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IgE peptide-specific CTL inhibit IgE production: A transient IgE suppression model in wild-type and HLA-A2.1 transgenic mice

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ABSTRACT

Effect of IgE peptide-specific CTL on IgE antibody production was studied in mouse models. CTL elicited in B6.A2Kb tg mice against a human IgE peptide nonamer, pWV, lysed human IgE-secreting U266 myeloma cells and inhibit IgE production by these cells. U266 transfected with mouse A2Kb transgene (U266-A2Kb) were optimally lysed by these CTL, because the $\alpha 3$ domain of A2Kb interacts well with the CD8 co-receptors. The CTL generated were more effective in inhibiting IgE production by U266-A2Kb cells than lysing these cells. IgE production by and progression of U266 myeloma were suppressed in B6.A2Kb tg mice rendered tolerant to these cells and vaccinated with pWV along with CpG. We also studied the CTL response elicited in wild-type mice by a mouse nonameric IgE peptide, PI-1, along with CpG. This treatment caused a transient suppression of the IgE response in mice previously sensitized to an antigen. In mice treated with this regimen repeatedly, the IgE response was fully recovered 20 days after each treatment. Notably, while IgE peptide/CpG-treated mice remained unresponsive to antigen challenge *in vivo*, antigen-specific IgE production can be elicited by antigen in cultured splenocytes from these mice. Moreover, IgE peptide/CpG also inhibited an on-going IgE response, including IgE production by bone marrow cells. Taken together, these observations indicate that a CTL-based IgE peptide vaccine targeting IgE-secreting B/plasma cells may be safely employed as a therapeutic approach for suppressing IgE production.

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1. Introduction

IgE-mediated allergic diseases, afflicting 25% of the US population, are manifested as atopic asthma, allergic rhinitis, food allergy, atopic dermatitis, and anaphylaxis. In addition to symptomatic treatments with various classical pharmaceutical agents, there are two main modalities of therapeutic treatment targeting directly the immune response: allergen desensitization or specific immunotherapy and anti-IgE passive antibody treatment. The first is a classic method, which is likely based on the induction of regulatory mechanisms such as the induction of specific anergy by regulatory CD4 T cells and immune deviation of effector CD4 T-cells [1]. Repetitive treatment with ascending doses of native or modified allergens favors Th1 induction and the production of blocking IgG4 antibodies. While this method has proven efficacy, it is associated with a risk of inducing ana-

phylaxis (5.4/million shots) [2]. This treatment requires the identification of the offending allergens. The second approach using MAb anti-IgE, is currently FDA approved for moderate to severe asthmatics [3,4]. While the treatment clearly results in symptomatic improvement in asthma, the effect is relatively modest. In addition, the current therapy is associated with a risk of anaphylaxis (3.14/1000 cases) higher than specific immunotherapy, probably due to reactivity to xenogeneic mouse sequences in the humanized monoclonal antibody [5,6].

Herein, we describe the development of an alternative approach of CTL-mediated, pan-IgE peptide therapy (PIT) that provides potential improvements over the above two therapeutic modalities. This method targets IgE and does not require identification of the offending allergens. Moreover, by targeting a consensus IgE epitope from the constant region of IgE heavy chain, the effect of dampening IgE levels, regardless of allergen specificities is similar to that by MAb anti-IgE; however due to the reactivity to IgE peptide presented by MHC I, the risk of anaphylaxis is unlikely.

Previously, it is well established that active immunization of mice by autologous IgE perinatally results in suppression of IgE production. This was due to production of anti-IgE antibodies as

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well as induction of CTL to IgE-producing cells [4,7–9]. Together, these two mechanisms are responsible for neutralizing peripheral or mucosal levels of IgE as well as central inhibition of IgE production by IgE-producing cells [7,10–14]. Indeed, IgE-specific CTL induced by mouse IgE peptides presented on antigen-presenting cells, inhibited and/or eliminated IgE-producing B and plasma cells in mice [9]. Herein, we showed that human IgE peptide-specific HLA-A2.1-restricted CTL, elicited in A2Kb¹ transgenic mice likewise inhibited human IgE production by U266, and eliminated A2Kb-transfected U266 myeloma cells both *in vitro* and *in vivo*. Furthermore, intermittent suppression of antigen-specific IgE production was observed in mice immunized with mouse IgE peptide administered together with CpG as adjuvant. Taken together, this study provides additional insights on development of a safe pan-IgE peptide vaccine based on the generation of IgE peptide-specific CTL that inhibit IgE production.

2. Materials and methods

2.1. MHC I binding and stabilization by IgE peptides determined by FACS and ELISA

The protein sequence of the myeloma IgE PS made of 428 amino acid residues from the VH to CH4 domains (HuMIGHAE2, Accession L0022 J00227 V00555) in the FASTA format, was analyzed by using the algorithms of the Bioinformatics & Molecular Analysis Section (BIMAS, National Institute of Health) and the University of Tübingen (<http://www.syfpeithi.de/>) for nonameric sequences. Twenty nonamer peptides with high scores of their canonical anchor residues for binding to HLA-A2.1 were selected and synthesized, at the core facility of University of North Carolina (Chapel Hill, NC). Decameric binders were selected by using the SYFPEITHI program and 10 peptides were synthesized. All 30 peptides were tested by FACS for their activities to induce HLA-A2Kb upregulation in A2Kb-transfected RMA-S ($\alpha 1\alpha 2$ from human HLA-A2.1, $\alpha 3$ from the H-2^b rodent) and HHD-transfected RMA-S-A2Kb (HHD: collinear construct of $\alpha 1\alpha 2\alpha 3$ with $\beta 2$ microglobulin) (the cell lines were kindly provided by P. Langlade-Demoyen at the Institute of Pasteur and M. Zanetti at UCSD, respectively [15,16]). These stable transfectants were grown in the presence of the IgE peptides, or with positive control canonical HIV peptide [16] at 10–100 $\mu\text{g}/\text{ml}$ for 8 h at 25 °C, then overnight at 37 °C, and the cells were analyzed for surface expression of HLA-A2.1 by using FITC-anti-HLA-A2.1 (G46-2.6, PharMingen). Alternatively, 96-well microtiter plates were coated with anti-HLA-A2.1 at 10 $\mu\text{g}/\text{ml}$ overnight and the coated plates were then layered with 3×10^5 cells for 30 min at r. t. The unbound cells were decanted and the wells were gently rinsed. Biotinylated anti-HLA-A2.1 at 1 $\mu\text{g}/\text{ml}$ was then added and plates were incubated for 30 min and rinsed. This was followed by the addition of HRP-streptavidin and the color at OD₆₁₅ developed after addition of the substrate was measured by an automated ELISA reader.

2.2. Preparation of A2Kb—and epsilon chain-transfected target cells

Construction of chimeric A2Kb in pDisplay-delta Vector: pDisplay with neomycin resistance gene (Invitrogen, SD, CA) was first

modified into pDisplay-delta vector by removing the C-terminus myc tag and the PDGF anchor sequences in order to permit the insertion of the native Kb transmembrane anchor for membrane expression. Full-length cDNA of HLA.A2 was cloned from U266 cells and full-length cDNA of H-2K^b was prepared from RMA-S cells. The $\alpha 1$ and $\alpha 2$ domain of HLA-A2, flanked by Bgl II and BamH I, was amplified, fused with murine $\alpha 3$ PCR fragments flanked by BamH I/Sal II, digested, and subcloned into pDisplay-delta vector in a three-piece ligation protocol with primers flanking Bgl II and Sal II. 2×10^5 U266 cells were transfected with 12 μg and 6 μg of A2Kb pDisplay-delta, and the translated products from the lysed cells were detected by immunoblotting using anti-HA antibody. For permanent transfection, 8 μg of the vector was mixed with 5×10^6 cells at 1 μM final concentration following the manufacturer's protocol (amaxa Biosystems, Köln, Germany). HA tag was detected in U266 cells stably transfected with A2Kb-pDisplay by staining with FITC anti-HA. Full length IgE cDNA (VH-CH ϵ 1-4), prepared from U266 cells was cloned into pTracer containing the zeocin resistance gene (Invitrogen, San Diego, CA) as pTracer/zeo-hulGE. For permanent transfection, 8 μg pTracer/zeo-hulGE was mixed with 5×10^6 EL-4-HHD (a gift of M. Zanetti at UCSD) at 1 μM final concentration and were selected by zeocin resistance. The resistant clones and subclones were detected by their intense fluorescence and expression of epsilon chain.

