell lung cancer, XAGE-1b (GAGED2a)

Introduction

Genetic variants of immunoglobulin G (IgG) heavy chains are called GM allotypes. They are encoded by three very closely linked genes – immunoglobulin heavy chain G1 (*IGHG1*), *IGHG2* and *IGHG3* – on chromosome 14q32. They are expressed on the constant regions of γ1, γ2 and γ3 chains. There are striking qualitative and quantitative differences in the distribution of GM allotypes among different racial groups. In addition, there is almost complete linkage disequilibrium between particular GM determinants within a race, and every major racial group is characterized by a distinct array of GM haplotypes [1,2]. Using hypothesis-driven candidate gene approaches, several studies have identified particular GM genes/genotypes as risk factors for many malignant diseases [2–7]. In lung cancer, a highly significant association was found between the GM 1,2 13,15,16,21 phenotype and susceptibility to this malignancy in a Japanese population [8]. The mechanism(s) underlying this association is not known.

One mechanism underlying the reported GM gene–lung cancer association could involve the contribution of GM determinants to humoral immunity to lung tumour-associated antigens, as GM genes are known to influence immunity to several self and non-self antigens, including tumour-associated antigens mucin 1 and human epidermal

growth factor receptor 2 [9–14]. In this investigation, we aimed to determine whether GM allotypes are associated with antibody responsiveness to XAGE-1b, a highly immunogenic lung tumour-associated antigen that belongs to the cancer-testis antigen gene families [15–17]. A recent comprehensive analysis of human gene expression has identified the Ig κ constant (*IGKC*) gene as a strong prognostic marker in human solid tumours, including lung cancer [18]. Identification of tumour-infiltrating plasma cells as the source of *IGKC* expression in this study strongly suggests a role for humoral immunity in lung cancer and provides a compelling rationale for investigating the role of KM alleles, genetic variants of *IGKC*, in humoral immunity to lung tumour-associated antigens.

There is increasing evidence that genes do not act in isolation, and that epistasis – modification of the action of a gene by one or more other genes – plays a significant role in human diseases. Genes expressed on the Ig heavy and light chains are probably some of the most likely candidates for gene–gene interactions in the human genome. Therefore, the aim of the present investigation was to determine whether GM and KM allotypes – individually or in particular epistatic combinations – contribute to antibody responsiveness to XAGE-1b in patients with non-small cell lung cancer (NSCLC).

Materials and methods

Blood samples

The study population is described in detail elsewhere [17]. The Institutional Review Boards of the respective institutions approved the study protocol. Blood samples from 89 Japanese patients with NSCLC were included in this investigation. Of these, 80 patients were diagnosed histologically examining available tumour specimens and nine were diagnosed cytologically using tumour cells in pleural effusion, sputum or bronchoalveolar fluid (BALF) because tumour tissue was not available.

Anti-XAGE-1b antibody determinations

These antibodies were measured by a previously described enzyme-linked immunosorbent assay (ELISA) [16,17]. Briefly, synthetic XAGE-1b (GAGED2a) protein (1 μg/ml) in coating buffer was adsorbed onto a 96-well ELISA plate (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Plates were washed with phosphate-buffered saline (PBS) and blocked with 5% fetal calf serum (FCS)/PBS (200 μl/well) for 1 h at 37°C. After washing, 100 μl of serially diluted serum was added to each well and incubated for 2 h at 4°C; horseradish peroxidase (HRP)-conjugated goat anti-human IgG (MBL) was then added to the wells, and the plates were incubated for 1 h at 37°C. After washing and development, absorbance [optical density (OD)] was read at 490 nm. Sera with OD values exceeding 1·0 at a dilution of 1:300 were considered positive for the XAGE-1b antibody, while those with OD values less than 0·2 were considered negative for this antibody. Patients who showed OD values between 0·2 and 1·0 were excluded. Of the 89 NSCLC patients, 29 were positive for the XAGE-1b antibody and 60 were negative.

