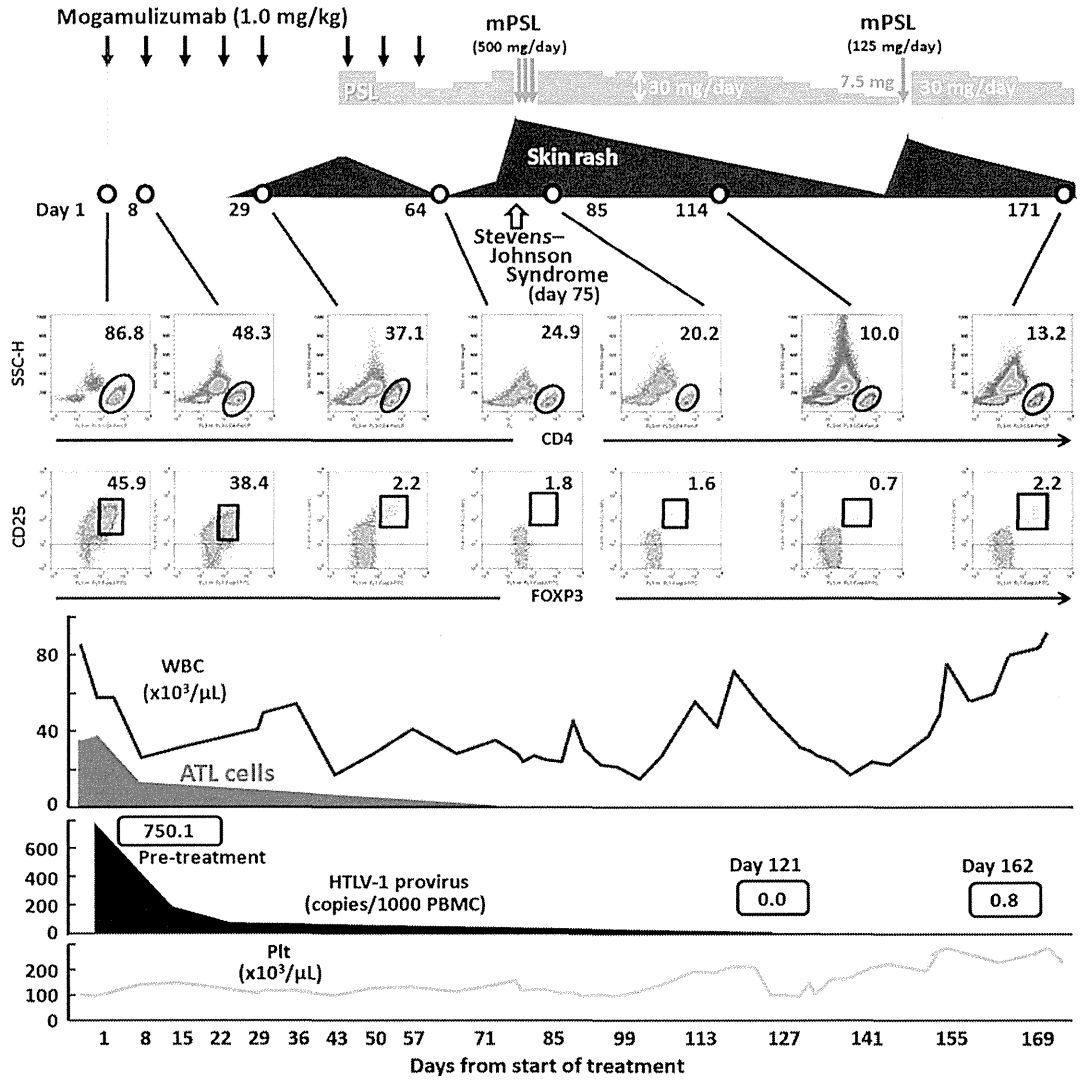

Fig. 1. Clinical course of an ATL patient receiving mogamulizumab monotherapy. ATL; adult T-cell leukemia/lymphoma; mPSL, methylprednisolone; Plt, platelet PSL; prednisolone; WBC, white blood cell.

load in asymptomatic carriers reported by other investigators is 18.0 copies/1000 cells.[6]

We also analyzed CD4, CD25 and FOXP3 expression by PBMC during and after antibody treatment (Fig. 1, middle panels). Before treatment, the majority of the patient's PBMC consisted of CD4-positive and CD25-positive ATL cells. Just before the 5th antibody infusion (day 29), around the time when her skin rash first appeared, the proportion of $CD25^{high}$-$FOXP3^{+}/CD4^{+}$ cells was markedly reduced, to 2.2%. This is low even compared to healthy individuals ($CD25^{high}$-$FOXP3^{+}/CD4^{+}$ cells, mean 3.3%, median 3.3%, range 2.6–4.4%) (Fig. 3). Around the time of SJS onset, the proportion of cells in the Treg subset was further reduced. The proportion of $CD25^{high}FOXP3^{+}/CD4^{+}$ cells at days 64, 85 and 114 was 1.8%, 1.6% and 0.7%, respectively. The striking reduction of the Treg subset persisted until 4 months after the last of the eight antibody infusions (day 171).

Discussion

Drugs often induce adverse cutaneous reactions of varying severity, ranging from simple uncomplicated eruptions to potentially fatal eruptions, such as SJS and TEN, within the spectrum of severe adverse reactions affecting skin and mucosa. Although many factors that might cause variability in the clinical course of such adverse reactions have been suggested, it remains unknown which factors are predominantly involved in these processes. The most prevalent severe drug eruptions are thought to be mediated by drug-reactive T-cells,[7] although we also need to be aware of the alternative view that severe drug eruptions are due to a dysregulated immune system. In this regard, an effect mediated by Treg cells is a likely candidate in severe drug eruptions. Indeed, it is reported that Treg cells can prevent experimentally-induced epidermal injury mimicking TEN in an animal model.[8] Furthermore, Takahashi *et al.* (2009) report that Treg cell function is profoundly impaired in patients with TEN.[9] Consistent with these reports, a marked reduction of the Treg subset was observed in the present case.

Mogamulizumab is the first therapeutic agent targeting CCR4, which is expressed on Treg cells,[10,11] to receive marketing approval anywhere in the world. The reduction of the Treg subset seen here was not specific to the present case, but is commonly observed in ATL patients receiving mogamulizumab. In fact, skin rashes were observed as a frequent non-hematologic adverse event (AE) (63%), mostly occurring

