

A Novel Multi-potential Dendritic Cell Targeted Gene Vaccination Platform; Application to Food Allergy and Beyond

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Introduction

IgE mediated severe food allergy is an increasing problem and an unmet need. It is well appreciated that anaphylactic allergy to foods is occurring with an increasing prevalence/incidence [1–3]. Food allergy affects up to ~3.7% of the overall US population and 6% to 8% of young children [4]. Peanut allergy, which affects around 1% of the population, is among the most severe food allergies and can cause life-threatening clinical manifestations, including death. In contrast to the expanding array of treatments available for inhalant and cutaneous allergic disorders, currently, the only available treatment for food induced severe allergic reactions when accidental peanut exposure occurs is rapid self-administration of injectable epinephrine. Strict avoidance to prevent reactions is not only nearly impossible but also a major lifestyle intrusion for both the patient and his/her family. Among IgE mediated food allergies, peanut, tree nut, egg and milk allergies are the most prominent, especially in children, of which peanut allergy is involved in more deaths than any other food [5]. Not only is there personal morbidity (and occasional mortality) from severe food allergy, but also there is a tremendous cost to the family members and particularly to parents of children who have this problem as each day their child must survive a potentially dangerous world with a host of hidden sources that could cause great harm.

Multiple therapeutic modalities, including classic allergen protein-based immunotherapy, have been tried for peanut and other severe food allergies but none have yielded satisfactory results. Of particular relevance to food allergies is that the conventional protein-based IT has proven repeatedly to be too dangerous to undertake in those with severe (anaphylactic) sensitivity as fatal reactions to IT have occurred even under investigational conditions. Therefore, other approaches, such as anti-IgE therapy [6], traditional and modified peptide immunotherapy [7,8], oral immunotherapy [9], mutated allergen proteins [10–12] and homologous proteins [13]; allergen immunotherapy plus Th1-response enhancing adjuvants, e.g. CpG

[14]; traditional Chinese medicine [15,16]; and allergen gene vaccination [17] have all been undertaken to try to overcome the problems accompanying allergen IT in severe food allergic patients. Anti-IgE can provide some level of ongoing protection for severe food allergy but this modality alone seems unlikely to become the long term “answer” as passive anti-IgE immunotherapy has only shown limited success in reducing allergic reactivity and would require uninterrupted life-long treatment. Studies are now ongoing testing whether prevention of the development of severe food allergy can be achieved in high-risk subjects (www.immunotolerance.org), but those approaches will not address the needs of those who are or who will become severely allergic to foods. For these reasons it is universally agreed that novel approaches for the treatment of established severe food allergy are urgently needed.

To address this unmet need, we have designed a novel human ligand (Fcε) based allergen gene vaccination platform that targets the allergen gene vaccine to DCs through high affinity interaction between a portion of the Fcε chain and the FcεRI displayed on the DC cell surface. The actual therapeutic is a naturally dimerized protein known as EPL comprised of the last three epsilon IgH constant region domains plus 55 repeat lysine bound electrostatically to DNA expression plasmids containing the gene of interest. This protein DNA complex is known as a “Polyplex”. While we are developing this platform for the treatment of severe food allergy, this same approach holds promise as a universal human gene delivery system that can be applied in infectious diseases, cancer and even autoimmune disorders.

Abbreviations: APC, antigen presenting cells; DC, Dendritic cells; EPL, Fcε-polylysine protein; FcεRI, High affinity receptor for IgE; “Allergy-humanized” mice, mice with the human Cε knock-in plus expressing the human FcεRIα transgene