2.3. Mice and Immunization

B6. A2Kb transgenic mice (with $\alpha 1\alpha 2$ from HLA-A2.1 and $\alpha 3$ from K end of the H-2^b haplotype, noncovalently attached to murine $\beta 2$) were kindly provided by Dr. Linda Sherman (TSRI, San Diego, CA) [17]. B6.A2Kb $\times$ B6.PL F1 were bred in-house. Female and male B6. A2Kb transgenic mice were immunized s.c. and i.m. with 30 μg human IgE peptide nonamer, pWV (593.12, WVDNKTFSV), plus 25 μg CpG in saline equalizer to a final volume of 100 μl [18]. Oligodeoxynucleotide (ODN) 1826 (type B/K) with CpG motifs underlined (5'-TCCATGACGTCCTGACGTT-3') and non-CpG ODN 1982 (5' TCCAGGACTTCTCTCAGGTT-3') were synthesized with nuclease-resistant phosphorothioate backbones by Tri-Link Biotechnology (San Diego, CA). The Na⁺ salts of the ODNs were resuspended at 5 $\mu\text{g}/\text{ml}$ in 10 mM Tris (pH 7.0), 1 mM EDTA, and diluted in 0.9% sodium chloride solution before injection. When boosting is required, mice were repetitively challenged, at intervals of 10 days, with a similar amount of antigens plus CpG or liposomes via similar dual routes.

Alternatively, B6.A2Kb mice were immunized with 30 μg pWV (593.12), 30 μg OVAp (ISQAVHAAHAEINEAGR) in liposomes in 200 μl delivered in equal volume via both s.c. route in the flank and the i.p. route as described [7,9,27]. IgE peptides and OVAp helper peptides were added to DOTAP liposomes in equal volumes, and incubated at r.t. for 1 h. Cationic liposomes, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), was purchased from Boehringer Mannheim (Indianapolis, IN). In the wild-type mouse model, female 8-week-old C57BL/6 mice (from the Jackson lab, Bar Harbor, Maine) were immunized with 30 μg PI-1 nonamer, LYCFYIGHI (p¹⁰⁹⁻¹¹⁷, restricted to both K^b and K^d) together with 25 μg CpG in 200 μl via both s.c. and i.p. routes.

2.4. ⁵¹Cr release assay (CRA)

For *in vitro* restimulation, single cell suspension of the spleen, mediastinal lymph nodes (MLN), or the lungs of IgE peptide-immunized mice were prepared. Lung lobes from 10 mice were pooled, flushed with 20 ml of PBS via the right cardiac ventricle to remove the intravascular blood and circulating leukocytes. Minced lungs were incubated for 90 min at 37 °C on a rocker, in 10% fetal bovine serum (FBS) in the presence of 100 U/ml DNase

¹ Abbreviations used: A2Kb, $\alpha 1\alpha 2$ of HLA2.1 with $\alpha 3$ of murine Kb haplotype; A2Kb (HHD), $\alpha 1\alpha 2$ of HLA2.1 with $\alpha 3$ of murine Kb haplotype covalently linked to human $\beta 2$ microglobulin; CRA, ⁵¹chromium release assay; CTL, cytotoxic T-lymphocytes; DOTAP, 1,2-Dioleoyl-3-Trimethylammonium-Propane; EL-4-HHD-A2Kb, A2Kb-linked $\beta 2$ transfected EL-4; IgE, immunoglobulin E; HRP-SA, horse radish peroxidase streptavidin conjugate; PIT, pan-IgE peptide therapy; pWV, WVDNKTFSV, a nonameric human IgE peptide; RMA-S-A2Kb, TAP-2 deficient RMA-S transfected with A2Kb; tg, transgenic; U266-A2Kb, A2Kb-transfected human IgE-secreting U266 myeloma.

Table 1Cell lines employed *in vitro* and *in vivo*

Cell lines	Gene modification	Purpose
RMA-S-A2Kb	Transfected with lab-constructed A2Kb chimeric gene (TSRI)	(i) Upregulation of HLA-A2.1 in Tap-2 deficient RMA-S; (ii) indicator target in CRA to maximize murine $\alpha 3$ of MHC I with murine CD8 interaction
RMA-S-HHD	Transfected with HHD form of A2Kb (Inst Pasteur)	Upregulation of HLA-A2.1 in Tap-2 deficient RMA-S
EL-4-A2Kb	Transfected with HHD form of A2Kb (Inst Pasteur)	Target indicators in CRA: to maximize murine $\alpha 3$ (Kb) of MHC I with murine CD8 interaction; enhanced covalently attached human $\beta 2$ association with A2Kb
EL4-IGE-A2Kb	Transfected with HHD form of A2Kb (Inst Pasteur) and lab-constructed VCH ϵ 1–4 (Fig. 2B)	Target indicators in CRA: to maximize murine $\alpha 3$ (Kb) of MHC I with murine CD8 interaction; enhanced covalently attached human $\beta 2$ association with A2Kb; natural human IgE peptide and $\alpha 1\alpha 2$ of HLA-A2.1 interaction
U266	None	Target indicator in CRA: natural human IgE peptide presented by homologous A2.1; mismatched $\alpha 3$ of A2.1 incompatible with murine CD8
U266-A2Kb	Transfected with lab-constructed A2Kb (Fig. 2A)	Target indicators in CRA: natural human IgE peptide presented by homologous A2.1 and transfected A2Kb; to maximize murine $\alpha 3$ (Kb) of MHC I with murine CD8

Six cell lines expressing HLA-A2.1 with homologous or heterologous $\alpha 3$ domain, and natural human IgE peptides are constructed and compiled.

I, and 250U/ml collagenase type I. Tissue debris was retained through a stainless steel mesh, followed by discontinuous Percoll gradient (35–55%). These cells were mixed with pWV-pulsed, 48h-LPS and dextran sulfate (DxS)-activated splenic cells as described [9]. 7 days later, the cells were mixed with different types of ^{51}Cr -labeled targets. To prepare as targets, 5×10^6 RMA-S-A2Kb cells or EL4-A2Kb cells were pulsed with pWV (10 $\mu\text{g}/\text{ml}$) for 1 h at 37 °C, washed, and then incubated with 0.6 μCi ^{51}Cr for 1 h at 37 °C, washed, and resuspended. To prepare IgE-secreting cells, or cells transfected with epsilon heavy chain as targets, U266, U266-A2Kb or EL-4-A2Kb-epsilon cells were incubated directly with ^{51}Cr for 1 h at 37 °C and then washed. Single cell suspension from spleens and MLN were prepared from immunized or control mice.

In vitro re-stimulated lymphocytes from spleens, MLN and lungs (5×10^6 splenic and MLN cells, 2×10^6 lung cells) were activated for 7 days *in vitro* with 10 $\mu\text{g}/\text{ml}$ pWV-pulsed, 48h-LPS/DxS-activated splenic APC from B6. A2Kb mice or B6 mice at a 3:1 ratio. Cells were then harvested and incubated with 1×10^4 ^{51}Cr -labeled target cells at different E/T ratios for 4h. The specific ^{51}Cr release was determined according to: (experimental release–spontaneous release/maximal release–spontaneous release) $\times 100$. Maximum release was determined by measuring ^{51}Cr in the supernatant of cells treated with 1% Triton in PBS. To evaluate the secreted levels of human IgE, supernatants were harvested from overnight cultures of the above restimulated splenic cells from B6.A2Kb mice incubated overnight with non-labeled U266 or U266-A2Kb cells at different E/T ratios.