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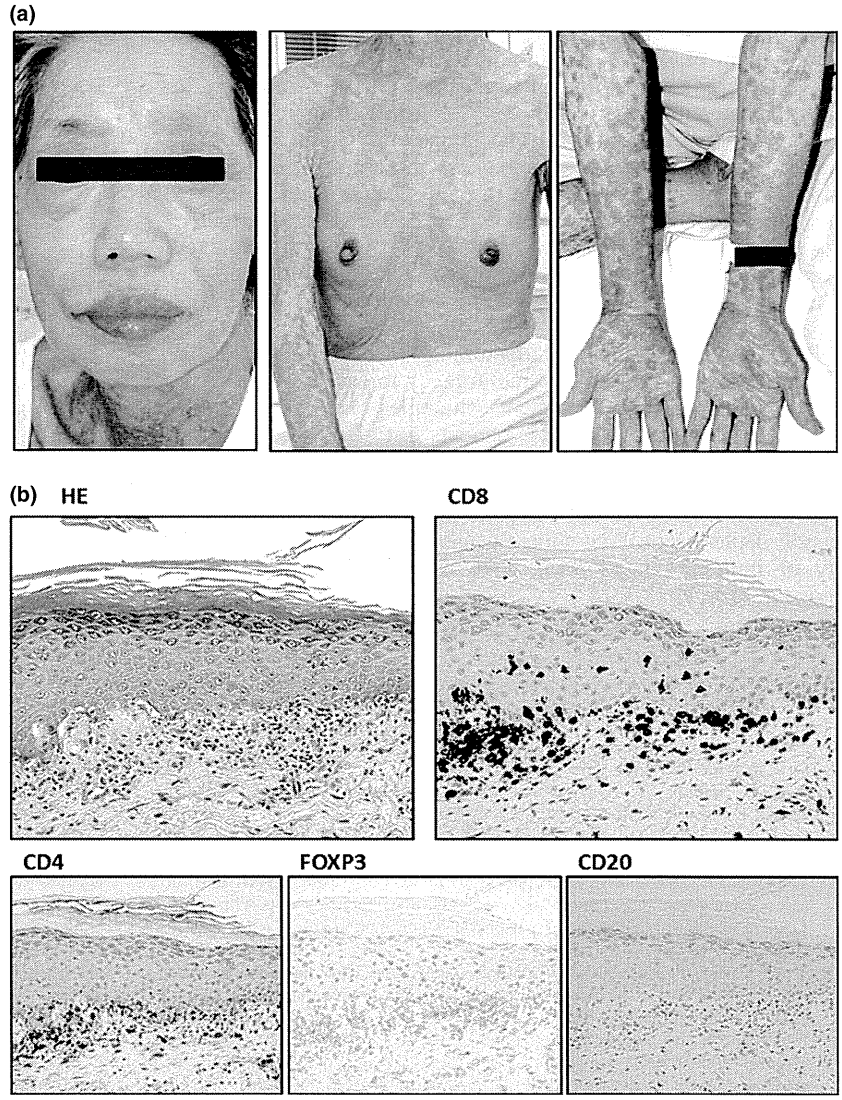
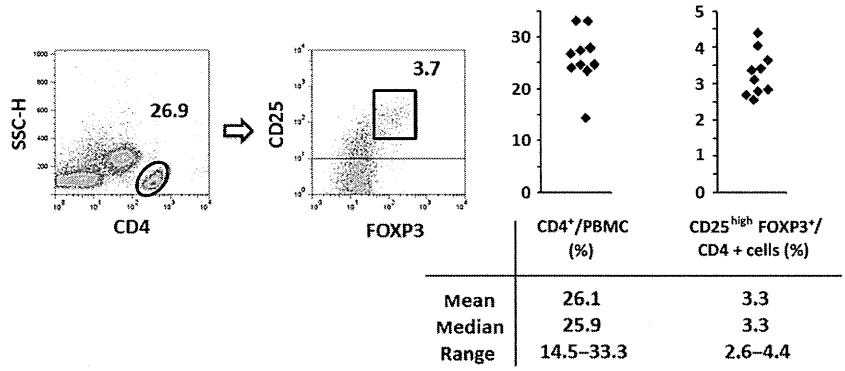


Fig. 2. (a) Macroscopic observations of the patient's skin on the day she was diagnosed with Stevens–Johnson Syndrome. (b) Corresponding skin biopsy showing liquefaction, degeneration and perivascular inflammation with dominant CD8-positive cells but almost no FOXP3-positive cells.



	$CD4^+$/PBMC (%)	$CD25^{high}$ $FOXP3^+$/ CD4 + cells (%)
Mean	26.1	3.3
Median	25.9	3.3
Range	14.5–33.3	2.6–4.4

Fig. 3. $CD4^+CD25^{high}FOXP3^+$ regulatory T cells in PBMC from healthy volunteers (n = 10).

after the fourth or subsequent infusions in the phase II study.[4] The present case was one of these patients. It has been reported that alterations in $CD4^+CD25^+FOXP3^+$ Treg cell frequencies and/or function may contribute to various types of autoimmune diseases.[12] Because the CCR4 molecule aids lymphocyte skin-specific homing,[13] it is not unexpected that skin rashes, which could be an immune-related AE, will be frequently observed in ATL patients receiving mogamulizumab. Because it is an urgent issue to identify which factors determine the severity of immune-related skin disorders associated with mogamulizumab treatment, further investigation on this matter are clearly warranted.

However, reduction of Treg cells is a promising strategy for boosting antitumor immunity in cancer patients, because these cells are increased in the tumor microenvironment and may play an important role in tumor escape from host immunity in several different types of cancer.[14,15] Thus, reduction of Treg cells by mogamulizumab in cancer patients would have both potential benefits leading to enhanced antitumor immunity, but also pose risks of autoimmune disease. The skin-related SAE, including SJS/TEN, are representative of the latter. Currently, several clinical trials of mogamulizumab are being conducted worldwide, not only for ATL, but also other types of lymphoma. In addition, we are currently conducting a clinical trial of mogamulizumab for CCR4-negative solid cancers (UMIN000010050), specifically aiming to deplete Treg cells. Therefore, it is a matter of some urgency to establish the safest and most effective treatment strategies for using mogamulizumab not only in ATL patients but also other types of cancer, to maximize benefit and minimize risk.

In summary, the present case should contribute not only to our understanding of human pathology resulting from therapeutic depletion of Treg cells, but also alert us to the possibility of immune-related SAE, such as SJS/TEN, when using mogamulizumab.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Which patients respond best to hepatitis B vaccination after a hepatitis B virus-related liver transplantation?

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Abstract
Background A combination of hepatitis B immunoglobulin and nucleos(t)ide analogues is the current standard of care for controlling hepatitis B recurrence after orthotopic liver transplantation (OLT). However, frequent immunoglobulin treatment is expensive and inconvenient. This study investigated the efficacy of hepatitis B virus (HBV) vaccination in preventing the recurrence of hepatitis B after living donor OLT.
Methods Twenty-seven patients who had undergone living donor OLT participated in the study; five had acute HBV infected liver failure (ALF-OLT) and 22 had HBV related liver cirrhosis (LC-OLT). Hepatitis B surface antigen (HBsAg)-containing vaccine was administered to them for at least 1 year after transplantation and continued once monthly for up to 36 months post-OLT. Patients who had anti-HBs antibody titers above 100 mIU/mL for a minimum of 6 months without immunoglobulin administration were defined as good responders; the others were defined as poor responders. Interferon-γ enzyme-linked immunospot assays against HBs and HBc antigens were used to assay cellular immune responses.
Results All five of the ALF-OLT patients had good responses after a median of four (range 2.5–5) vaccinations. Nine of the 22 LC-OLT patients had good responses after a median of 19 (range 11.5–30) vaccinations. Among the LC-OLT group, those with livers donated by relatively higher-aged, marital and high-titer anti-HBs antibody donors were good responders. LC-OLT patients classed as good responders showed interferon-γ responses comparable to those of the ALF-OLT patients.
Conclusions The ALF-OLT and LC-OLT patients who received livers from relatively higher-aged, marital, high-titer anti-HBs antibody donors were the best candidates for HBV vaccine administration. Boosting donors before transplantation may facilitate later vaccine response of the recipients.

Keywords Vaccination · Living donor liver transplantation · Hepatitis B immunoglobulin · Marital donor · Immune response

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Introduction

Prior to the introduction of effective post-transplantation antiviral prophylaxis, liver transplantation for hepatitis B virus (HBV)-related disease was usually followed by immediate HBV reinfection of the allograft, resulting in a fatal hepatitis B recurrence [1–3]. Recent studies have found that treatment with a combination of hepatitis B

immunoglobulin (HBIg) and nucleos(t)ide analogues decreases the risk of hepatitis B recurrence, and achieves a higher rate of graft survival [4–8]. However, long-term administration of HBIg is associated with several unresolved issues, including limited availability and extremely high cost, so several protocols for treatment with low-dose HBIg in combination with nucleos(t)ide analogue have been reported [9–12]. Previously, we reported that treatment with high-dose HBIg in the early period post-transplantation followed by low-dose HBIg with nucleos(t)ide analogues offers reliable, cost-effective control of hepatitis B recurrence [13]. However, even with such a simplified protocol, patients would still need to receive a drip infusion or intramuscular injection of hundreds to thousands of units of HBIg every 2–3 months.

