

IgE-mediated allergen gene vaccine platform targeting human antigen-presenting cells through the high-affinity IgE receptor

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Background: Treatment of IgE-mediated food allergy with standard protein-based allergen immunotherapy has proved both unsuccessful and hazardous. Allergen gene vaccination represents a promising alternative, but difficulties in gene targeting and expression in antigen-presenting cells represent a major limitation for efficient gene vaccination.

Objective: We sought to construct a genetically engineered human ϵ -polylysine (EPL) fusion protein that binds allergen gene expression systems and targets the gene vaccine complex to antigen-presenting cells through the interaction of EPL and the high-affinity receptor for IgE for efficient allergen gene vaccination.

Methods: Genetic engineering was used to design and produce the EPL fusion gene, consisting of the human CH ϵ 2-4 linked to 55 lysine residues, and the conventional approaches were used to characterize the biologic features of EPL.

Results: EPL was assembled as functional dimers and capable of binding DNA plasmids in both an EPL protein and plasmid DNA concentration-dependent manner. EPL targeted plasmid DNA to the high-affinity receptor for IgE on cell surfaces and increased the model gene uptake/expression. The EPL-DNA complexes were shown not to trigger mast cell degranulation.

Conclusion: EPL is able to function as a gene carrier system to target allergen gene to the high-affinity receptor for IgE-expressing cells through ligand receptor-mediated interactions. (J Allergy Clin Immunol 2009;123:1-11.)

Key words: Gene vaccination, human IgE, dendritic cells, food allergy, IgE high-affinity receptor

Abbreviations used

APC: Antigen-presenting cell
CHO: Chinese hamster ovary
DC: Dendritic cell
EPL: ϵ -Polylysine
GFP: Green fluorescent protein
NP: 4-hydroxy-3-nitrophenacetyl
PLL: Poly-L-lysine

DNA-gene vaccination has attracted a great deal of interest in a broad range of biomedical areas, including allergy, autoimmunity, cancer, and infection. It has become well established as a therapeutic approach in a variety of animal systems.¹ DNA vaccination with allergen genes represents a particularly promising therapeutic strategy for food allergy, a problem of increasing magnitude²⁻⁴ in which traditional protein-based immunotherapy has proved too dangerous. However, one major hurdle for gene vaccination is its low immunogenicity, resulting in relatively weak immune responses. This less than optimal immunogenicity appears to result primarily from the fact that the injected DNA is taken up mainly by cells other than professional antigen-presenting cells (APCs). Thus it has become clear that effective DNA vaccination will also require delivering sufficient DNA to APCs that play a crucial role in the induction of effective cellular and humoral immune responses, as well as maximizing gene expression. A variety of approaches to selectively direct the DNA expression systems to APCs are being undertaken, including targeting APC surface molecules, such as DEC 205,⁵ and the use of viral vectors.⁶

We have approached this issue by selectively targeting allergen gene vaccine to human dendritic cells (DCs) through their expression of the high-affinity receptor for IgE, Fc ϵ RI. DCs as professional APCs can initiate T cell-mediated immunity and control a substantial part of the adaptive immune response and have proved to be of crucial importance for the initiation of transgene-specific immune responses for all methods of DNA vaccination.⁷ DCs in human subjects, as opposed to mice,⁸ normally express Fc ϵ RI, and the level of expression is increased in allergic human subjects.⁹⁻¹⁸ Our overall strategy was to genetically engineer and produce a carrier protein that would bind plasmid DNA and direct its expression to DCs through ligand-mediated targeting. Thus we designed and produced an ϵ immunoglobulin heavy chain polylysine fusion protein (ϵ -polylysine [EPL]) composed of the last 3 constant-region domains of the human ϵ heavy chain plus a 55-amino-acid

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The fundamental construct was modified by the insertion of an HIV tat peptide sequence (GRKKRRQRRR) to potentially enhance cellular uptake and a nuclear localization signal peptide sequence (PKKKRKV) to enhance nuclear transport to produce a second generation of EPL, or the EPL⁺ protein.^{20,21} These