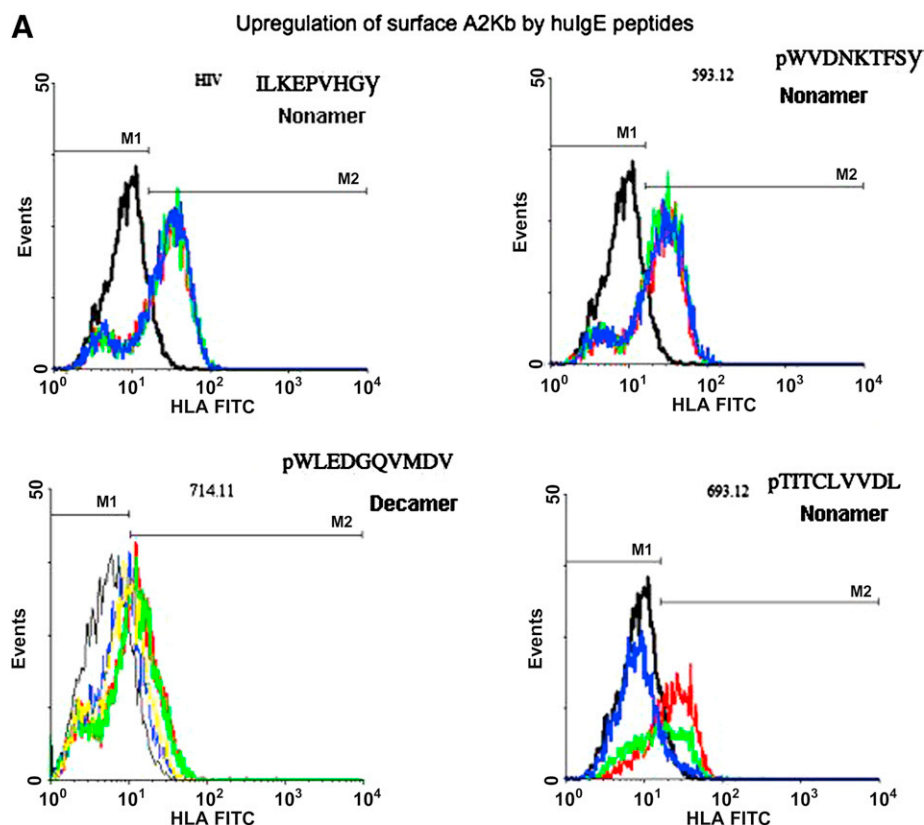


Fig. 1. (A) RMA-S-A2Kb cells were incubated with peptides at 26 °C/5% CO_2 as described. Cells were stained with FITC-G46-2.6, analyzed by FACScan (BD), blue (100 $\mu\text{g}/\text{ml}$), green (25 $\mu\text{g}/\text{ml}$), and red (10 $\mu\text{g}/\text{ml}$). (B) Cell-based ELISA is described in Material and methods. (C) Two groups of five mice each were immunized with 30 μg pWV plus 25 μg CpG s.c./i.m. or intranasally: 5×10^6 spleen and MLN cells (group 1), and 2×10^6 lung cells (group 2) were restimulated with 10 μg pWV-pulsed LPS/DxS-activated splenic APC at a 3 to 1 ratio for 7 days, and CRA was performed.

2.5. IgE and IgG assays

Passive cutaneous anaphylaxis (PCA) reactions, total IgE measurement, and antigen-specific IgG assays were described previously [7,19]. The concentrations of secreted JW chimeric IgE were determined by using a double MAb-based sandwich assay for human IgE (BD Pharmingen, San Diego, CA). For mouse specific antigenic responses, antigen-specific IgG was determined by using plates coated with KLH, and the bound antibodies were detected by biotinylated goat anti-mouse IgG, followed by HRP-SA and substrate, and OD₆₁₅ was recorded. Total mouse IgE was determined by a sandwich assay with EM-95 and biotinylated BF-815 developed in our laboratory [37]. KLH-specific ELISPOT was determined by a method developed in the laboratory [20].

2.6. Pre-treatment of A2Kb neonates with U266-A2Kb cells and survival analysis

U266-A2Kb at 5×10^6 /ml or IgE-secreting murine hybridomas 26.82 as control [21] were first treated with mitomycin C (Sigma-Aldrich, St Louis, MO) at 10 µg/ml for 30 min. Cells were osmotically lysed with water, microfuged at 10,000 rpm for 30 min, and the pellets were resuspended at 5×10^6 cells per 100 µl PBS, and stored frozen at -70°C . B6.A2Kb mice received three weekly injections, starting from the first week of life, of the above cell preparation of 5×10^6 U266-A2Kb cells in 100 µl, or the same number of IgE-secreting murine hybridomas as control via the flank muscle into the intraperitoneal cavity. 5×10^6 /ml human PBMC (of unspecified

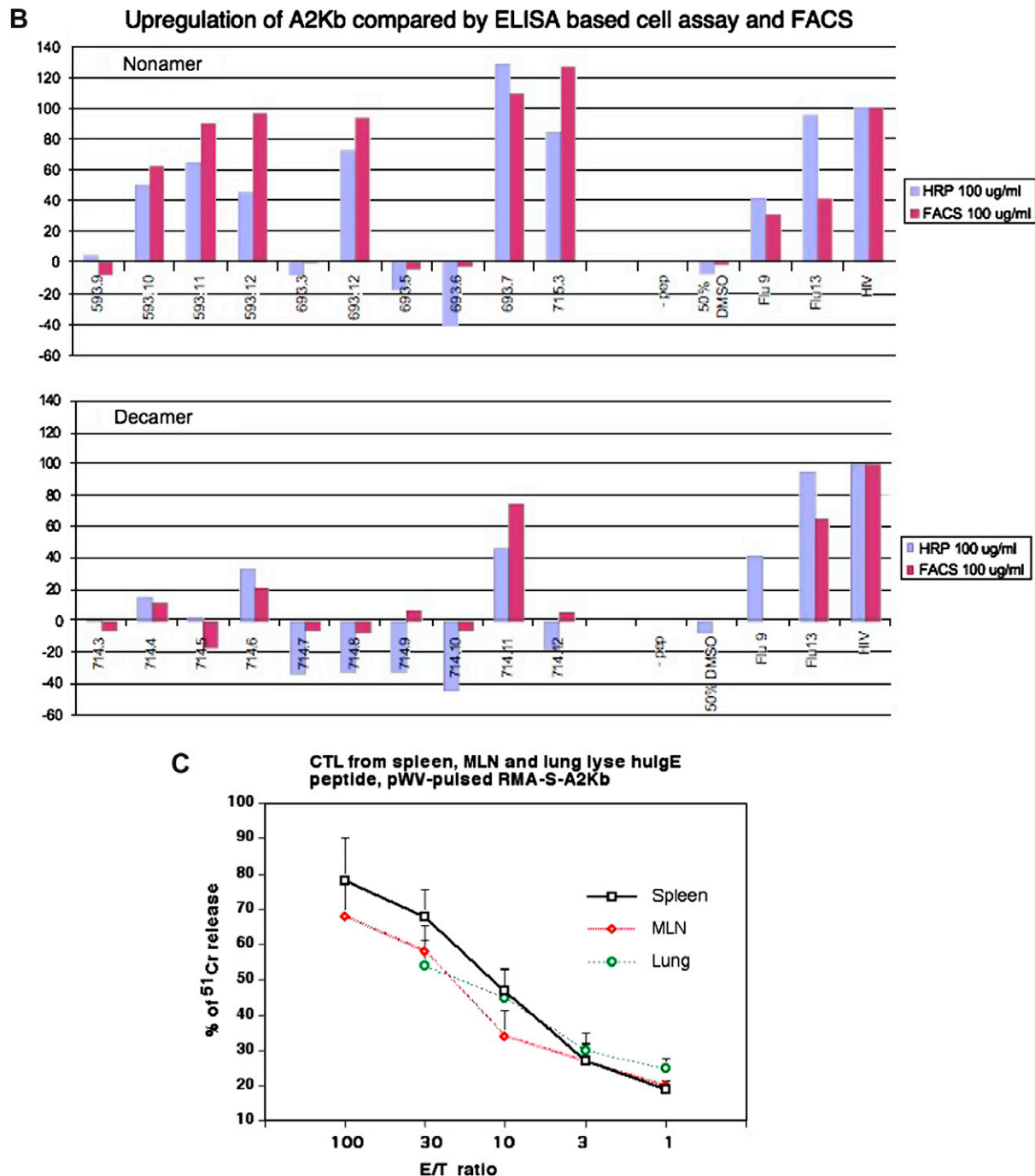


Fig. 1 (continued)