Active immunization of post-orthotopic liver transplantation (OLT) recipients with HBV vaccine is a recently emerging approach. However, most studies report low response rates, even with double concentration of vaccines or prolonged vaccination regimens [14, 15]. Patients who had not been HBV carriers [e.g., acute liver failure (ALF) patients following sexual transmission of HBV as an adult; or non-chronic HBV carrier patients who received hepatitis B core antibody (HBcAb)-positive livers] are accepted as good candidates for vaccine administration [15, 16]. Vaccination in patients who have been HBV carriers or liver cirrhosis (LC) patients typically yields disappointing results [14, 15]. Understanding how different cohorts respond to HBV vaccination is critical to the design of safe, cost-saving, and custom-designed prophylaxis protocols.

It remains unclear to what extent cellular immune responses may contribute to protection from HBV reinfection. Since non-carrier patients respond well to the HBV vaccination, immune tolerance is expected to play a large role in this process. Yet only a few reports have mentioned T cell immune reaction after HBV-related OLT [14].

In this report, we assessed a monthly, long-term vaccination protocol starting 1 year after OLT, to investigate those characteristics that could discriminate between the vaccine-responsive and non-responsive patients. In addition to anti-hepatitis B surface (anti-HBs) antibody titer due to a humoral immune response, CD4 T cell immune responses to hepatitis B surface antigen (HBsAg) were used to assess the cellular immune response to vaccination in immunocompetent patients.

Methods

Patients

From October 1996 to June 2011, OLT was performed in 264 adults at Okayama University Hospital. Of these, ten patients had ALF due to acute HBV infection. Thirty-seven patients had end-stage LC due to chronic life-long HBV infection. Five-year survival rates were 88 and 87 % for HBV-related ALF patients and for HBV-related LC patients, respectively.

The HBV vaccine was administered to five ALF patients (ALF-OLT) and 22 LC patients (LC-OLT). The general characteristics of the patients included in this study are summarized in Table 1. All of them received living donor liver transplantation (LDLT). The numerical data are expressed as median and interquartile range values, and categorical data are presented as positive counts or percentages in all tables.

For analysis of the HBV-specific cellular immune response (Table 2), the study enrolled all five ALF-OLT patients, along with 15 of the 22 LC-OLT patients. Additionally, 11 healthy volunteers who had received the HBV vaccine and developed a successful anti-HBs antibody response (termed 'Healthy vaccine'), ten patients with chronic hepatitis B (termed 'Chronic hepatitis'), and five patients who recovered from acute hepatitis B (termed 'Self-limited') were enrolled as controls. The five patients who recovered from acute hepatitis B had a history of acute hepatitis B diagnosed with high-titer IgM-HBc antibody response, and presented as HBsAg negative, anti-HBs antibody positive, anti-HBc antibody positive at the time of

Table 1 Patient characteristics

N	ALF 5	LC 22
Recipient related factors		
Age at OLT	29 (27–46)	53 (47–56)
Age at start of vaccine	36 (30–51)	56 (49–59)
Sex (M)	1 (20 %)	19 (86 %)
HBsAg at OLT	0.7 (0–1)	2000 (100–2000)
HBV DNA at OLT (≥3.7)	0 (0 %)	8 (36 %)
MELD at OLT	21 [19–21]	15 [9–18]
HCC at OLT (+)	0 (0 %)	15 (68 %)
Donor related factors		
Age at OLT	32 (27–44)	46 (31–49)
Sex (M)	4 (80 %)	9 (40 %)
ABO (identical)	4 (80 %)	12 (54 %)
Blood relation (no)	0 (0 %)	8 (36 %)
Anti-HBs antibody (>100)	1 (20 %)	9 (40 %)
Anti-HBc antibody (+)	1 (20 %)	11 (50 %)
Anti-HBc(+)/anti-HBs(+)	1 (20 %)	10 (45 %)
Anti-HBc(+)/anti-HBs(−)	0 (0 %)	1 (4 %)
Anti-HBc(−)/anti-HBs(+)	0 (0 %)	0 (0 %)

ALF acute liver failure, *LC* liver cirrhosis, *OLT* orthotopic liver transplantation, *MELD* Model for End-stage Liver Disease, *HCC* hepatocellular carcinoma

Table 2 Characteristics of the cases for HBV antigen-specific T cell response

N	Healthy vaccine	Chronic hepatitis	Self-limited	ALF-OLT	LC-OLT-good	LC-OLT-poor
	11	10	5	4	8	7
Age	29 (28–31)	53 (42.5–61)	67 (58.5–77)	41.5 (37.2–47.2)	60 (53–62)	55 (40–58)
Sex [M (%)]	10 (91)	7 (70)	2 (40)	0 (0)	8 (100)	7 (100)
HBs Ag (+)	0	10 [titer 2000 (1893–2000)]	0	0	0	0
HBs Ab (IU/l) (>100/≤100)	8/3	0/10	2/3	2/2	4/4	1/6

LC-OLT-poor patients received HBIG within 3 months

Age and HBsAg were shown as median (interquartile range)

ALF-OLT acute liver failure patients who received OLT, *LC-OLT-good* liver cirhosis patients who received OLT and had a good vaccine response, *LC-OLT-poor* liver cirrhosis patients who received OLT and had a poor vaccine response

the study. The chronic hepatitis B patients were followed for several years at our hospital and all were HBsAg positive with a median HBV-DNA titer of 2.5 (interquartile range 2.1–4.2) logcopies/mL. The healthy volunteers had no HBsAg and anti-HBc antibodies, and the median anti-HBs antibody level was 240 (interquartile range 100–797) mIU/mL.

Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the approval by the Ethics Committee at the Okayama University Hospital.

Antiviral prophylaxis

Our HBV prophylaxis protocol was as follows. We administered HBIg at 200 IU/kg intraoperatively. Recipients were administered another 2000 IU/week HBIg for an additional 1 week post-operatively. HBIg (2000 IU) was administered thereafter only when anti-HBs antibody titers fell below 100 mIU/mL. After 6 months, HBIg was administered only to maintain anti-HBs antibody titers at >10 mIU/mL. We measured levels of HBsAg and anti-HBs antibody and/or HBV-DNA every month for 6 months after LDLT, and every 2–3 months thereafter. Three of the ALF-OLT patients were anti-HBs antibody positive at the time of OLT, these patients were not administered nucleos(t)ide analogues. The remaining two ALF-OLT patients, and all of the LC patients were given nucleos(t)ide analogues. The two ALF-OLT patients were given lamivudine (LAM), and of the 22 LC-OLT patients, 14 received LAM, six were given LAM + adefovir dipivoxyl (ADV), and two received entecavir (ETV). Administration of nucleos(t)ide analogues was started a minimum of 1 month pre-operatively, when possible.

Post-OLT re-activation of HBV was defined as continuous positivity for serum HBsAg and/or serum HBV-DNA.

HBV vaccine protocol

HBV vaccine administration was initiated at least 1 year after OLT, and when patients showed no active infection or rejection episode in the preceding month. The vaccine consisted of recombinant purified HBsAg (Bimmugen; Kaketsuken, Kumamoto, Japan). Ten micrograms were administered every 1–2 months. Based on the effect of the vaccine, patients were classified as "good responders; LC-OLT good" or "poor responders; LC-OLT poor". Patients who showed anti-HBs antibody titers above 100 mIU/mL without HBIg for a minimum of 6 months were defined as good responders, since all of these patients did not need HBIg administration for an additional 2 years (median) of follow-up. All other patients were defined as poor responders. Patients who showed a good response within 36 months were given additional vaccinations when their anti-HBs antibody titer decreased, whereas vaccination was stopped in patients who showed no good response after 36 months.