Immunohistochemistry

Tumour specimens from 80 patients were also examined by immunohistochemistry. Surgically resected tissues were fixed with buffered formalin and embedded in paraffin. Five-micrometre sections were deparaffinized with xylene and ethanol. Antigen retrieval and inactivation of endogenous peroxidase have been described previously [16]. After incubation with 0·1% Tween 20/5% FCS/PBS for 1 h, the USO 9–13 monoclonal antibody (mAb) was placed at a concentration of 2 μg/ml and incubated for 1 h at room temperature. Immunofluorescence staining was performed as described above. For intracellular localization, rhodamine-conjugated wheat germ agglutinin (WGA) (Vector Laboratories, Burlingame, CA, USA) and 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) were used. The stained cells were visualized under a digital high-definition microscopic system (model BZ-9000 for the magnification of ×40; Keyence, Osaka, Japan).

Of 80 patients, 46 were XAGE-1b antigen-positive and 34 were antigen-negative. Detailed clinical information was not available for three antigen-positive patients. Of the remaining 43 antigen-positive patients, 26 were antibody-positive and 17 were antibody-negative.

GM and KM allotyping

Serum samples were typed for G1M (1/a, 2/x, 3/f, 17/z), G2M (23/n), G3M (5/b1, 13/b3, 21/g) and KM 1 and 3 allotypes by a standard haemagglutination-inhibition method [19]. In brief, a mixture containing human blood group O rhesus-positive (ORh^+) erythrocytes coated with anti-Rh antibodies of known GM/KM allotypes, the test sera and monospecific anti-allotype antibodies were incubated in a microtitre plate. Test sera containing IgG of the particular allotype inhibited haemagglutination by the anti-allotype antibody, whereas negative sera did not. The notation for GM allotypes follows the international system for human gene nomenclature, in which haplotypes and phenotypes are written by grouping together the markers that belong to each IgG subclass, by the numerical order of the marker and of the subclass; markers belonging to different subclasses are separated by a space, while allotypes within a subclass are separated by commas.

Three alleles – KM 1, KM 1,2 and KM 3 – segregate at the KM locus on chromosome 2p12. More than 98% of people positive for KM 1 are also positive for KM 2. The KM 1

Table 1. Distribution of GM* and KM phenotypes in the XAGE-1b antibody-positive and -negative patients with lung adenocarcinoma ($n = 89$).

	XAGE-1b antibody				
Phenotype	Positive ($n = 29$)	(%)	Negative ($n = 60$)	(%)	*P*-value
GM 1,17 21	0	0	10	16·7	0·027
GM 1,2,17 21	15	51·7	19	31·7	0·06
GM 1,17 13,21	0	0	4	6·7	0·30
GM 1,2,17 13,21	1	3·4	8	13·3	0·26
GM 1,2,3,17 23 5,13,21	2	6·9	5	8·3	1·0
Other GM	11	37·9	14	23·3	0·21
KM 1	3	10·3	6	10·0	0·61
KM 1,3	8	27·6	26	43·3	0·15
KM 3	11	62·1	28	46·7	0·17

*Fisher's exact test (6×2), $P = 0·023$.

allele, without KM 2, is extremely rare. Here, and in most other investigations, positivity for KM 1 includes both KM 1 and KM 1,2 alleles.

Statistical analysis

The significance of the association between GM and KM phenotypes and the prevalence of antibodies to XAGE-1b in NSCLC patients was analysed using Fisher's exact test and Pearson's χ^2 test. Subjects with very unusual GM phenotypes and those whose frequency was <4% were combined as 'other', in order not to have a test with too many degrees of freedom. Associations between the prevalence of antibodies and GM phenotypes and patient survival were assessed using a Cox regression model. Statistical significance was defined as $P < 0·05$. All reported *P*-values are two-sided.

Results

Table 1 presents the distribution of GM and KM phenotypes in XAGE-1b antibody-positive and -negative patients with lung adenocarcinoma. The majority of the subjects possessed typical Japanese GM phenotypes, which can be explained by postulating the segregation of four haplotypes present in this population: GM 1,17 21, GM 1,2,17 21, GM 1,17 13 and GM 1,3 23 5,13. The frequency of KM phenotypes observed was also typical of this population.