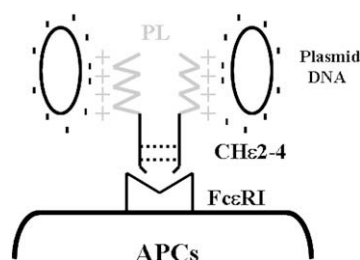


FIG 1. Schematic diagram of the concept for IgE-directed allergen gene vaccination targeting FcεRI-expressing APCs through EPL. The positively charged polylysine tract (PL) of EPL binds to the negatively charged plasmid DNA through electrostatic interaction to form EPL-DNA polyplexes. The CHε2-4 dimer in EPL targets polyplexes to FcεRI-expressing APCs with extremely high affinity.

additional sequences were inserted between the CHε2-4 and polylysine tract sequences to produce the EPL⁺ protein (Fig 2, A, lower panel). The EPL⁺ protein expression level was much lower than that of the EPL protein in the stably transfected clone so that the amount of the affinity-purified EPL⁺ protein was not sufficient to be clearly visualized in the Coomassie staining (data not shown). However, Western blot analysis demonstrated that the EPL⁺ protein was also expressed as dimers, as was the EPL protein (Fig 2, C; the positions for EPL⁺ dimers and monomers are noted, and the nonspecific band is identified with an asterisk). Because of its low expression level, we have not extensively tested the functional properties of the EPL⁺ protein.

EPL proteins bind plasmid DNA

To verify that EPL protein binds DNA and therefore can function as a ligand-mediated gene carrier, we mixed differing amounts of the EPL protein with varying amounts of plasmid DNA, as indicated in Fig 3, and ran the samples in gel retardation assays. Binding of protein (EPL or otherwise) to DNA will retard DNA migration in the gel. Poly-L-lysine (PLL) served as a positive control, whereas the purified IgE alone served as a negative control (Fig 3, A). The EPL protein showed significant DNA binding (retardation in lanes 4 and 5) in a manner similar to PLL (lane 3) and in contrast to the IgE protein itself, with which retardation was not observed (lane 2 compared with lane 1). These experiments demonstrated the DNA-binding ability of our EPL construct and showed that this binding is both EPL protein (Fig 3, B; the EPL/DNA molar ratio was 100:1 [lane 2], 50:1 [lane 3], 25:1 [lane 4], and so on) and plasmid DNA concentration dependent (Fig 3, C). Although it is not possible to exactly quantify the DNA-binding ability of the EPL protein, these data do provide a general relationship between EPL protein amounts and DNA binding.

EPL polyplexes bind to human FcεRI

EPL-DNA polyplexes with a GFP expression plasmid were prepared and incubated with either CHO cells, which express a transfected human FcεRIα chain (3D10 cells), or Ku812 cells, a human basophil-like cell line that expresses the entire FcεRI receptor complex.¹⁹ Binding was detected with fluorescein isothiocyanate-labeled rabbit anti-human IgE by means of FACS analysis. As shown in Fig 4, EPL-DNA polyplexes bound to both 3D10 cells (Fig 4, upper panel) and Ku812 cells (Fig 4, lower panel) at a level equivalent to native IgE, demonstrating the key property of FcεRI binding. Binding was not observed with CHO cells that did not express the FcεRIα chain.