Immune suppression

Patients were treated using a standard immunosuppressive regimen (tacrolimus or cyclosporine A with steroids and/or mycophenolate mofetil). One patient was free from calcineurin inhibitors at the time of vaccine administration.

Routine laboratory tests and serum HBV-DNA assay

Hepatitis B surface antigen, anti-HBs antibody, hepatitis Be antigen (HBeAg), and anti-HBe antibody (HBeAb) levels were measured routinely using a commercially available chemiluminescent enzyme immunoassay system (Lumipulse System; Fujirebio, Tokyo, Japan). HBV-DNA levels were measured using a transcription-mediated amplification assay (TMA) (SRL, Tokyo, Japan), a polymerase chain reaction (PCR) assay (Amplicor HBV

Monitor assay; Roche Diagnostics, Tokyo, Japan), or a real-time PCR assay (COBAS TaqMan HBV Test; Roche Diagnostics).

HBV recombinant proteins for cellular immune response analysis

Hepatitis B virus recombinant protein HBsAg was purchased from Advanced ImmunoChemical, Inc. (Long Beach, CA). Recombinant protein hepatitis B core antigen (HBcAg) was purchased from the Institute of Immunology (Tokyo, Japan). These proteins were used as stimulating antigens at 1 μg/mL for the enzyme-linked immunospot (ELISPOT) assay.

CD14-positive monocyte isolation and myeloid DC generation

Mononuclear cells were separated from peripheral blood by centrifugation on the Ficoll-Hypaque density gradient (Amersham Pharmacia, Uppsala, Sweden), as previously described. CD14-positive monocytes were purified using microbeads (Miltenyi Biotec, Auburn, CA) in accordance with the protocols of the manufacturer. Subsequently, CD4-positive T cells (T4) were positively sorted in the same way. T4 cells were frozen immediately. CD14-positive cells were cultured at 1×10^6/mL in RPMI containing 5 % heat-inactivated human AB serum (ICN Biomedicals; Aurora, OH) supplemented with 100 ng/mL of granulocyte macrophage colony-stimulating factor (kindly provided by Kirin Pharma, Tokyo, Japan) and 50 ng/mL of interleukin-4 (kindly provided by Ono Pharmaceuticals, Osaka, Japan) at 37 °C in 5 % CO_2 for 5 days. Cells were confirmed to be CD11c-positive myeloid immature dendritic cells (DC).

Interferon-γ (IFNγ) ELISPOT assay with myeloid DC and CD4-positive T-cells

The immature DC cultures were exposed to recombinant HBsAg and HBcAg (1 μg/mL each) for 1 day. To mature the DCs, 1 ng/mL of lipopolysaccharide (LPS) (Sigma, St. Louis, MO) was added to the culture 1 day after HBV protein addition. On the same day, mouse anti-human interferon-γ antibody (MABTECH, Sweden) was diluted to 5 μg/mL with ELISPOT buffer (0.159 % Na_2CO_3, 0.293 % $NaHCO_3$) and coated overnight at 4 °C onto 96-well filtration plates (Millipore, Billerica, MA) at 100 μL per well. The coated plate was washed with phosphate-buffered saline (PBS) and blocked with 10 % fetal calf serum in RPMI1640 medium for 1–2 h. Myeloid DCs were counted and seeded at 5×10^3/well. Cryopreserved T4 cells were thawed, counted, and seeded at 2×10^5/well. On the next day, the plate was washed six times with PBS. Wells were coated with rabbit anti-interferon-γ serum (diluted to 1/800 in PBS), and the plate was incubated at 37 °C for 2 h. The plate was washed six times with PBS and coated with goat anti-rabbit immunoglobulin G-alkaline phosphatase (IgG-AP; Southern Biotech, Birmingham, AL) diluted to 1/2000 with PBS. After a 1 h incubation at 37 °C, the plate was washed six times with water and spots were developed using 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitroblue tetrazolium chloride (BCIP/NBT) as a substrate. Spot development was stopped after 10 min by washing with distilled water. The spots were viewed and counted under a microscope.

Statistical analysis

Statistical comparisons were performed using JMP version 9 (SAS Institute, Cary, NC, USA). The Wilcoxon rank-sum test was used to compare the continuous data and the Chi-square test was used to compare categorical data. For multivariate analysis, logistic regression analysis was used. The Steel–Dwass test was used for multiple group analysis. A p value of <0.05 was considered significant.

Results

The effects of HBV vaccination

None of the patients in the ALF-OLT group showed reactivation of the virus. One patient of the LC-OLT group showed transient positive responses for HBsAg and HBV DNA, however, these became negative again with frequent HBIg administration. At the final observation point, no patients showed HBsAg or HBV DNA-positive response. All five ALF-OLT patients had good responses to vaccination (Table 3). A median of four (range 2.5–5) vaccinations were sufficient to induce a good response. In contrast, LC-OLT patients were less responsive, with only nine of 22 displaying a good response. Additionally, these nine good responders required a median of 19 (range 11.5–30) vaccinations before these patients could be weaned from HBIg administration (Fig. 1).

Table 3 Results of HBV vaccination

N	ALF 5	LC 22
Response to vaccination (good/poor responders)	5/0	9/13
Number of vaccinations require before ceasing HBIg treatment	4 (2.5–5)	19 (11.5–30)

HBIg Hepatitis B immunoglobulin

Vaccine safety

None of the patients showed any adverse reactions as judged by their general condition, or by laboratory examination. One patient reported itchiness after injection of the eighth vaccination dose, although the symptom subsequently stopped.

The characteristics of vaccine responsiveness in LC-OLT patients

To determine the characteristics for defining a good response in LC-OLT patients, clinical data from recipients and donors were investigated (Table 4). The background data of the recipients, including HBV-DNA levels, HBeAg positive reactions, HBsAg levels at the time of OLT, and the anti-HBs antibody titer at the time of the initial vaccination did not differ between the good and poor responder groups (Table 5). However, the donor-related factors did differ. Notably, the good responders' donors were relatively high in age ($p = 0.019$) and not blood relatives of the recipients ($p < 0.001$). These donors (to good responders) showed high anti-HBs antibody titers at the time of OLT ($p = 0.038$). Since all of the patients in this study received LDLT, non-blood-related donors all corresponded to spouses of the OLT recipients. Multivariate logistic regression analysis was carried out with the following variables: donor age at OLT $\geq$47, non-blood-related donor, donor anti-HBs antibody titer >100 mIU/mL (Table 6). A status of non-blood-related donor was identified as a significant independent predictor of a good response to vaccination. Since the donor anti-HBs antibody was one of the factors associated with a good response, we asked whether the donors had received vaccination, and found that none of them had ever received an HBV vaccine. As shown in Table 4, none of the donors showed the anti-HBc antibody-negative, anti-HBs antibody-positive condition which indicates vaccine-induced seropositivity to the HBs antigen.

HBV antigen-specific immune responses

To determine the effectiveness of vaccine-induced cellular immune responses in post-OLT patients, we used the IFN-γ ELISPOT assay. First of all, we analyzed the clinical characteristics of those patients showing strong HBsAg-specific T cell immune responses when compared with those of non-transplanted patients, and vaccine-induced anti-HBs antibody-positive, healthy volunteers (Fig. 2). The patients with stronger HBsAg-specific CD4 T cell IFN-γ responses (equal or more than the median; 7 spots) showed lower levels of HBV DNA, lower HBsAg, higher anti-HBs antibody titer, and higher HBcAg-specific immune responses. The HBsAg and HBcAg-specific CD4 T cell immune response under different clinical conditions is shown (Fig. 3). Volunteer controls who were positive for anti-HBs antibodies (as a result of previous vaccine administration) showed numerous HBsAg-specific IFNγ spots. Spot numbers were reduced in control chronic hepatitis B patients, but remained high (against both HBsAg and HBcAg) in acute resolved hepatitis B patients. The ALF-OLT and LC-OLT good responders had relatively higher HBsAg-specific T-cell immune responses than LC-OLT poor responders. The LC-OLT patients with successful vaccine-induced humoral immune responses also showed higher cellular immune responses than control chronic hepatitis B patients. The LC-OLT patients with poor vaccine responses also had low cellular responses, similar to those seen in chronic hepatitis B patients.