A global Fisher's exact test, considering all GM phenotypes, shows that there is a significant difference in the distribution of various phenotypes between the XAGE-1b antibody-positive and -negative groups of patients ($P = 0·023$). Further dissection of this association elucidates that the discrepancy in the distribution of GM 1,17 21 and GM 1,2,17 21 phenotypes contributed most to the total variation. None of the subjects with the GM 1,17 21 phenotype was positive for the XAGE-1b antibody ($P = 0·027$). The frequency of the GM 1,2,17 21 phenotype in the antibody-positive group was higher than in the antibody-negative group, but it did not reach statistical significance (52 *versus* 32%; Pearson's $\chi^2 = 3·3$; $P = 0·06$). However, in subjects who were also homozygous for the KM 3 allele, this GM phenotype contributed significantly to the antibody responsiveness: subjects with GM 1,2,17 21 and KM 3,3 phenotypes were almost four times [odds ratio (OR) = 3·8] as likely to be positive for the XAGE-1b antibody as the subjects who lacked both these phenotypes (Table 2). No other significant interactions were found. Also, none of the KM phenotypes alone was associated with anti-XAGE-1b antibody responsiveness.

Subsequent analyses were restricted to patients with XAGE-1b antigen-positive advanced (IIIB/IV) lung cancer. The clinical and demographic characteristics of these patients are presented in Table 3. The prevalence of anti-XAGE-1b antibodies was higher in patients with less advanced disease ($P = 0·030$). Other characteristics, except age, were not significantly different in the two groups of patients. A global Fisher's exact test, considering all GM phenotypes, shows that there is a significant difference in the distribution of various phenotypes between the XAGE-1b antibody-positive and -negative groups of patients with XAGE-1b antigen-positive advanced lung cancer ($P = 0·007$). There were only three patients with the GM 1,17 21 phenotype in this group, and all were negative for the XAGE-1b ($P = 0·055$, Table 4). The prevalence of GM 1,2,17 21 was significantly higher in the antibody-positive group than in those who lacked the XAGE-1b antibody (54 *versus* 18%; $P = 0·026$). The only allotype different between the responder and non-responder phenotypes is the γ1 determinant GM 2, prompting us to analyse the

Table 2. Distribution of combined GM 1,2,17 21 and KM 3,3 phenotypes in antibody-positive and -negative patients in relation to existence of XAGE-1b antibody ($n = 89$).

	XAGE-1b antibody			
Phenotype	Positive $n = 29$ (%)	Negative $n = 60$ (%)	OR (95% CI)	*P*-value
GM 1,2,17 21(+)/KM 3,3 (+)	11 (37·9)	9 (15·0)	3·8 (1·1–13·1)	0·04
GM 1,2,17 21(+)/KM 3,3 (−)	4 (13·8)	10 (16·7)	1·3 (0·3–5·3)	1·0
GM 1,2,17 21(−)/KM 3,3 (+)	7 (24·1)	19 (31·7)	1·2 (0·3–3·9)	1·0
GM 1,2,17 21(−)/KM 3,3 (−)	7 (24·1)	22 (36·7)	1·0	

CI: confidence interval; OR: odds ratio.

Table 3. Characteristics of the patients with XAGE-1b antigen-positive advanced lung cancer ($n = 43$).

	XAGE-1b antibody		
Characteristic	Positive ($n = 26$)	Negative ($n = 17$)	*P*- value
Sex, no. (%)			
Male/female	13/13 (50·0)	13/4 (76·5)	0·11
Age, years			
Mean	76·5 ± 7·6	69·8 ± 10·1	0·018
Smoking status, no. (%)			
Never smoked	10 (38·5)	5 (29·4)	0·75
ECOG performance status score, no. (%)			
0–1	21 (80·8)	11 (64·7)	0·30
Clinical stage, no. (%)			
IIIB/IV	10/16 (38·5)	1/16 (5·9)	0·030
Brain metastasis, no. (%)			
positive/negative	9/17 (34·6)	5/12 (29·4)	0·75
EGFR mutation, no. (%)			
Positive/negative	13/13 (50·0)	4/13 (23·5)	0·12

ECOG: Eastern Cooperative Oncology Group; EGFR: epidermal growth factor receptor.

interindividual variation in antibody responsiveness in relation to the GM 2 status of the subjects. No significant associations were found in the whole group ($P = 0·34$) as well as in the XAGE-1b antigen-positive group ($P = 0·18$). Thus, it appears that the influence of GM 2 on antibody responsiveness is manifested only when it is in a complex with γ1 determinants GM 1 and 17 and the γ3 determinant GM 21. Although a significant interactive effect of GM 1,2,17 21 with KM 3 homozygosity was observed (OR = 10; $P = 0·04$), this association should be viewed with caution, as the number of subjects in some categories was very small, resulting in a wide confidence interval (data not shown).