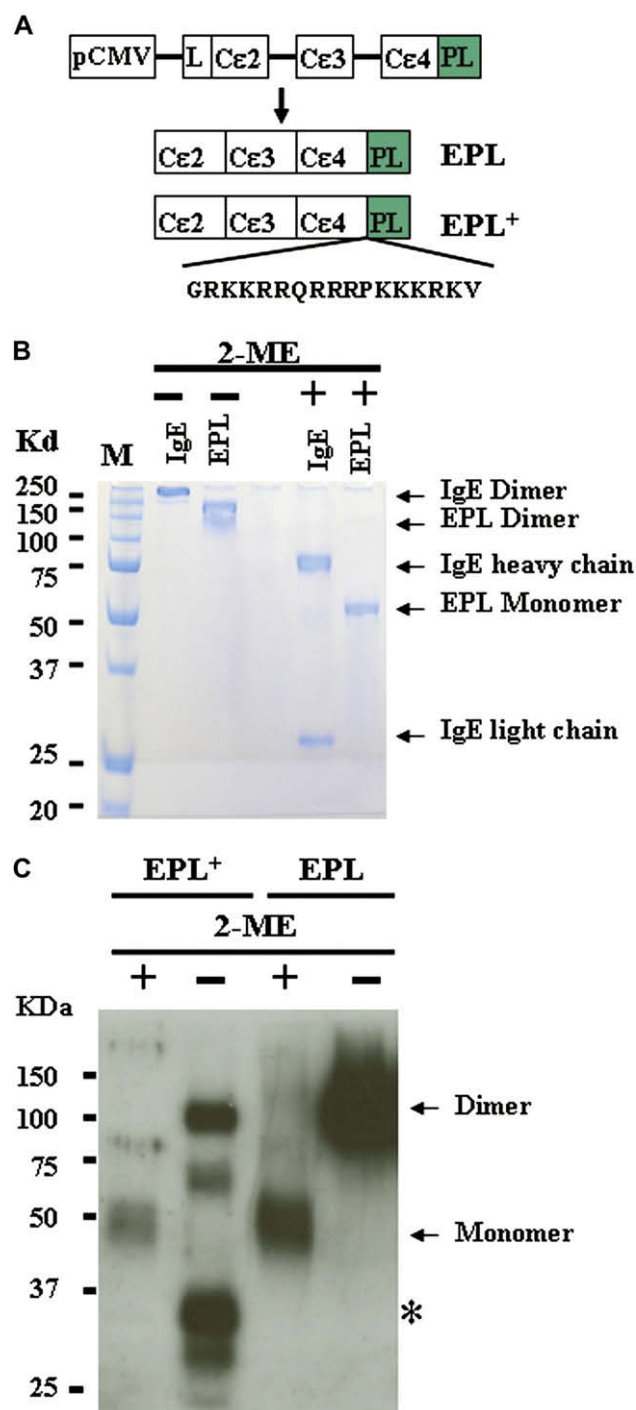


FIG 2. Construction and expression of EPL and EPL⁺ proteins. **A**, Diagram of the EPL and EPL⁺ protein expression constructs. **B**, Characterization of the affinity-purified EPL protein by means of SDS-PAGE stained with Coomassie blue. **C**, Characterization of the expressed EPL⁺ protein, with EPL protein as a control for comparison. 2-ME, 2-Mercaptoethanol.

EPL polyplexes enhanced cellular uptake and expression of the plasmid DNA

The ability of EPL protein to enhance the plasmid uptake through IgE-FcεRI binding was tested in the control CHO cells versus CHO cells expressing the human FcεRIα chain (3D10). Cells were incubated with either a GFP expression plasmid alone