Discussion

In this study we found that HBV vaccination was effective in OLT patients whose donors were relatively high in age, marital (non-blood-related), with high-titer anti-HBs antibodies. The multivariate analysis revealed that a marital (non-blood-related) donor was the only factor that associated strongly with a good response to vaccine. Among these OLT recipients, a good response to vaccination included effective responses in both the humoral and cellular arms of the immune system.

Controlling HBV reactivation after OLT is critical. In the absence of prophylaxis, hepatitis B recurs very frequently and results in early graft failure. The prophylaxis protocols have progressed from HBIg immunoprophylaxis in the early 1990s, to lamivudine in the late 1990s, to the more recent application of HBIg combined with nucleos(t)ide analogues. In 1991, Muller et al. [17] reported the first use of long-term HBIg immunoprophylaxis, reducing the HBV recurrence rate to 25 % after 6 months of OLT and 18 % after 12 months. A multicenter study revealed that the three-year risk of HBV recurrence was 75 ± 6 % without HBIg, 74 ± 5 % with short-term (2-month) HBIg, and 36 ± 4 % with long-term (>6-month) HBIg treatment [18]. Patients who were positive for HBeAg or HBV-DNA displayed the greatest risk of recurrence (83 %); patients with acute fulminant liver failure showed the lowest risk (16 %).

In 1996, Grellier et al. [19] reported a trial of LAM as a prophylactic treatment, achieving 18 % recurrence of HBV at 6 months after OLT. However, the long-term recurrence rate at 3 years after OLT progressed to 41 %, indicating that LAM monotherapy is not recommendable for post-transplantation prophylaxis.

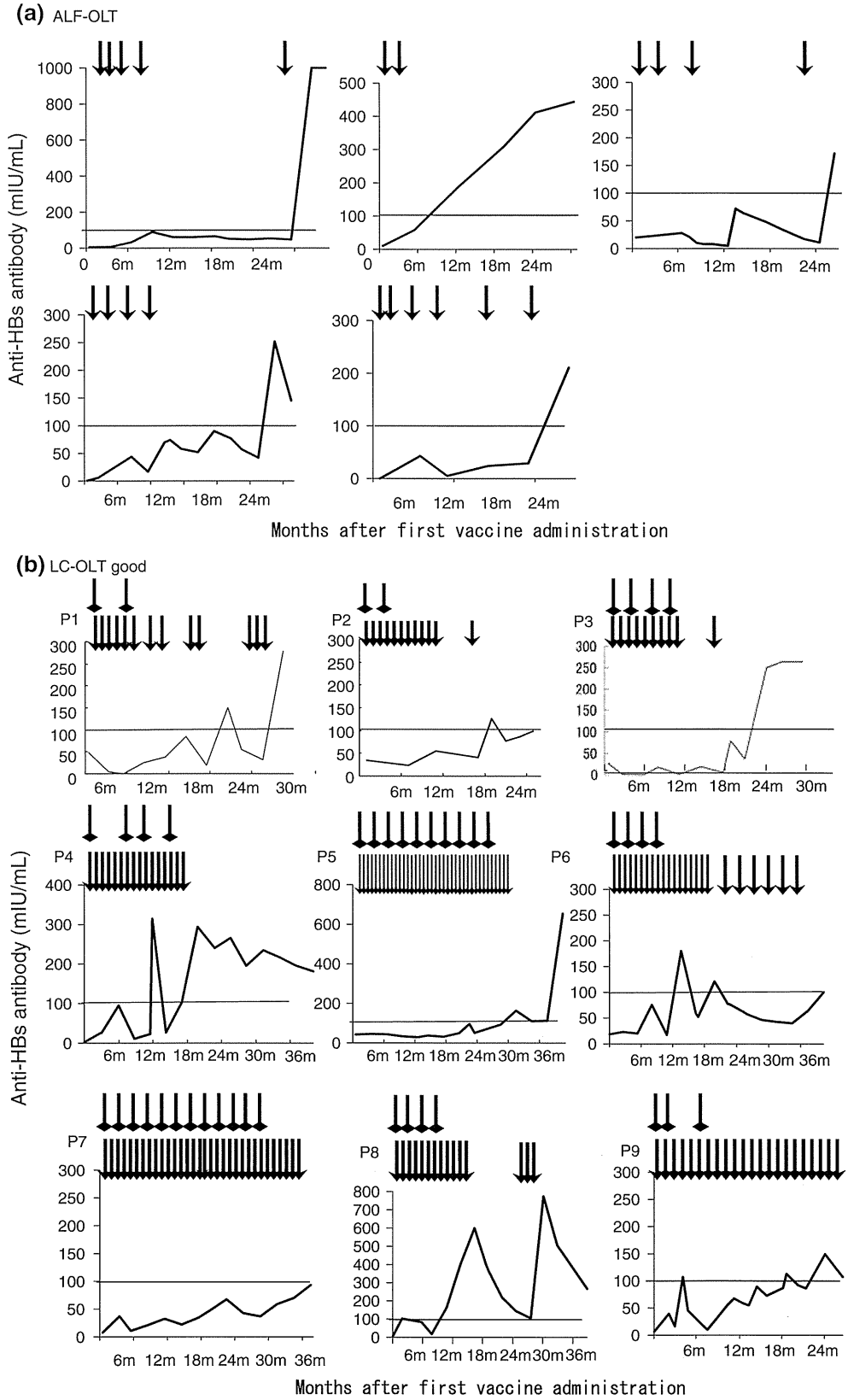

Fig. 1 Individual patients' timecourse of anti-HBs antibody titer after vaccine administration. The timecourse of the anti-HBs antibody titer after the first vaccine administration is shown. The *arrowhead* indicates a vaccine administration point, and the *square head* indicates an HBIg administration point. **a** Patients who received orthotopic liver transplantation (OLT) due to hepatitis B-related acute liver failure (ALF-OLT). All patients had a good response to vaccination. **b** Patients who received OLT due to liver cirrhosis with a good response to vaccination (LC-OLT good). **c** LC-OLT patients with a poor response to vaccination (LC-OLT poor)

Although monotherapy with HBIg or LAM resulted in a high rate of recurrence, a combination of these agents has been administered with reasonable success. In 1998, Markowitz et al. [20] reported no recurrences after 1 year of combination therapy. Since HBIg is very expensive, several reports have described modified combination