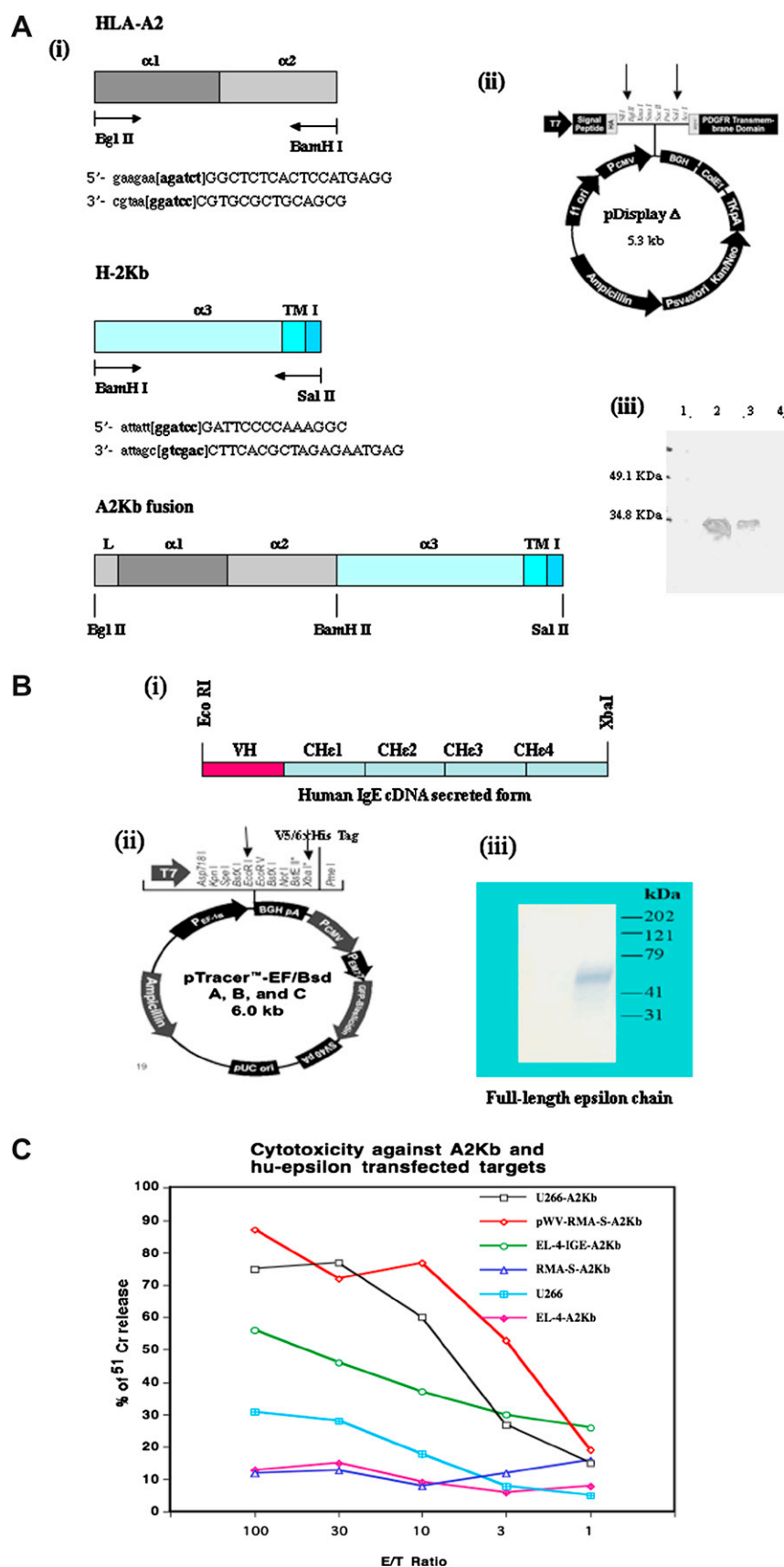


Fig. 2. (A) Construction of A2Kb fusion cDNA (i), its subcloning into pDisplay-delta vector (ii), and transfection into U266 cells (iii) are described in Section 2. (B) Construction of pTracer-epsilon chain cDNA. VH-CH ϵ 1-4 (i); its cloning into pTracer as pTracer/zeo-huIgE; (ii) Western blot of human IgE in pTracer/zeo-huIgE transfected HHD-EL-4 and A2Kb-RMA-S cells (iii) are described in Section 2. (C) B6.A2Kb mice were immunized with 30 μ g pWV (593.12), 30 μ g OVAp in DOTAP liposomes for 10 days. Spleen cells *in vitro* were restimulated with 10 μ g pWV and OVAp-pulsed APC, or OVAp-pulsed APC. ^{51} Cr-labeled EL-4-A2Kb (β 2-HHD)/pTracer-huIgE and U266-A2Kb (cDNA), and U266 cells were left non-pulsed with pWV as targets.