Allergen gene vaccination

Allergen gene vaccination represents a promising alternative to protein-based allergen-specific IT in terms of safety and efficacy. Optimally in this approach, the allergen gene of interest is delivered to, taken up by and expressed solely by appropriate APC. No allergen proteins are administered exogenously and only small amounts of protein-derived peptides are predicted to be displayed on the surface of the antigen presenting cells that express the injected gene. This approach has been shown to be safe and effectively inhibit allergen-specific IgE production and suppress Th2 while reciprocally enhancing Th1 responses [18–20]. While allergen gene vaccination has been shown to modulate allergic responses in animal models, the current state of the art requires greatly improved targeting. When effective, allergen vaccination has achieved more balanced Th2/Th1 responses, including suppression of Th2 responses and IgE production, and enhancement of IFN-γ, IgG2a and Th1 responses [18–22]. In addition, successful allergen gene-based vaccination also has been shown to reduce the number of mast cells at allergic inflammatory sites such as the lung [23].

Allergen genes have been previously administered as naked plasmid DNA by various routes, including intramuscular or intradermal injection, biolistic transfection via the “gene gun”, or orally as plasmid DNA-polymer complexes [17–22]. DNA immunization by injection has been shown to be effective in inhibiting devel-

opment of antigen specific IgE production. In contrast, the ability of DNA vaccination with allergen-encoding vectors to suppress already established IgE immune responses remains controversial [18]. A major hurdle for these and all allergen gene vaccination approaches has been the poor efficiency of DNA transfer to antigen presenting cells (APCs) necessary for subsequent allergen presentation to immune competent cells so as to drive efficient modulation of allergic diseases. Dendritic cells (DCs) as professional APCs are crucial for the initiation of transgene-specific immune responses for all methods of DNA vaccination [24]. However, none of the current gene-transfer methods for allergen gene vaccination specifically target the gene to these critical DCs. The poor efficiency of many of the prior attempts at gene vaccination is thus likely related to the low efficiency of vaccine gene delivery to appropriate DCs. Indeed, we would compare the current state of the art for DNA vaccination in allergy to ‘carpet bombing’ where massive amounts of ordinance (DNA) were dropped (injected); the overwhelming number of weapons simply missed the targets (APCs). As discussed below, use of an FcεRI targeting for gene vaccination represents a quantum leap forward and is the equivalent of using laser guided ‘smart bombs’ in that the epsilon Fc targets the DNA to the IgE receptor bearing APC while addition of a DC specific promoter restricts expression to the desired target cells. The potential advantages of our EPL based allergen gene vaccine platform are listed in Table 1.

FcεRI expression on human APCs provides a ligand-mediated mechanism by which to target human Fcε protein:allergen DNA polyplexes to dendritic cells as an immunotherapeutic vaccine.

Although a primary function of IgE is to mediate the effector phase of allergic reactions, in humans IgE plays an important role in antigen capture and presentation to APC leading to enhanced antigen-specific immune responses and/or immune tolerance. This occurs primarily through IgE-dependent antigen focusing via the picomolar affinity interaction between IgE

1.	Genetically engineered single fusion protein providing for reproducible production
2.	Can serve as a universal carrier for gene expression plasmids
3.	Uses easily produced gene expression plasmid requiring only cloning-in the gene of interest, most of which are known and cloned
4.	Does not require the production of multiple recombinant proteins.
5.	Preparation of protein carrier (EPL) DNA polyplexes accomplished by simply mixing in the desired ratio.
6.	Targets key dendritic cells via picomolar ligand interaction between epsilon IgH and FcεRI
7.	Can be targeted to distinct DC populations via employing different routes of administration, e.g. intradermal, sub-cutaneous, sub-lingual and even intravenously.
8.	Can readily prepare polyplexes containing a number of genes of interest to direct immune response to a profile of relevant targets, e.g. multiple peanut allergens, multiple HIV mutants of the same gene (GP 120) or multiple distinct HIV genes (GP120 and GP41).

Table 1. *Enabling features of epsilon-polylysine (EPL) polyplex gene vaccination platform.*

and the high affinity FcεRI on APCs. This extraordinarily high affinity of the Fcε-FcεRI ligand-receptor interaction likely accounts for the special place this interaction has in enhancing antigen/allergen presentation and modulation of immune/allergic responses.