Of the 43 patients with antigen-positive tumours, 17 were negative for the XAGE-1b antibody; however, only one of these belonged to the clinical stage IIIB, the rest being clinical stage IV. Therefore, survival curves were plotted with the stage IV patients as well as with the combined group of patients with clinical stages IIIB and IV. As shown in Fig. 1, the anti-XAGE-1b antibody positivity was associated significantly with enhanced overall survival in both groups of patients, the antibody-positive subjects surviving more than twice as long as those who lacked this antibody (stage IIIB/IV: 33 *versus* 14 months, $P = 0·007$; stage IV: 33 *versus* 13 months, $P = 0·039$). Although not statistically significant (due possibly to the small sample size), stage IIIB/IV subjects with the GM 1,2,17 21 phenotype, which was associated with a higher prevalence of anti-XAGE-1b antibodies, survived longer than those expressing the GM 1,17 21 phenotype, which was associated with the lack of antibodies to XAGE-1b (31 *versus* 15 months, $P = 0·29$, Fig. 2).

Discussion

The results presented here show that the Ig GM 1,2,17 21 phenotype is associated with the presence of naturally occurring antibodies to the cancer-testis antigen XAGE-1b, while the GM 1,17 21 phenotype is associated with the lack of such antibodies. One mechanism underlying this association could involve GM allotypes being part of the recognition structures for the immunogenic epitopes of the XAGE-1b protein. Perhaps membrane-bound IgG (mIgG) molecules with GM 1,2,17 21 allotypes are more efficient in the uptake, processing and subsequent presentation of XAGE-1b epitopes to the collaborating T cells, resulting in strong humoral immunity, whereas the mIgG molecules with the GM 1,17 21 phenotype form a lower affinity receptor for the critical epitopes of this protein. Additionally – and contrary to the prevalent belief in immunology – these constant-region determinants could directly influence anti-XAGE-1b antibody specificity by causing conformational changes in the antigen-binding site in the Ig variable region. There is convincing evidence that the Ig constant region can influence antibody affinity and specificity [20]. Thus, constant regions expressing different GM allotypes, even when combined with identical variable region sequences, can generate new antibody molecules with new functions. They could also influence the expression of idiotypes involved in XAGE-1b immunity. The contribution of both variable and constant regions in the formation of idiotypic determinants has been clearly documented for the T15 system in mice, and such isotype-restricted idiotypes have been postulated to be involved in the regulation of class-specific antibody responses [21].

We also found that subjects with GM 1,2,17 21 and KM 3,3 phenotypes were significantly more likely to generate anti-XAGE-1b antibodies than subjects who lacked both these phenotypes. The simultaneous involvement of both GM and KM alleles on antibody responsiveness would

Table 4. Distribution of GM* and KM phenotypes in the XAGE-1b antibody-positive and -negative patients with XAGE-1b antigen-positive advanced lung adenocarcinoma ($n = 43$).

	XAGE-1b antibody				
Phenotype	Positive ($n = 26$)	(%)	Negative ($n = 17$)	(%)	*P*-value
GM 1,17 21	0	0	3	17·6	0·055
GM 1,2,17 21	14	53·8	3	17·6	0·026
GM 1,17 13,21	0	0	1	5·9	0·40
GM 1,2,17 13,21	1	3·8	4	23·5	0·07
GM 1,2,3,17 23 5,13,21	1	3·8	1	5·9	1·0
Other GM	10	38·5	5	29·4	0·75
KM 1	3	11·5	2	11·7	1·0
KM 1,3	8	30·8	7	41·2	0·53
KM 3	15	57·7	8	47·1	0·55

*Fisher's exact test (6 × 2), $P = 0·007$.