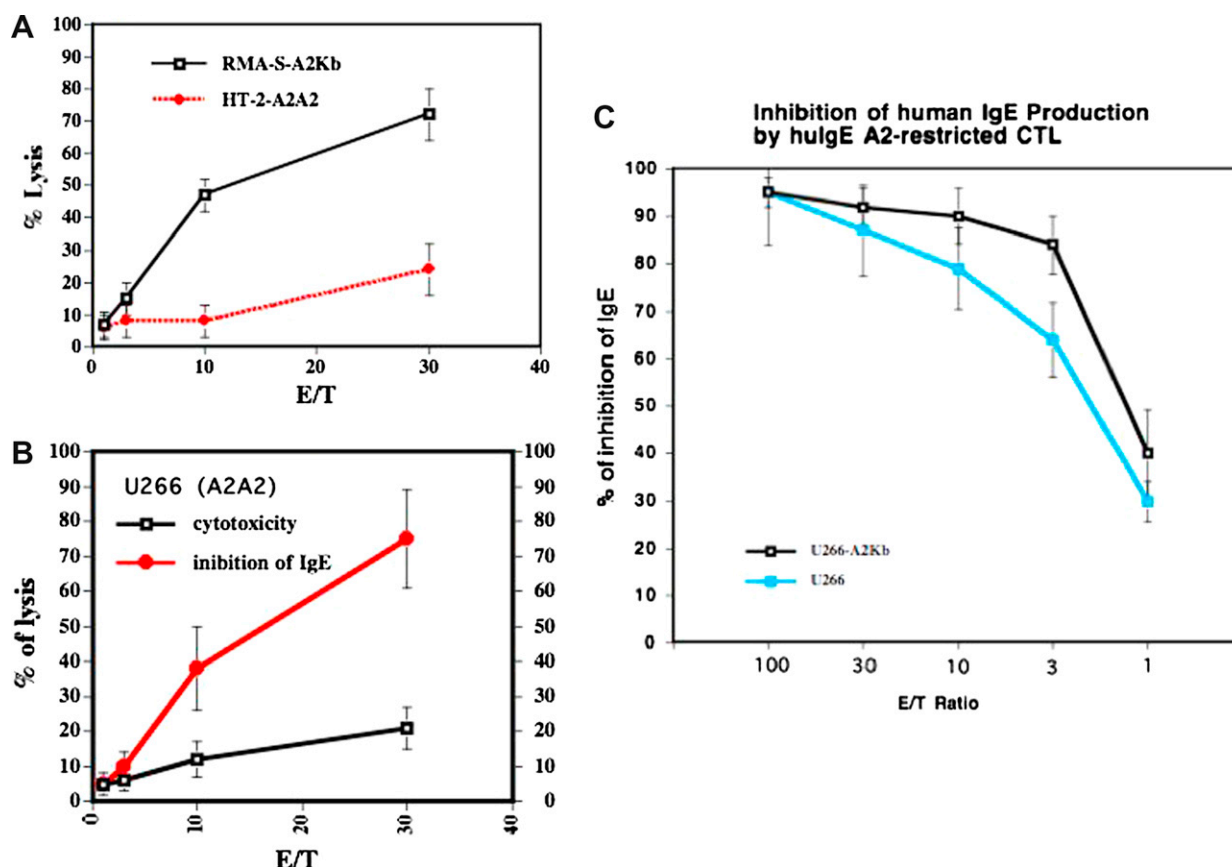


Fig. 3. (A) ^{51}Cr labeled pWV-pulsed RMA-S-A2Kb cells and HT-2 cells were employed as targets. (B) $(2 \times 10^4)^{51}\text{Cr}$ labeled U266 cells were employed as targets in a 4-h ^{51}Cr release assay; alternatively, 2×10^5 non-labeled U266 cells were incubated with restimulated spleen cells overnight and the supernatants were tested for levels of human IgE. (C) About 2×10^5 non-labeled U266-A2Kb vs. U266 cells were incubated overnight, and the supernatants were tested for IgE levels.

haplotype donor (C.T.L., LLP Cleveland, OH), U266-A2Kb, and normal B6.A2Kb splenocytes were incubated with $10 \mu\text{g}/\text{ml}$ mitomycin C for 30 min, washed, and prepared as stimulators. In mix lymphocyte cultures [22], 2×10^5 responder splenic lymphocytes from perinatally treated and control B6.A2Kb mice were stimulated with mitomycin-treated stimulated cells, human PBMC (unknown haplotype, from C.T.L., Cleveland, OH), or U266-A2Kb at ratios of responders/stimulators of 20, 6.6, 2, and 0.66 for 7 days, and then pulsed with $1 \mu\text{Ci}$ thymidine overnight. Two-month old mice were immunized with $25 \mu\text{g}$ of CpG and $30 \mu\text{g}$ pWV s.c. and i.m. and boosted with the above mixture seven days later along with 5×10^5 tumor cells injected s.c.

The Prism program creates survival curves using the method of Kaplan and Meier that calculate the 95% confidence interval for fractional survival at any particular time [23]. The portion of all individuals surviving as of that time was plotted on the X-axis. Two survival curves obtained from perinatally U266-A2Kb treated mice immunized with pWV in CpG vs. CpG control were compared according to the log-rank test. Chi square, p -value and mean survival days were determined based on computation of the two groups of data (d.f. = 1).

3. Results

3.1. Lysis of U266 cells and inhibition of hulgE production in vitro by human IgE peptide-specific CTL

Table 1 summarizes the rationales of employing each respective cell line for conducting binding of human IgE peptide to MHC I and inhibition and/or lysis of human IgE peptide pulsed target indicators.

We synthesized human IgE peptides selected according to the algorithms of binding to HLA-A2.1 and tested for their binding to A2Kb-transfected RMA-S cells with TAP 2 mutation [16,24]. Fig. 1A shows that a nonamer, pWV (593.12), upregulated HLAA2.1 on the cell surface by 10 fold at $10\text{--}100 \mu\text{g}/\text{ml}$, comparable to that induced by a positive control HIV peptide. Another nonamer, pTI (693.12) also induced HLAA2.1 expression at $10 \mu\text{g}/\text{ml}$, but not at $100 \mu\text{g}/\text{ml}$. A decameric peptide, pWL, induced a significant upregulation of A2Kb at all doses tested, albeit at lower levels. Moreover, Fig. 1B shows a strict concordance between FACS-based and ELISA-based analyses. Six out of 10 nonamers tested were scored positive by both FACS and ELISA, while two out of 10 decamers tested were positive. The sensitivities of detection of upregulated surface MHC I by two methods are comparable for all IgE peptides tested and the positive control HIV peptide.

We also tested the effect of IgE peptides in induction of CTL. pWV was administered into mice along with CpG either subcutaneously or intranasally. Fig. 1C shows that CTL specific for peptide-pulsed A2Kb-transfected target cells were detected in the spleen and MLN of mice in the former group and in the lungs of mice in the latter group.

Homologous species-specific $\alpha 3$ domain/CD8 interaction, in addition to TCR and peptide/MHCI recognition, enhances CTL-mediated lysis against targets [25]. Two constructs were prepared: First, a chimeric A2Kb construct composed of a fusion cDNA product from HLA-A2.1 cDNA ($\alpha 1\alpha 2$ domain) and rodent K^b ($\alpha 3$ of K^b) was cloned into the p-Display vector (Fig. 2A, Panel i, ii). The vector was used to transfect U266 cells. The translated chimeric A2Kb product was detected by immunoblotting analysis using anti- K^b antibody (Panel iii). Second, A2Kb (HHD)-transfected EL-4 cells were further