The FcεRI on human APC is composed of αγ subunits as opposed to αβγ subunits on mast cells and basophils. FcεRI (αγ) is expressed on APCs including macrophages/monocytes, blood DCs, follicular DCs, and Langerhans cells [25–30]. Antigens captured on some APC subsets via inducible FcεRIs and IgE-dependent antigen focusing can elicit heightened immune responses under specific circumstances and anatomical locations when the concentration of antigen/allergen is below that for effective presentation via conventional antigen capture and presentation pathways [31,32]. In contrast, DC subsets with constitutive FcεRI expression in some specialized anatomic locations, such as plasmacytoid DCs in the blood [33] and DCs in the oral and nasal mucosa [30,34], are associated with the induction and maintenance of allergen-specific tolerance [35]. The extraordinarily high affinity of the Fcε-FcεRI ligand-receptor interaction likely accounts for the special place this interaction has in enhancing antigen/allergen presentation and modulation of immune/allergic responses. This mechanism appears to play a particularly important role in allergic responses in atopic patients, as the APCs from atopic patients express much higher levels of FcεRI than those in non-allergic subjects [25–35].

Design, production and in vitro evaluation of a universal FcεRIα targeting protein that carries gene expression plasmids to human APCs

We reasoned that an effective way to markedly enhance the efficiency of human gene vaccination and allergen gene vaccination in particular would be to specifically target allergen genes to human DCs through their expression of FcεRI. Ligand-mediated gene delivery has been undertaken to enhance the efficacy of gene vaccination by targeting selected APC types that express a specific cell surface marker [6]. The extremely high affinity interaction between IgE and FcεRI provides a unique opportunity that we could exploit in developing an efficient allergen gene ligand mediated delivery platform for allergen gene IT. Thus the combined EPL:plasmid DNA polyplexes are directed to human dendritic cells through high affinity ligand binding of EPL to DC FcεRI (diagrammed in Figure 1) and thereby overcome the current major obstacle of DC targeting. This provides for a particularly effective IgE-mediated allergen gene IT to target DCs in allergic patients as their APCs and particularly their DCs and Langerhans cells express enhanced levels of FcεRI [25–35]. Using this approach, we are pursuing the construction of EPL polyplexes carrying the *Ara h* 2, 3 and 6, the major allergens in peanuts. However, FcεRIα is expressed to a variable degree on APCs from normal individuals such that this approach for gene delivery to DCs is projected to be efficacious in non allergic-atopic subjects as well. Thus the epsilon – FcεRIα ligand mediated targeting is expected to be applicable in infectious, malignant and autoimmune diseases across populations regardless of their allergic status.

Thus we designed a gene and then produced an epsilon IgH-based bifunctional fusion protein, termed Epsilon-PolyLysine (EPL) as part of a platform for human allergen gene vaccination. The epsilon IgH Cε2–Cε3–Cε4 region was linked with a