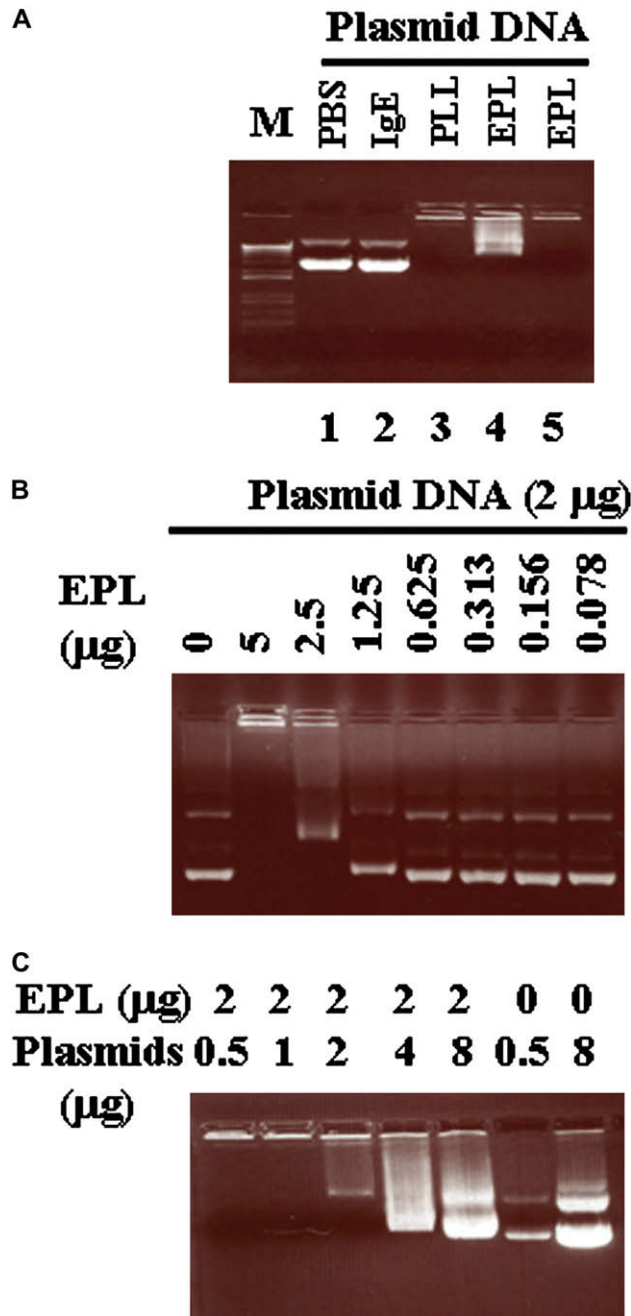


FIG 3. DNA-binding capacity of EPL in the agarose gel retardation assay. **A**, EPL binds to plasmid DNA. **B**, EPL protein concentration-dependent DNA binding. **C**, Plasmid DNA concentration-dependent EPL binding.

or an EPL-GFP plasmid polyplex. GFP protein expression, as evidence of the plasmid uptake and expression, was monitored by using fluorescent microscopy and FACS. FACS analysis showed that EPL protein significantly enhanced the frequency of GFP-positive cells (Fig 5) because the significantly enhanced GFP expression frequencies were observed in GFP complexed with EPL (0.75%, panel III) but not in the GFP plasmid alone in the Fc ϵ RI α -expressing 3D10 cells (0.03%, panel II) or in the CHO cell controls (0.01%, panels V and VI). These results were further confirmed by using fluorescence microscopy (data not shown). These *in vitro* results show that the EPL-GFP polyplex led to increased plasmid DNA uptake and expression through Fc ϵ RI targeting.

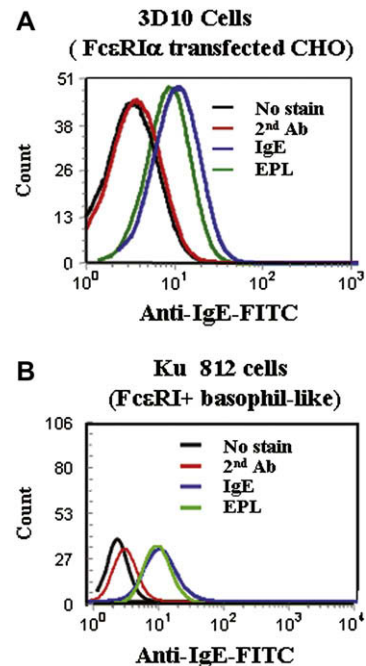


FIG 4. Binding of EPL to cell-surface Fc ϵ RI. EPL binds to the human Fc ϵ RI α chain with similar capacity to that of IgE in 3D10 cells (**A**), and in Fc ϵ RI-expressing Ku812 cells (**B**) it was detected by flow cytometry. The results represent one of the 2 experiments performed. Ab, Antibody; FITC, fluorescein isothiocyanate.