Fig. 1 continued

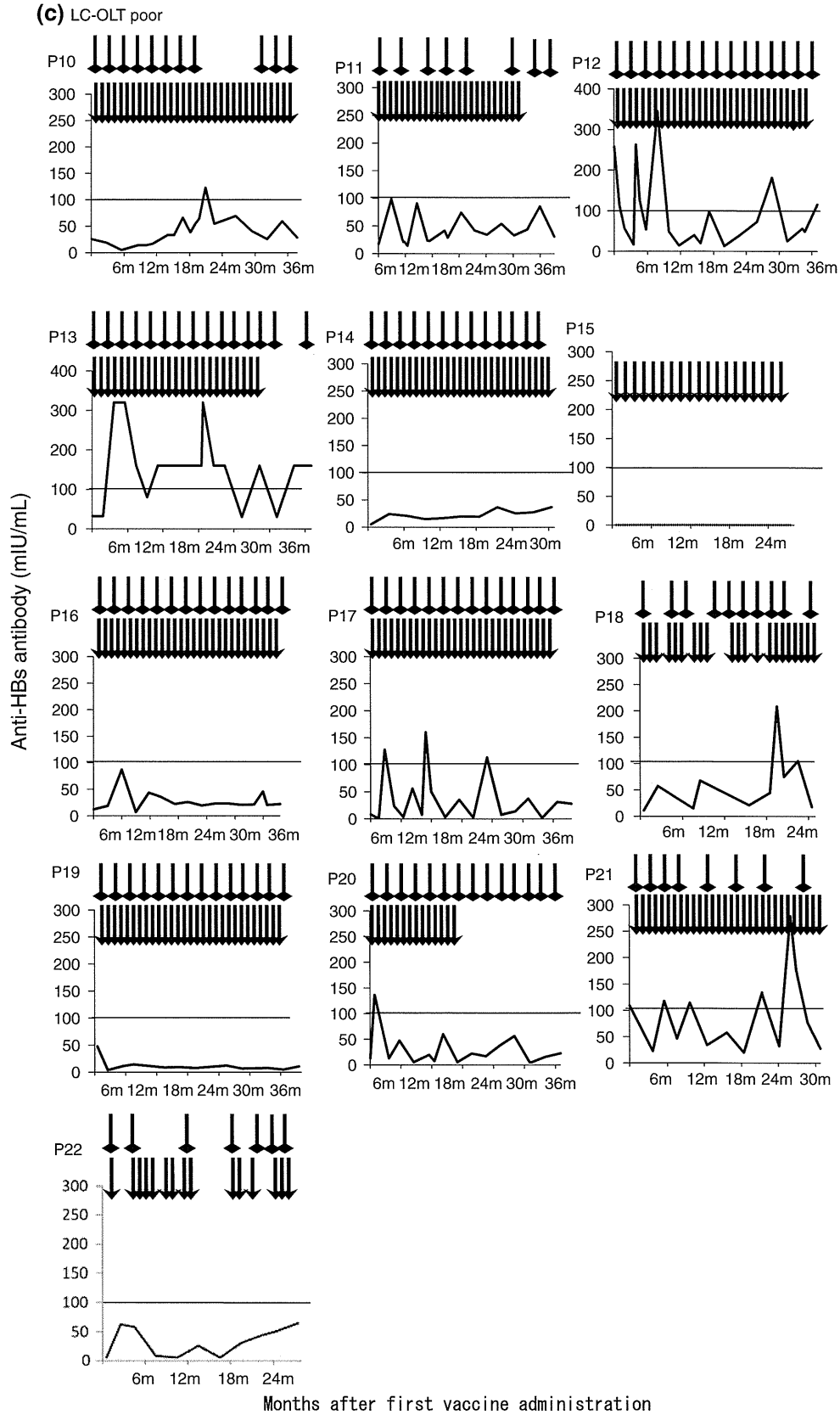

Table 4 LC patient characteristics

Characteristics of recipients													Characteristics of donors						
Patient's number	Response to vaccine	Age (year) at OLT	Sex	HBsAg (mIU/mL) at OLT	HBsAb at OLT	HBeAg/HBeAb at OLT	HBV DNA (logcopies/mL) at OLT	MELD at OLT	HCC at OLT	Time of vaccination (months post-OLT)	HBsAb (mIU/mL) at vaccine	NA at vaccine	Age at OLT	Sex	Blood relation	ABO compatibility	HBcAb	HBsAb	HBsAb (mIU/mL)
1	Good	56	M	100	–	–/+	<3.7	17	+	51	49	LAM	52	F	–	Compatible	–	–	<0.1
2	Good	48	M	>2000	–	+/+	3.5	20	+	24	23	LAM	46	F	–	Compatible	+	+	134
3	Good	44	M	100	–	+/–	<3.7	12	–	55	1	LAM	48	F	+	Identical	+	+	189
4	Good	50	M	>2000	–	+/–	3.4	9	+	42	25	LAM + ADV	48	F	–	Compatible	+	+	627
5	Good	54	M	>2000	–	–/+	3.8	15	–	40	43	LAM + ADV	48	F	–	Compatible	–	–	<0.1
6	Good	57	M	>2000	–	–/+	2.7	15	+	45	18	LAM	53	F	–	Identical	–	–	<0.1
7	Good	48	M	642	–	+/–	4.8	17	–	29	7	LAM	44	F	–	Compatible	+	+	179
8	Good	47	F	>2000	–	+/–	4.5	12	–	19	6	LAM	50	M	–	Compatible	+	+	1000
9	Good	55	M	>2000	–	+/–	6.1	21	+	49	6	LAM + ADV	48	M	+	Identical	+	+	133
10	Poor	52	M	>2000	–	+/–	5.3	8	+	25	4	LAM	21	M	+	Compatible	+	+	1000
11	Poor	62	M	>2000	–	–/+	<2.6	8	+	13	17	LAM + ADV	36	M	+	Identical	–	–	<0.1
12	Poor	39	M	>2000	–	+/–	<2.6	7	–	30	169	LAM	35	F	+	Identical	–	–	<0.1
13	Poor	49	M	100	–	–/+	4.0	21	+	107	32	LAM	22	F	+	Identical	–	–	<0.1
14	Poor	26	M	100	–	+/–	5.5	20	+	75	30	LAM	53	M	+	Identical	+	+	397
15	Poor	54	F	100	–	+/–	4.6	22	+	55	1	LAM	28	M	+	Identical	–	–	<0.1
16	Poor	50	M	160	–	–/+	2.7	18	+	38	6	LAM	25	M	+	Compatible	+	–	<0.1
17	Poor	44	M	>2000	–	–/+	<2.6	15	–	32	14	LAM	47	F	+	Compatible	–	–	<0.1
18	Poor	55	F	>2000	–	+/–	2.8	10	+	19	10	LAM + ADV	51	F	+	Identical	+	+	44
19	Poor	54	M	>2000	–	–/–	<2.6	8	+	18	47	ETV	49	F	–	Compatible	+	+	1000
20	Poor	63	M	1740	–	–/+	<2.6	12	–	17	42	LAM + ADV	36	M	+	Identical	–	–	0.2
21	Poor	58	M	35	–	–/+	<2.6	16	–	16	19	ETV	33	F	+	Identical	–	–	0.3
22	Poor	61	M	>2000	–	–/+	2.9	15	+	68	5	LAM	26	M	+	Identical	–	–	<0.1

NA nucleos(t)ide analogue, *LAM* lamivudine, *ADV* adefovir dipivoxyl, *ETV* entecavir, *HBcAb* anti-HBc antibody, *HBsAb* anti-HBs antibody

Table 5 Patient characteristics according to vaccine responsiveness in LC (univariate analysis)