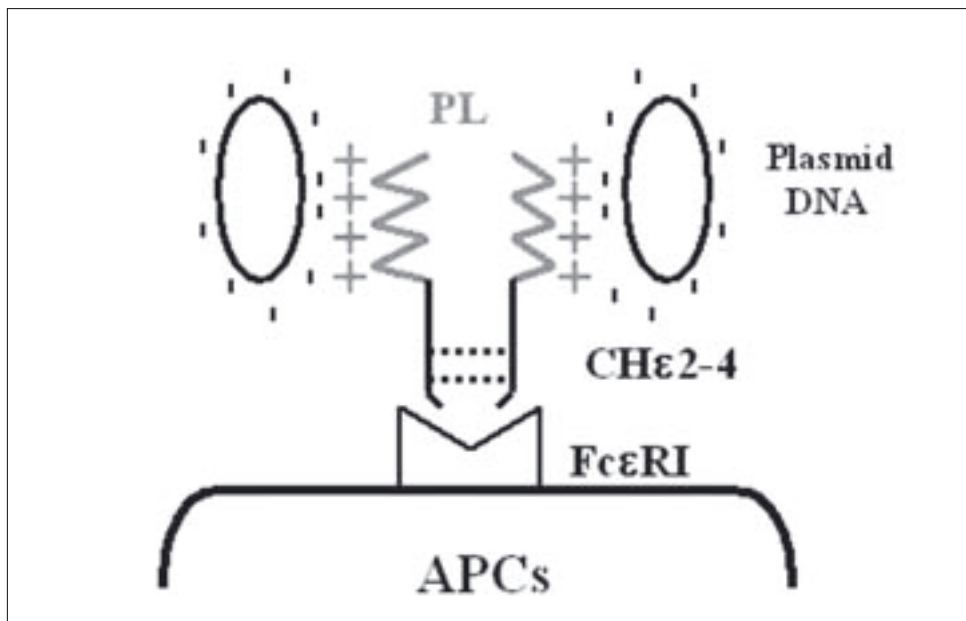


Figure 1. Diagram of the epsilon-mediated targeting of the EPL polyplex gene DNA to dendritic cells via epsilon-FcεRI binding. PL = polylysine

synthesized DNA coding for 55 repeat lysine residues to create the EPL expression vector. The linearized plasmid DNA was stably transfected into CHO cells, and the EPL expressing cells, were identified by ε-ELISA screening, isolated, cloned and subsequently used for production in roller bottle cultures. EPL was purified from the cell culture supernatants by an anti-human IgE affinity chromatography. The resulting fusion EPL protein consists of the CHε2–3–4 Fc portion of human ε IgH plus a highly positively charged polylysine tail designed to electrostatically bind gene expression plasmids.

We have documented that this EPL protein naturally dimerizes *in vitro*, a feature critical to maintain high affinity binding to FcεRI. In addition, we have shown that EPL binds to cells expressing FcεRIα in a fashion equivalent to native IgE. Production of EPL by genetic engineering as opposed to chemical linking ensures that each epsilon molecule chain in the final naturally dimerized EPL is uniformly associated with the same amount of polylysine thereby overcoming issues about the reproducibility and uniformity of the product that will be critical in obtaining regulatory approval for human use. It also obviates any concerns of accidental epsilon dimer cross-linking that might lead to immediate mediator release.

Experiments with EPL and plasmid DNA showed, as predicted, that EPL binds DNA in a dose dependent fashion. This was observed across at least a two log fold range of protein to DNA ratios from at least 1:1 to 20:1 (EPL:DNA ratio). Such protein-DNA complexes are termed “polyplexes”. Having shown that polyplexes

were appropriately formed by EPL and DNA, we have gone on to demonstrate *in vitro* that such polyplexes are preferentially taken up and expressed in FcεRIα expressing cells. Thus EPL polyplexes containing a GFP expression plasmid showed preferential expression in CHO cells transfected with the human FcεRIα gene and in Ku812, an FcεRI bearing human mast cell-like line.

Another notable feature of the approach is the ease by which polyplexes directed to a series of allergens may be produced. This can be accomplished by simply using the same *fascin* driven expression system to produce expression plasmids for several allergens, e.g. *Ara h 2*, *Ara h 3*, and *Ara h 6* and either mixing these with one batch of EPL to make a set of mixed polyplexes or mixing each alone with EPL and then combining them as desired.