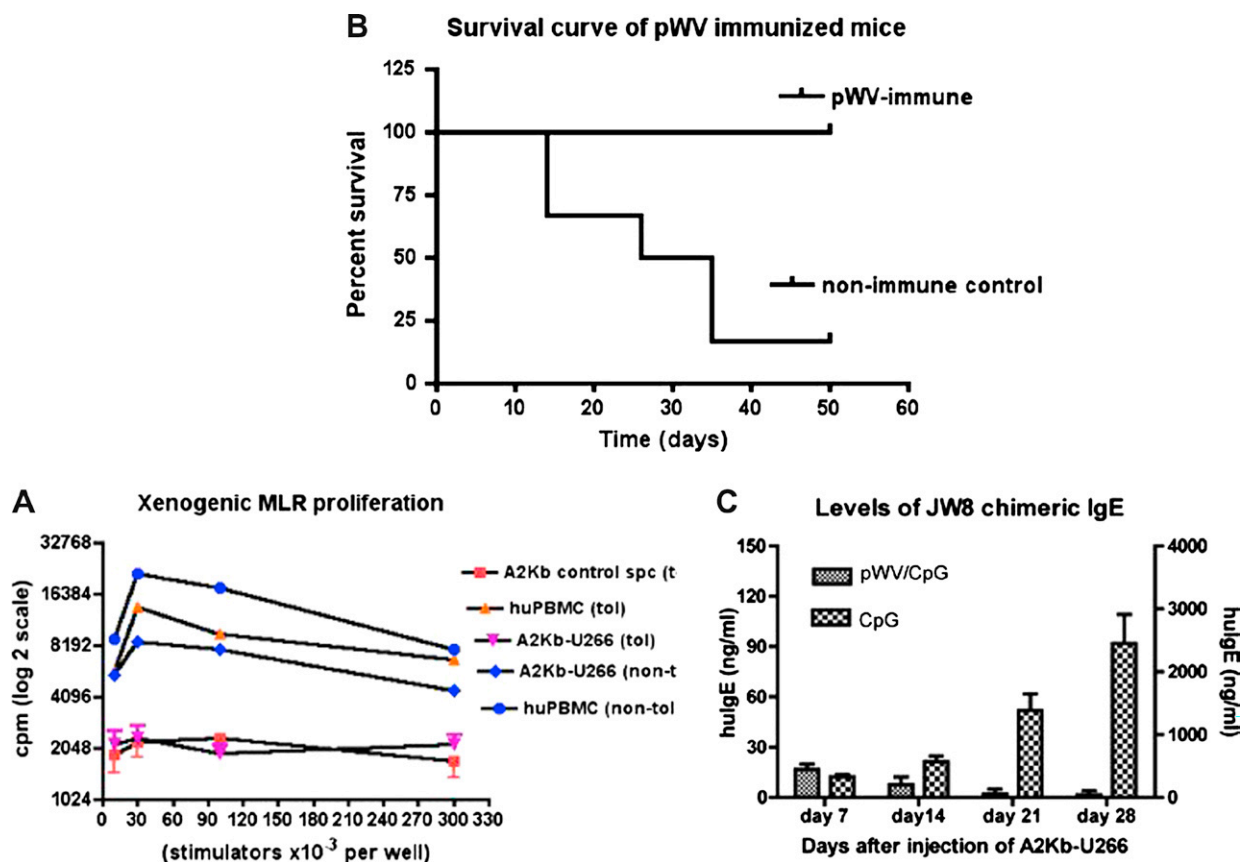


Fig. 4. Immunization with pWV inhibits IgE production and suppressed the growth of U266-A2Kb myeloma *in vivo*. A2Kb mice were injected with U266-A2K cells or control 26.82 hybridomas according to Section 2. Responder splenic lymphocytes 2×10^5 were stimulated with mitomycin-treated stimulator cells for seven days *in vitro*, pulsed with 1 μ Ci thymidine (panel A). Perinatally treated mice were immunized with pWV peptide plus CpG s.c. and i.m., or with CpG alone, and seven days later boosted at the same site along with s.c. injection of 1×10^6 U266-A2Kb cells. Survival of mice was monitored and the data were analyzed by Kaplan–Meier survival curve (panel B). Individual sera were collected and pooled from the surviving mice on specified weekly intervals, and the total human IgE was determined (panel C).

transfected with full-length human epsilon chain cDNA prepared from U266 cells and cloned into p-Tracer (Fig. 2B, Panel i, ii). The IgE epsilon heavy chain was detected in the lysate of p-Tracer-transfected EL-4 cells by immunoblotting analysis using anti-human IgE antibody (Panel iii).

Fig. 2C shows that pWV-induced CTL lyse appropriate targets. Thus, *in vitro* restimulated splenocytes from pWV-treated A2Kb tg mice lysed A2Kb-transfected U266 to the same extent as pWV-pulsed RMA-S-A2Kb targets. This strongly suggests that an endogenously processed pWV in IgE-secreting U266 is naturally exhibited on the surface A2Kb molecules and recognized by CTL.

In comparison, CTL were less efficient in lysing non-transfected U266 cells due to the mismatched human $\alpha 3$ domain of MHC I in interaction with murine CD8. In contrast, efficient lysis was observed in EL-4-A2Kb cells that were transfected with human pTracer-hulGE (VH-CH ϵ 1-4) as anticipated. As control, EL-4-A2Kb cells not expressing the IgE heavy chain was not lysed. Likewise, EL-4-A2Kb and RMA-S-A2Kb cells not pulsed with pWV peptide were not lysed. Taken together, these observations indicate that the endogenously processed IgE peptide presented by $\alpha 1\alpha 2$ domain of MHC I recognized by TCR as well as cognate interactions between murine CD8 and murine $\alpha 3$ domain of MHC I together determine an efficient CTL-mediated target lysis.

Next, we evaluated the sensitivity thresholds of inhibiting IgE production vs. CTL-mediated lysis. Fig. 3A shows that CTL lysed pWV peptide-pulsed RMA-S-A2Kb cells more efficiently ($\sim 70\%$ at $E/T=30$) than peptide-pulsed HT-2 cell expressing A2A2 ($\sim 20\%$ at $E/T=30$).

Importantly, the magnitude of inhibiting of IgE production by non-A2Kb-transfected U266 cells was more sensitive ($\sim 75\%$ at $E/T=30$) than that of lysis of non-A2Kb-transfected U266 cells ($\sim 20\%$ at $E/T=30$). Fig. 3B shows that comparable extent of inhibition of human IgE production was observed in U266 cells transfected with A2Kb vs. that of non-transfected U266. These observations indicate that inhibition of IgE production offers a more pertinent criterium for immunization efficacy as compared to lysis of IgE-producing cells.

3.2. Inhibition of human IgE production and protection against U266 myeloma cells *in vivo* in IgE peptide-immunized mice

To determine the protective effect of human IgE peptide vaccination against the growth of human IgE-producing myeloma in HLA-2.1 mice [26], we first rendered these mice tolerant to the xenogeneic transplants by treating with U266-A2Kb perinatally. These mice subsequently would succumb to injection of U266-A2Kb cells. In contrast, untreated control mice or those perinatally treated with murine 26.82 IgE-secreting hybridomas would reject the xenogeneic U266-A2Kb graft. Thus, A2Kb transgenic mice were treated starting the first week of life with three weekly i.p. injections of pellets of lysed U266-A2Kb, or mouse 26.82 IgE hybridomas as control [26–28]. Fig. 4A shows that spleen cells from U266-A2Kb-treated mice did not proliferate upon incubation of mitomycin-treated U266-A2Kb cells, while those from control mice mounted three to four fold higher proliferative responses upon challenge with mitomycin-treated U266-A2Kb cells. Cells from both groups responded to mitomycin-treated normal PBMC (panel A).