N	Good responders 9	Poor responders 13	*p* value
Recipient related factors			
Age at OLT	50 (47–55)	54 (46–59)	0.546
Sex (male)	8 (88 %)	11 (84 %)	0.774
Time of vaccination (months after OLT)	42 (26–50)	30 (17–61)	0.442
HBsAg at OLT (≥1500 IU/l)	6 (66 %)	8 (61 %)	0.805
HBeAg positive at OLT	6 (66 %)	5 (38 %)	0.190
HBV DNA at OLT (≥3.7 logcopies/mL)	4 (44 %)	4 (30 %)	0.513
MELD at OLT	15 [12–18]	15 [8–19]	0.480
Child-Pugh score at OLT	10 [8–10]	9 [6–11]	0.845
HCC at OLT (+)	6 (66 %)	9 (69 %)	0.899
Anti-HBs antibody titer at the start of vaccination	18.6 (6.4–34.6)	17.4 (5.9–37.1)	0.920
Nucleos(t)ide analogue (LAM/LAM + ADV/ETV)	6/3/0	8/3/2	0.312
TacrolimusCyclosporinA	6/3	11/1#	0.148
Tacrolimus level (ng/mL)	4.7 (3.0–5.6)	3.8 (2.9–5.8)	0.744
Donor-related factors			
Age at OLT	48 (47–51)	33 (25–48)	0.019*
Sex (M)	2 (22 %)	7 (53 %)	0.138
ABO (identical)	3 (33 %)	9 (69 %)	0.093
Blood relation (no)	7 (77 %)	1 (7 %)	<0.001*
Anti-HBs antibody titer (>100)	6 (66 %)	3 (23 %)	0.038*
Anti-HBc antibody (+)	6 (66 %)	5 (38 %)	0.190
Anti-HBc(+)/anti-HBs(+)	6 (66 %)	4 (30 %)	0.093
Anti-HBc(+)/anti-HBs(−)	0 (0 %)	1 (7 %)	0.297
Anti-HBc(−)/anti-HBs(+)	0 (0 %)	0 (0 %)	–

MELD Model for End-stage Liver Disease, *HCC* hepatocellular carcinoma, *LAM* lamivudine, *ADV* adefovir dipivoxyl, *ETV* entecavir

One patient received no calcineurin inhibitor

Table 6 Multiple logistic analysis of factors associated with good responses to HBV vaccine in LC

N	Odds ratio	95 % CI	*p* value
Age at OLT (>47)	5.4	0.300–214.000	0.244
Blood relation (no)	29.4	2.551–984.110	0.005*
Anti-HBs antibody titer (>100)	5.0	0.343–149.947	0.233

Note: Variables significant at $p < 0.05$

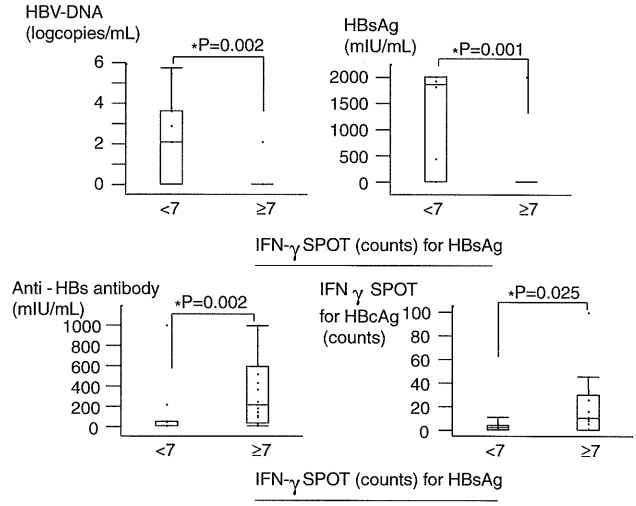

Fig. 2 The clinical characteristics of the non-OLT patients with strong HBsAg-specific T cell interferon-γ response. The clinical characteristics of the non-OLT patients showing strong HBsAg-specific T cell immune responses by enzyme-linked immunospot (ELISPOT) assay are shown. Those patients with stronger HBsAg-specific CD4 T cell IFN-γ response (equal or more than the median; 7 spots) showed lower HBV DNA, lower HBsAg, higher anti-HBs antibody titer, and higher HBcAg-specific immune responses

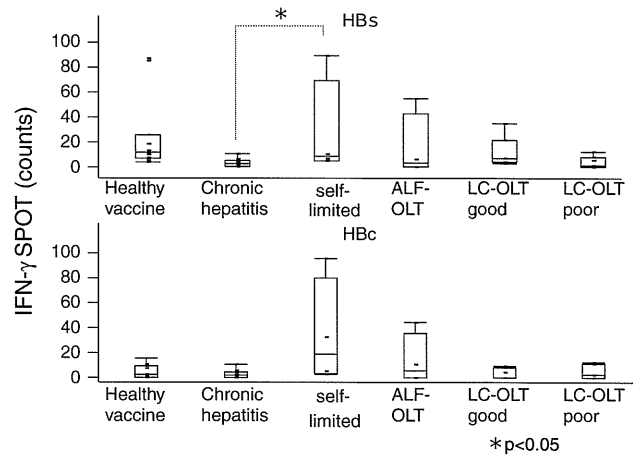

Fig. 3 Cellular immune responses against HBsAg including OLT patients. The number of spots due to interferon-γ response in the ELISPOT assay for HBsAg (*upper figure*) and HBcAg (*lower figure*) is shown. *1* Healthy vaccine: healthy controls who were positive for anti-HBs antibodies with HBV vaccine ($n = 11$). *2* Chronic hepatitis: chronic hepatitis B patients ($n = 10$). *3* Self-limited: self-limited acute hepatitis B patients who showed serum anti-HBs antibody-positive/HBcAb-positive with no HBsAg or HBV-DNA ($n = 5$). *4* ALF-OLT: post-OLT acute liver failure patients ($n = 4$). *5* LC-OLT good: post-OLT liver cirrhosis patients who showed good response to vaccine ($n = 8$). *6* LC-OLT poor: post-OLT liver cirrhosis patients who showed poor response to vaccine ($n = 7$). Values are plotted as median (range)

therapies. We previously have shown that long-term LAM with short-term, high-dose HBIg followed by low-dose HBIg (sufficient to maintain an anti-HBs antibody titer of >10 mIU/mL) is cost-effective and powerful enough to control HBV recurrence after LDLT [13]. With this cost-saving method, no clinical evidence of HBV recurrence has been seen.

In 2000, Sanchez-Fueyo et al. [21] reported an 82 % response to HBV vaccination after OLT. These researchers

used three cycles of double-dose recombinant HBsAg vaccine for immunization over 6 months, with a target antibody titer of >10 mIU/mL. The cohort included six acute infected patients and 11 chronic carriers. However, recent reports show that chronic HBV carrier recipients did not respond well, with response rates ranging from 7.7 to 12.5 % [22, 23]. Acute HBV-infected patients who underwent OLT were often positive for the anti-HBs antibody even before OLT, with strong immune responses. Such patients might be expected to respond well to vaccination, since these individuals (unlike chronic carriers) have not developed a tolerance to HBV. In our patients, five acute infected patients showed good responses to vaccination, responding after a median of only four vaccinations. These results indicate that while acute HBV-infected patients are good candidates for HBV vaccination post-OLT; chronic HBV carriers are poorer candidates for this protocol. However, as some HBV carriers did respond to vaccination; further studies should be performed to clarify the differences between the good and poor responders.

Several reports have identified the differences between good responders and poor responders in non-HBV-infected patients who received HBcAb-positive donor livers. Lacking previous HBV exposure, these recipients should not have developed tolerance to the virus and so should have been good responders. Of these, good responses were seen in pediatric cases where the recipients had higher anti-HBs antibody titers at the time of OLT and lower tacrolimus levels at the time of vaccination [24]. The present study revealed that repeated vaccine administration resulted in successful immunization in 40 % of the LC-OLT recipients. For these recipients, the strength of the response did not correlate with recipient characteristics, not even with age, one of the most important factors for successful immunization [25]. In contrast, the characteristics of the donor were important. The good responders' donors were relatively high in age, non-blood-related and had high anti-HBs antibody titers before donation. Note that, in our trial, the term "non-blood-related donor" indicates the spouse of the recipient, since deceased donor liver transplantation is not widely accepted in Japan [26]. The donors with high-titer anti-HBs antibody probably were infected with HBV by the recipients after their marriage, resulting in the anti-HBs antibody boost. These donors' immune systems should not have developed tolerance to the virus. This elevated immunity might be the reason why our patients had relatively better outcomes following vaccination than those of previous reports [27]. Adoptive immune transfer of HBV-specific immune response could be possible [28]. For successful transfer of immune memory to the recipients, the anti-HBs antibody titer of the donors should be high, and vaccine-induced anti-HBs antibody might be less effective than antibodies produced in a previous self-limited infection. Luo et al. [29] have shown that a particularly high anti-HBs antibody titer (>1000 IU/L) in the donor is essential for adoptive immune transfer. The results of the present study suggest that HBV vaccination of non-blood-related living donor candidates having a lower anti-HBs antibody titer (<100 mIU/mL) might facilitate improved vaccine response post-OLT in LC recipients.