Production of a second-generation EPL protein for enhanced polyplex uptake and gene expression

We have now produced a second-generation EPL protein, termed EPL+ and designed to increase DC uptake and expression of the polyplex gene. We modified the basic EPL gene sequence through insertion of two additional sequences between the 3' end of the epsilon heavy chain and 5' end of the polylysine sequence. This new sequence encodes the part of the HIV tat-peptide (GRKKRRQRRR) and a Nuclear Localization Sequence (NLS). The tat-peptide has been shown to enhance the transcellular internalization of a number of agents including small peptides, proteins and polymers, oligonucleotides and phage vectors [36,37]. The exact mechanism of internalization driven by the TAT peptide is not known but it is thought to be via micropinocytosis [38,39]. NLS is well known to play an important role in the nucleocytoplasmic protein transport and drives nuclear accumulation via enhanced trafficking of various substrates from the cytoplasm into the nucleus through nuclear pore complexes [40]. Whether EPL+ is functionally superior to EPL remains to be demonstrated.

Limiting gene expression to dendritic cells

Use of DC cell type specific promoter allows targeted DNA vaccine to be expressed in DC in a cell type restricted fashion. As a consequence of FcεRI targeting, in addition to DCs, EPL:DNA polyplex vaccines will target (1) basophils and mast cells, (2) potentially, albeit unlikely, cells expressing the low affinity FcεRII (CD23) or (3) be taken up non-specifically by cells at the site of vaccination. To address this issue, we have built in a further level of cell specificity by making use of the DC type specific *Fascin* promoter in the construction of the expression vector system we employ *in vivo*. *Fascin* expression is limited to maturing and mature DCs, follicular DCs and Langerhans cells but it is not expressed in mast cells or basophils [41,42]. Thus by employing *Fascin* controlled gene expression plasmids in our polyplex vaccine platform, we expect to restrict expression of the targeted allergen to DCs rather than other FcεRI-bearing cells, e.g. mast cells and/or basophils.

EPL polyplexes do not directly induce mast cell mediator release

One potential concern with the EPL polyplex platform is that polyplexes will form that carry multiple EPL proteins that would then induce mast cell release by cross-linking FcεR1s. To test this, mice transgenic for the human FcεRI alpha chain were used to measure the ability of EPL or EPL polyplexes to induce mast cell degranulation. Various amounts of EPL, alone or mixed with plasmid DNA, and positive and negative controls, were injected intradermally into the back skin of the mice, followed by an intravenously challenge with 1% Evan's blue dye. After 30 minutes, mast cell release was visualized by leakage of blue dye into the skin at the injection sites. EPL alone or EPL:DNA polyplexes formed at different molar ratios were unable to trigger local mediator release while human IgE:allergen complexes induced marked mediator release as positive controls.

"Allergy-humanized" mice: an animal model in which to test the abilities of the EPL:gene polyplex vaccine platform.

IgE ligand mediated DC antigen presentation cannot be tested in wild type mice for mice, in contrast to humans, do not to display FcεRI on their APCs [43]. However, Dr. Jean-Pierre Kinet has derived a strain of Balb/C mice that express the human FcεRIα chain transgene. Fortunately, the upstream control elements are contained in the human α chain transgene and these mice express the human α/murine γ chain FcεRI on their APCs in a fashion analogous to that in humans [43] in addition to expressing the full α(human)βγ FcεRI on their basophils and mast cells. These mice have the ability to be used for FcεRI APC targeting. However, since the Fcε portion EPL is of human origin, the mice recognize the human ε protein sequence as foreign and generate an immune response to it – a response that will confound any results with repeated vaccinations with EPL based polyplexes. To overcome this, Dr. Kinet's laboratory has now knocked-in the human epsilon IgH in place of its murine counterpart in the human FcεRIα transgenic mice and thereby derived mice that are both human FcεRIα and human IgE positive; mice we have termed "allergy-humanized". Our human IgE-based allergen gene vaccines are expected to be able to be repeatedly administered to such mouse without the confounder of inducing an immune response to the human epsilon chain as in them the human epsilon chain is an endogenous rather than a "foreign" protein. These mice with their human IgE and human FcεRIα provide the appropriate animal system for testing the *in vivo* abilities of the human EPL based polyplexes with peanut allergens as there are well established protocols for inducing and eliciting peanut allergy in mice. We have already constructed the appropriate expression vectors for peanut allergens for this undertaking