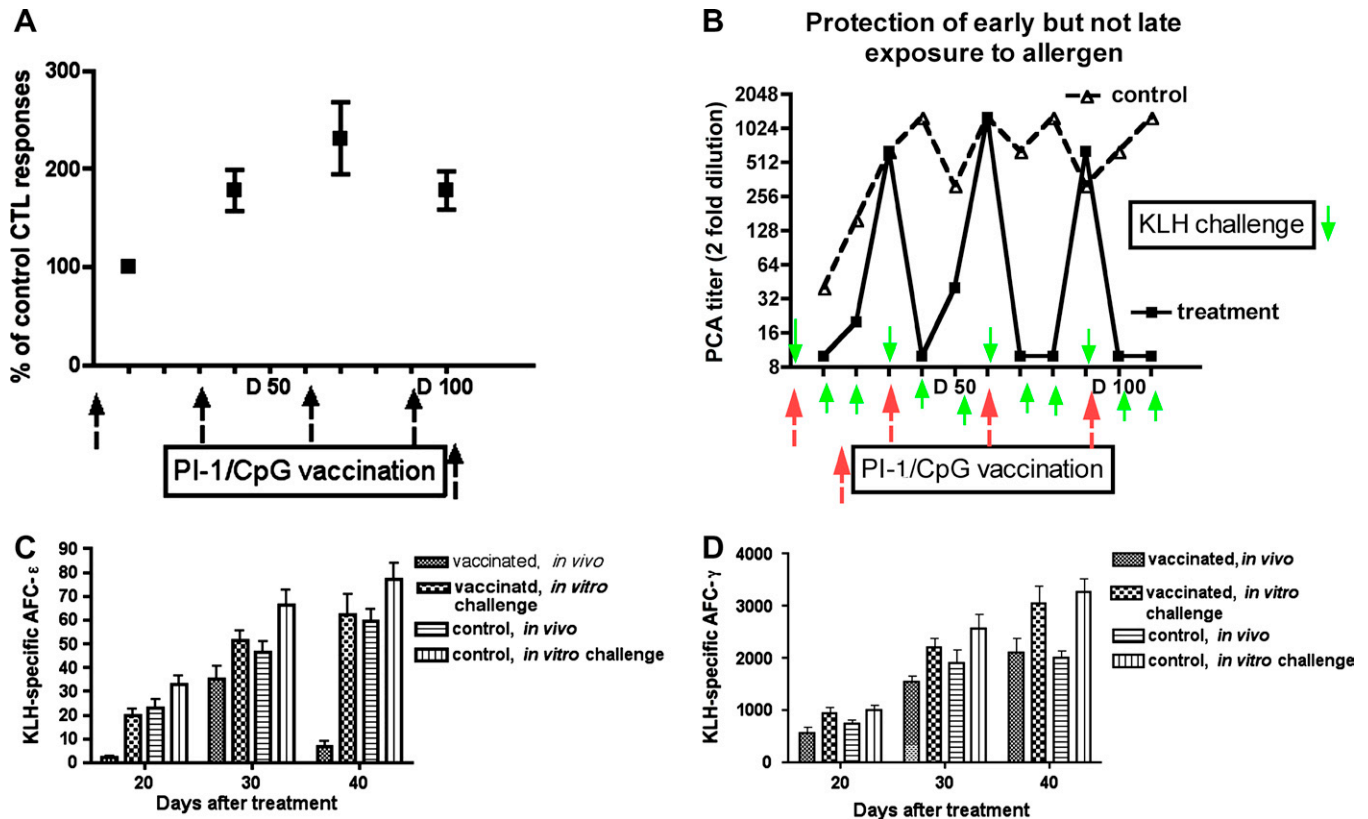


Fig. 5. (A) Four groups of age-matched B6 mice were immunized with PI-1 peptide in CpG s.c./i.m. as described in Results. Spleen cells from individual mice were mixed with PI-1-pulsed EL-4 indicator cells at an *E/T* ratio of 30. Group 1 serves as the baseline response (100%). (B) Two groups of mice (10 each) were treated with either PI-1 plus CpG (treatment) or injected only with CpG (control). These mice were then sensitized with KLH antigen according to the same schedule, sera were collected and analyzed for PCA responses as described. (C) Three groups of mice (5 each) received the same immunization and antigen stimulation protocol up to 40 days similar to Panel B. IgE-producing cells were determined *in situ* and *in vitro* after fresh antigenic challenge. (D) The same mice described in Panel C were prepared and IgG-producing cells measured by ELISPOT.

Next, human IgE production and survival were evaluated in perinatally U266-A2Kb-treated mice that were immunized with pWV along with CpG. Fig. 4B shows that pWV-immunized, U266-A2Kb-tolerant mice (7/7) were all protected from U266-A2Kb metastasis during the observation period of 50 days after live tumor injection, according to Kaplan–Meier survival curve analysis. In contrast, only one out of six control U266-A2Kb tolerant mice, immunized with CpG adjuvant survived up to day 50, and the group had the

mean survival time of 30.5 days, (chi square difference=9.141; *p*-value=0.0025 with d.f=1 of the two groups). As shown in Fig. 4C, although there was an initial spike of residual serum IgE (~16 ng/ml) in perinatally tolerized and IgE peptide/CpG-treated group, the levels of IgE are continually diminished. In contrast, a rapid rise of circulating myeloma IgE (500–3200 ng/ml) of non-treated mice was observed: four mice on day 14 (4/6), four mice on day 21 (4/6), and two mice on day 28 (2/6).

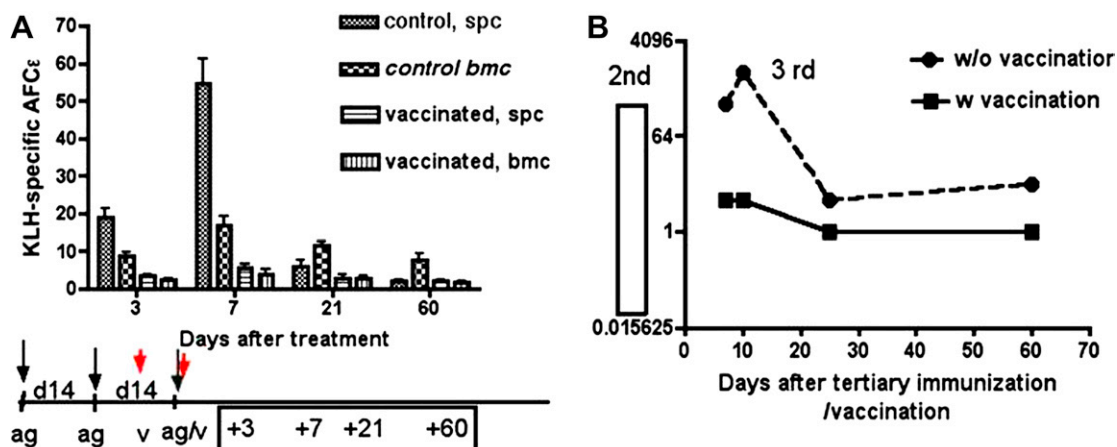


Fig. 6. Protection of on-going antigen-specific IgE responses. (A) Mice were sensitized with 1 µg KLH in 2 mg alum i.p. (black arrow), and immunized with 30 µg PI-1 and 30 µg CpG s.c./i.m. (red arrow), and mice challenged with KLH and reimmunized. Spleen cells (spc) and bone marrow cells (bmc) were individually prepared from each group, and the numbers of IgE-producing cells (EPs) were enumerated. (B) Anti-KLH PCA titers were measured in the immunized group (w) and control group (w/o).

3.3. Prevention of IgE responses by immunization with IgE peptide with CpG adjuvant

3.3.1. PI-1 peptide plus CpG

CTL induced against a murine IgE peptide nonamer, PI-1 ($p^{109-117}$ of the murine CH ϵ 2 sequence), is known to suppress IgE production *in vivo* [7,9]. Further, immunization with certain IgE peptides with CpG in the absence of helper peptide is documented to induce primarily augmented effector CTL responses [29]. Thus, four groups of age-matched B6 mice were immunized with PI-1 together with CpG s.c./i.m. on day 0 (Group 1), day 0 and –30 (Group 2), day 0, –30 and –60 (Group 3), and day 0, –30, –60 and –90 (Group 4), and *in situ* CTL responses were enumerated day 7 after the last boost. The responses in Group 1 served as the baseline (100%). Fig. 5A shows that the magnitude of the *in vitro* CTL response day 7 following different booster doses was 1.7–2.3-fold higher than that of mice immunized only once.

3.3.2. Period of transient protection

To test duration of protection by IgE peptide immunization, a group of ten mice was treated repetitively with PI-1 together with CpG on day 0, 30, 60 and 90. Mice were primed also with 2 μ g KLH in 2 mg alum on day 0 i.p. and challenged with KLH/alum intermittently at intervals of 10 days. As a control, another group of 10 mice were treated with CpG alone in saline, and immunized with KLH/alum according to the same schedule. Fig. 5B shows that after the first IgE peptide/CpG treatment on day 0, KLH-specific PCA titers on day 10 and day 20 (PCA ~8–16) from PI-1/CpG-treated mice were significantly depressed as compared to those of CpG-treated controls (PCA ~32–128). In contrast, serum PCA responses on day 30 in both PI-1/CpG-treated and control mice were comparable when tertiarily challenged with KLH in alum on day 20.

Since the breakthrough of antigen-specific IgE responses occurred on the third KLH challenge following the primary vaccination, re-immunization with PI-1/CpG was required on day 30 (Fig. 5B). Thus, upon re-immunization, KLH-specific IgE responses were protected with the same duration upon the fourth KLH/alum challenge (day 40 PCA ~8 in re-immunized vs. 1024 in control mice) and fifth KLH/alum challenge (day 50 PCA ~32 in re-immunized vs. 512 in control mice). Furthermore, this periodicity of protection was patently evident upon the third (day 60) and fourth (day 90) re-immunization resulting in a period of nearly complete protection for additional 20 days with greatly diminished PCA titers (~8). These observations indicate importantly that suppression of IgE responses due to IgE peptide PI-1 immunization is transient and IgE responses are subsequently recovered approximately 20 days following each immunization.

3.3.3. Capacity of IgE production *in vitro*

Three groups of mice were immunized with IgE peptide/CpG and sensitized with antigens up to 40 days with a group of control as described in Fig. 5B. Mice were sacrificed on day 7 after the last KLH/alum challenge and *in situ* splenic IgE-producing ELISPOT were measured. In addition, cultures of spleen cells were initiated and stimulated with 1 μ g/ml KLH *in vitro*. Fig. 5C shows a near absence of *in situ* KLH-specific IgE-producing plasma cells in spleens from mice following the day 20 and day 40 regimens of IgE peptide/CpG initiation and re-immunization. In contrast, *in situ* IgE-producing ELISPOT were significantly recovered in spleens from mice following the day 30 regimen (~35 IgE-secreting AFC/10⁶). Interestingly, ELISPOT were detected on day 20 and day 40 splenocytes, challenged with soluble KLH *in vitro*, comparable to those of non-immunized control mice. Thus, Fig. 5C indicates that despite the suppressed IgE responses *in vivo*, antigen-specific precursor B-cells remain intact and are free to respond to antigen stimulation in dispersed cultures *in vitro*.