The present study of HBV vaccine efficacy in ALF-OLT and LC-OLT patients revealed that the vaccine response depended on the immune tolerance to the virus in both recipients and donors. The liver is the biggest immune organ in the abdomen and so can play a critical role in immune responses. Multiple populations of non-hematopoietic liver cells, including sinusoidal endothelial cells, stellate cells located in the subendothelial space, and liver parenchymal cells, take on the roles of antigen-presenting cells [30]. The viral-specific immune competence of the grafted liver might overcome the general immunotolerance to the virus in chronic HBV carriers.

In conclusion, patients who received OLT due to acute infection of HBV were good candidates for HBV vaccination. The chronic HBV carrier recipients who received livers from donors who were non-blood-related (i.e, the recipient's spouse) and who harbored high anti-HBs antibody titers were the best candidates for HBV vaccine administration. Vaccine-induced, HBV-specific immune responses were strong enough to induce not only humoral but also cellular responses in vitro.

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Conflict of interest The authors declare that they have no conflict of interest.

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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

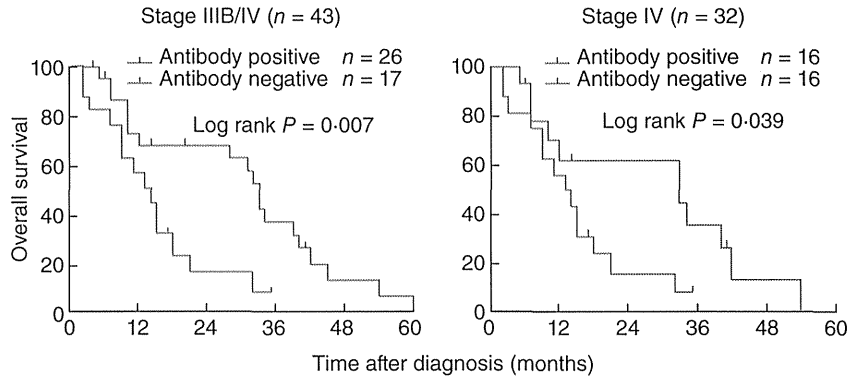
The results presented here show that the Ig GM 1,2,17 21 phenotype is associated with the presence of naturally occurring antibodies to the cancer-testis antigen XAGE-1b, while the GM 1,17 21 phenotype is associated with the lack of such antibodies. One mechanism underlying this association could involve 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), $P = 0·007$.



Stage	Overall survival	Antibody		*P*-value
		Positive	Negative	
Stage IIIB/IV	Median, month	33·0	14·0	0·007
	95% CI	30·3–35·7	8·2–19·8	
Stage IV	Median, month	33·0	13·0	0·039
	95% CI	0–66·1	7·1–18·9	

Fig. 1. Kaplan–Meier survival plots of XAGE-1b antigen-positive advanced lung adenocarcinoma patients as a function of XAGE-1b antibody.

suggest that the association of γ and κ chains in IgG antibodies directed against XAGE-1b might not be random. Only γ and κ chains carrying specific GM and KM allotypes might form a paratope with the necessary quaternary structure for an effective recognition of the XAGE-1b epitopes. Non-random pairing of heavy and light chains has been reported in experimental animals [22,23].

As mentioned previously, the XAGE-1b antigen is highly immunogenic and, therefore, an excellent vaccine candidate for active immunotherapy. In XAGE-1b antibody-positive patients, specific T cell responses were also frequently observed [17]. If the results presented here are confirmed in an independent study, they could aid in identifying subjects (GM 1,2,17 21) who are more likely to benefit from XAGE-1b-based vaccines. For those with the non-responder (GM 1,17 21) phenotype, XAGE-1b could be fused with appropriate adjuvants, such as heat shock proteins or flagellin, to overcome the allotypic restriction in immune responsiveness. It is relevant to note that antibody responses to certain heat shock proteins as well as to flagellin are also influenced by GM genotypes [24,25], making it conceivable to formulate a fusion XAGE-1b–heat shock protein/flagellin vaccine that could potentially generate high antibody responses in the majority of the population. Identification of the natural responders/non-responders to XAGE-1b would also be helpful in the proper evaluation of any future vaccine efficacy trials.

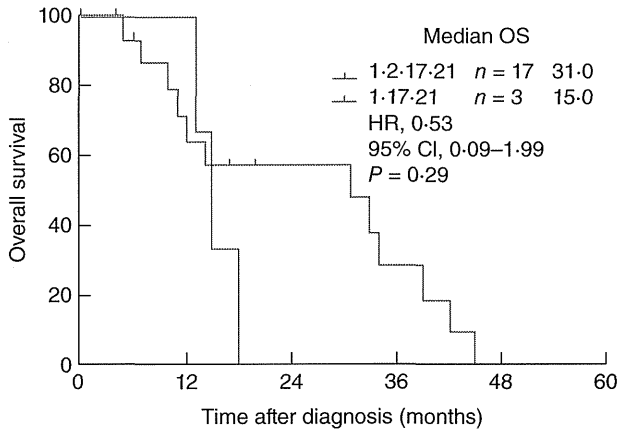


Fig. 2. Kaplan–Meier survival plots of XAGE-1b antigen-positive stage IIIB/IV lung adenocarcinoma patients as a function of GM 1,2,17 21 and GM 1,17 21 phenotypes.

Associations observed in this report can also be explained by postulating as-yet unidentified immune response genes for XAGE-1b whose alleles might be in linkage disequilibrium with those of GM and KM loci.

Although the results reported here are statistically significant, they could also be the result of chance fluctuations, as the *P*-values for the associations were not adjusted for multiple testing. Such adjustment is controversial [26], and in the present investigation would be overly punitive, as the multiple tests performed are not independent due to significant linkage disequilibrium in the GM gene complex. This is the first study of its kind, and needs to be replicated and extended by independent investigations.

It is relevant to point out that the highly significant GM phenotype–lung cancer association that was reported more than three decades ago [8] has not been confirmed or refuted by modern genome-wide association studies (GWAS) of this malignancy [27]. One contributing factor for this omission might be the absence of GM gene probes in most genotyping platforms. GWAS are assumed to be able to detect/tag all single nucleotide polymorphisms (SNPs) in the genome whose frequency is at least 5%. This, however, is not true. Most GM alleles are common within a racial group (some with allele frequency >70%), but the *IGHG* gene segments harbouring them are highly homologous and apparently not amenable to the high-throughput genotyping technology used in GWAS. Because these genes

were not typed in the HapMap or the 1000 Genomes projects, they cannot be imputed or tagged (through linkage disequilibrium) by any SNPs that are included in the genotyping platforms. Therefore, a candidate gene approach would be necessary to confirm/refute the findings reported here.

It is hoped that these results, coupled with those identifying the *IGKC* gene as a strong prognostic marker in human solid tumours [18], would inspire large-scale studies to determine conclusively the contribution of Ig GM and KM alleles in humoral immunity to XAGE-1b. It would also be of interest to investigate the role of these determinants in immunity to NY-ESO-1, a prototype cancer-testis antigen. Results from such investigations would be extremely valuable in devising novel immunotherapeutic interventions in patients with lung adenocarcinoma.

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Disclosure

The authors have no conflicts of interest to declare.

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