EPL polyplexes do not induce direct mediator release

A potential concern with the EPL protein polyplexes is whether the negatively charged DNA plasmids would cross-link EPL proteins and thereby have the potential to trigger mediator release of the mast cells and basophils. To examine this possibility directly *in vivo*, we tested the ability of EPL-DNA polyplexes to construct a wide variety of ratios to trigger mast cell release when injected into the skin of human Fc ϵ RI α transgenic mice. As shown in Fig 6, a positive control mixture of human anti-NP IgE and its corresponding antigen, NP30-BSA, induced marked blue staining as evidence of IgE-mediated local mediator release (Fig 6, A), whereas the negative controls, saline (Fig 6, B) or DNA plasmid alone (Fig 6, C), did not trigger mediator release. Neither EPL protein alone (Fig 6, D) nor EPL-DNA polyplexes formed at molar ratios of 1:1, 4:1 10:1, or 20:1 (Fig 6, E–H, respectively) triggered local mediator release, indicating that neither EPL protein alone nor EPL-DNA complexes were able to trigger mast cell mediator release.

DISCUSSION

DNA vaccination is an attractive approach for food allergy because it has been shown to result in a more balanced T_H2/T_H1 response, including suppression of T_H2 responses and IgE production and enhanced IFN- γ , IgG2a, and T_H1 responses.^{22–27} However, poor immunogenicity of the naked plasmid DNA vaccines remains a major obstacle to realizing the considerable potential of this approach. We used an IgE-focused DNA vaccination strategy to enhance the DNA vaccination efficiency. Such a method takes advantage of the extraordinarily high picomolar affinity interaction between IgE and Fc ϵ RI, which in human

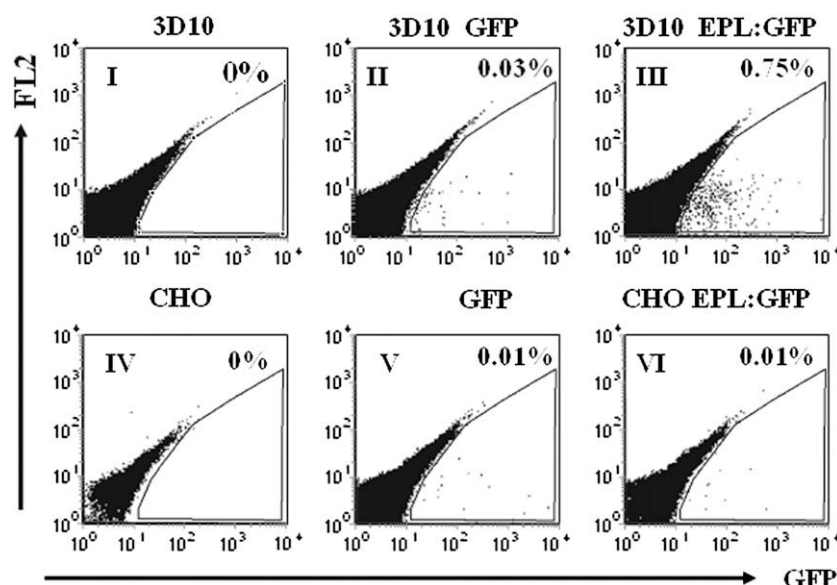


FIG 5. Facilitation of the GFP plasmid uptake expression by EPL protein. GFP plasmid uptake and expression, in the presence or absence of EPL, was determined with CHO cells and CHO cells expressing human FcεRIα (3D10 cells) by means of FACS analysis. The frequencies of the GFP-positive cells were displayed in each panel. The results represent one of the 2 experiments performed.