3.3.4. IgE Isotype-specific suppression

The suppressive effect of IgE peptide/CpG immunization is restricted to the IgE response. As shown in Fig. 5D, despite the suppressed *in vivo* IgE responses on day 20 and day 40 *in situ*, *in vitro* re-challenged KLH-specific IgG responses in IgE suppressed mice remained comparable to those of control mice.

3.4. Effect of IgE peptide/CpG immunization on on-going IgE production

To evaluate whether on-going IgE responses can also be suppressed by IgE peptide/CpG immunization, antigen-specific IgE responses were first initiated on day 0 and day 14 by KLH/alum, mice were then vaccinated on day 21, followed by concomitantly reboosting with IgE peptide/CpG and KLH on day 28. Fig. 6A shows that IgE-producing ELISPOT in the spleens of normal mice peaked on day 7, was diminished on day 21 and day 60, while bone marrow ELISPOT peaked on day 7 and persisted up to day 60. Notably, immunization on day 21 and day 28 significantly dampened on-going IgE responses in both spleens and bone marrows throughout day 3 to day 60 following the third KLH challenge on day 28. Moreover, Fig. 6B shows in parallel that serum PCA titers of immunized mice, day 3 to day 60 after antigenic re-challenge were significantly lower than those of control mice.

4. Discussion

In summary, this study has demonstrated two major concepts underlying a cell-mediated pan-IgE vaccine: (i) Identity of natural IgE peptides as protective peptides, i.e., Principle of Correspondence (POC). Protective human IgE vaccine epitopes are natural IgE peptide that induce IgE peptide-specific CTL targeting not only peptide-pulsed indicator cells but also IgE-producing plasma cells, such as human IgE-producing U266 cells (Figs. 1–4). In the A2Kb tg model, the interactions of murine CTL and U266 targets are optimized via both TCR and murine CD8 interactions with the invariant murine α 3 domain of A2.1Kb on A2Kb-transfected human IgE-producing U266 cells *in vitro* (Figs. 2 and 3) and *in vivo* (Fig. 4); (ii) Recovery of IgE responses post vaccination, i.e., Periodicity of Protection (POP). IgE peptide administered together with CpG can prevent the development of an IgE response and suppress an on-going IgE response (Figs. 5 and 6). The vaccine is effective in causing a suppression of IgE of approximately three weeks. The vaccine is also safe; and since following this transient suppression, the IgE response is recovered in the absence of re-vaccination (Fig. 5). Thus a permanent suppression of an IgE response caused by vaccination of human IgE peptide with CpG is unlikely.

The present approach of a pan IgE active vaccine offers three alternative improvements in preventing or controlling an on-going IgE response over the existing methods of treatment [1–6]. First, the IgE peptide vaccine appears to target allergen-activated cells expressing IgE without affecting antigen-specific B-cell precursors. Our data showed a diminished number of IgE-producing plasma cells by ELISPOT in both spleens and bone marrows as well as IgE-secreting myelomas/hybridomas. We reason that IgE-switched B-cells or plasmablasts that are immediate precursors of IgE-secreting plasma cells may also be affected. In contrast, resting primary B-precursors that have not undergone IgE switch are not affected. Moreover, antigen-specific memory B-cells (B220⁺, CD138⁺), which do not exhibit surface IgE [31] and IgE peptide biomarkers, will likewise be spared by this approach. Thus, the vaccine-activated CTL affect allergen-activated IgE-B cells and plasma cells without compromising the antigen-specific IgG responses or affecting precursor B-cells specific for parasitic or tumor antigens [30,32–34].

The second improvement of IgE peptide/CpG vaccine resides in its induction of augmented effector CTL but not long-term

memory CTL [35–40]. Raz, and Rouse and colleagues showed that CpG and cytotoxic peptides can directly license APC for inducing effector CTL, while bypassing helper peptide-specific cognate helper T-cells [35,36]. Previously, we showed that administration of IgE peptides in conjunction with OVA helper peptide (OVAp) in DOTAP liposomes led to induction of memory CTL responses [9]. This may raise a concern whether memory CTL may exert prolonged downregulation of IgE production. On the other hand, long-term inhibition of IgE responses by memory CTL may be advantageous for patients with severe chronic allergic asthma, while transient inhibition by effector CTL may actually be more desirable for controlling acute IgE-mediated allergic responses. Herein, effector CTL can be repetitively restimulated by the peptides along with CpG with similar magnitude [41]. This observation indicates that stimulation with IgE peptides along with CpG in the absence of helper T-cell peptide leads to effector but not memory CD8 T-cell development. Harty and colleagues showed that CpG can uncouple development of effector CTL from memory CTL via induction of type I IFN- $\alpha\beta$ [29,35]. Thus, we propose that a safe and effective pan-IgE allergy vaccine may be designed by a meticulous regimen of administering IgE peptide together with CpG in the absence of helper peptide. It may be pointed out that the effect of anti-IgE is also transiently protective within a period of two to three weeks following its administration. Nevertheless, in eliciting CTL responses, IgE peptide vaccine is unlikely to invoke a xenogeneic human anti-mouse antibodies (HAMA)-mediated anaphylactic response.

Although in this study, type B/K 1826 ODN was employed with adequate 'just in time' protection without a prolonged effect, it is possible type A/D ODN may provide an even higher safety margin since A/D type stimulates higher levels of type I IFN- $\alpha\beta$ [42], which further deviates memory CTL development. Thus, as shown in Fig. 5B and C, effector CTL inhibited IgE production during an acute allergen exposure without compromising the subsequent allergen-specific IgE response. Moreover, suppression of KLH-specific IgE responses is not due to CpG-mediated immune deviation [43], since control CpG injected mice exhibited sustained IgE responses upon allergen/alum injection i.p. (Fig. 5B) [44]. In future studies, we wish to extend the analysis of effect of repetitive vaccination of pWV with CpG in inhibiting chimeric human-mouse IgE antibody production in vivo. To this end double transgenic mice expressing both A2Kb and NP-specific human IgE heavy chain transgene [18] will be a suitable mouse model for these studies.

The third potential improvement resides in redistribution of IgE peptide-specific CTL to both systemic and mucosal lymphoid tissues (Fig. 3C). In contrast to passive anti-IgE treatment, mucosal immunization of an active IgE peptide vaccine is likely to protect mucosal organs such as lungs and the GI tract that may be sequestered from the MAb-based anti-IgE therapy [45,46]. Thus it is possible that reduced mucosal IgE may thus facilitate dissociation of receptor-bound IgE from mucosal mast cells of the lungs and the GI tract [47,48], while bioavailability of passive MAb anti-IgE in summary, we propose a transient IgE suppression model via induction of IgE peptide-specific effector but not memory CTL. In the CD4-independent pathway, CpG and IgE peptide license APC for direct induction of primary effector CTL without engagement of helper peptide-specific cognate CD4 T-cells [35,36]. In this model, CTL are superimposed in phase with the acute IgE-producing cells expressing high-density natural IgE peptides on surface MHC I. These 'just-in-time' effector CTL cause a transient but not a long-lasting suppressive effect on IgE production. In conclusion, IgE peptide-based pan-IgE peptide therapy (PIT) provides potential improvements over the existing therapeutic modalities of specific immunotherapy and passive anti-IgE therapy. It is possible that the vaccine studied herein can alter the natural course of IgE-med-

iated allergic diseases, including allergic asthma, via regimens of both short-term and long-term preventive and therapeutic vaccine delivery.

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