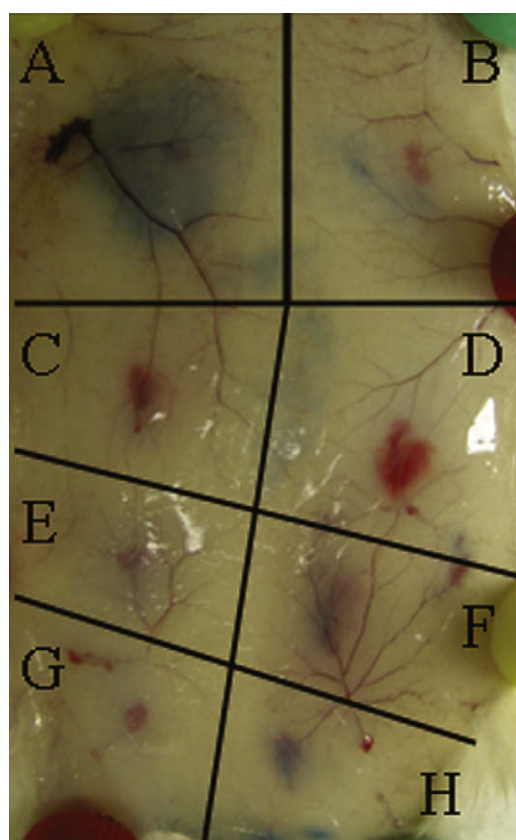


FIG 6. Testing of the potential for EPL-triggered mast cell mediator release. The back skin of a FcεRIα transgenic mouse was injected with an anti-NP IgE and NP30-BSA mixture (A), saline (B), GFP plasmid (C), EPL alone (D), and EPL-DNA polyplexes at molar ratios of 1:1, 4:1, 10:1, and 20:1, respectively (E-H). Only Fig 6, A, showed a positive reaction.

subjects, as opposed to mice, is expressed on APCs. In fact, FcεRI is not only expressed on human APCs in general but also expressed in even higher levels on APCs of atopic individuals. This differential FcεRI expression pattern on human APCs between atopic and nonatopic individuals provides the rationale for the design of an IgE-based allergen gene vaccination strategy. The successful construction and production of EPL and the subsequent characterization of EPL features in various experimental assays have provided the solid basis for moving this platform forward.

Chemical cross-linking has been used to couple various protein ligands to PLL to prepare ligand-mediated gene transfer reagents.²⁸ However, this method has serious shortcomings. First, interprotein cross-linking is likely to occur. Such a potential is especially a concern in the case of IgE or Fc fragment of IgE because such cross-linking of IgE would be expected to trigger mast cell and basophil mediator release. Furthermore, the degree of the cross-linked ε-PLL complexes is difficult to control because of the nature of the chemical reaction, resulting in size and composition variations that would result in batch-to-batch variation; variation would make the development of a true human therapeutic difficult scientifically and highly problematic at a regulatory level. Our genetic engineering-expressed EPL overcomes this problem because the Fcε and its fused polylysine tract are expressed as a single and uniform molecule, thereby avoiding the issue of molecular heterogeneity. Indeed, using *in vivo* skin testing, we have demonstrated that over a wide range of doses and ratios, EPL-DNA polyplexes were not able to trigger mast cell mediator release and should be safe from this vantage point.

Allergy effector cells, such as mast cells and basophils, will also be targeted by our EPL proteins because they express abundant surface FcεRI. To avoid allergen gene vaccine expression in these cells, we have designed an expression system that addresses this issue *in vivo*. We have incorporated the DC cell type-specific promoter fascin into the vaccine gene expression

plasmid. Only maturing and mature DCs, follicular DCs, and Langerhans' cells express fascin.^{29,30} It is not present in mast cells, basophils, or both. Thus use of a fascin-controlled allergen gene expression vector will therefore ensure the selective DC-specific expression of the targeted allergen genes.

Up to this point, we have necessarily evaluated the characteristics of this EPL platform primarily *in vitro*. Although we can use the hFcεRIα transgenic mice to measure the acute effects of EPL, such as skin test reactivity after exposure to EPL, both wild-type and hFcεRIα⁺ mice generate an anti-human ε IgH response that will confound the interpretation of any findings of a vaccination protocol with EPL. Fortunately, investigation of the *in vivo* effects of EPL and EPL⁺ proteins have now become feasible, because Dr Kinet and colleagues have very recently constructed BALB/c mice that are hFcεRIα transgenic and have the human IgH ε gene knocked in (personal communication). In those "allergy-humanized" animals, we will be able to repeatedly inject our EPL polyplexes without the complicating anti-human ε response. Those future experiments in allergy-humanized mice will extend the current *in vitro* work to a suitable *in vivo* experimental model for testing the efficiency of this novel Che-targeted allergen gene vaccine platform as a potential immunotherapeutic intervention in IgE-mediated food allergy.

Clinical implications: The EPL platform can function as a universal human FcεRI⁺ APC targeting system for gene delivery and efficient allergen gene vaccination to food allergies, including peanut allergy.

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