Future Directions

At this point, we are poised to test the *in vivo* immunogenicity and immunomodulatory properties of the EPL:peanut allergen DNA vaccine platform. As this is an entirely new approach to food allergen vaccination, we will have to define changes in the humoral and cellular immune response induced by modification of (1) EPL dose, (2) EPL:DNA complex ratio, (3) number/timing of immunizations and (4) differences related to the site/route of immunization. It is likely that outcomes as disparate as induction of strong Th1 immunity *vs.* immune tolerance may occur depending on the specific vaccination conditions employed. The efficacy of naked

DNA vs. EPL vs. EPL+, the latter containing the membrane fusion and nucleus localization sequences, will also be determined so as to determine if EPL and EPL+ gene vaccination show the advantages *in vivo* that they do show *in vitro*.

Once these dose, composition and route testing studies are completed, we will then apply that information to the gene vaccination in peanut allergy to define the ability of this platform to modulate peanut-specific responses in animals prior to peanut sensitization and most importantly in animals with established IgE anaphylactic sensitization to peanut.

As noted earlier, this approach, if successful, could be readily applied to any allergen, however complex, once the genes for the relevant allergens are known. It does not require the GMP manufacture of recombinant allergens, just the availability of GMP EPL and that the sequence of the allergen(s) in question be known. Thus it is not difficult to imagine having a set of on the shelf allergen expression plasmids that could be added to an EPL carrier system to make an allergen gene vaccine mixture that is individualized to a patient's needs. While we are focused on using this gene vaccine strategy in the arena of food allergy, this system is just as likely to find applicability the arena of infectious diseases, cancer immunotherapy or even autoimmunity. In infectious diseases, this would allow the production of vaccines to multiple antigens and/or antigenic variants simultaneously. In cancer, this approach could be used to induce or enhance immune responses to weak antigens. For autoimmunity, by modifying the route and dose of vaccination, it may be possible to induce tolerance rather than a robust T or B cell response. Clearly, we are just at the beginning of our understanding of the potential and problems associated with FcεRI ligand mediated gene vaccination as we have designed it.

Conclusion

As treatment for severe food allergy, standard allergen protein-based desensitization has been both unsuccessful and fraught with adverse events. Allergen gene vaccination is a promising approach to the unmet need of an effective intervention in severe food allergy as well as gene vaccine immunotherapy in infectious, malignant and autoimmune diseases. However, this approach has been limited by difficulties in gene targeting to and expression in antigen presenting cells. Thus we have genetically engineered two IgE-polylysine fusion proteins, termed EPL and EPL plus, capable of binding to DNA vaccine and targeting to FcεRI⁺ positive dendritic cells as a platform to drive efficient peanut allergen gene vaccination. We have shown that EPL platform is able to function as a gene carrier system targeting allergen genes to FcεRI⁺ cells and enhance their expression therein. Thus this platform has the potential to facilitate vaccine gene targeting to FcεRI⁺ DCs for efficient gene vaccination, not only for allergen genes in allergic disease but for any condition in which gene delivery to and expression in antigen presenting cells is desired.

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Discussion

Kleine-Tebbe: There are only a few open receptors. So, what do you think how will you really attack effector cells considering that there is just a very small proportion of constantly expressed Fc?

Saxon: I think once the IgE is bound to the receptor it really never probably comes off, but there is always some cycling out there. And the way you going to get it on there is not by giving it i.v., you have to give it at a high local concentration. So that is why I like the idea, realizing the concentration of IgE in the serum is really low. So if you give μg doses, either intradermally, sublingually or intranodally, you have a high concentration and you do not need a thousand μg . This is not a stoichiometric event, all I need do is to get that gene in there and get it expressed.

Kleine-Tebbe: But you are relying on the on the constant turnover of IgE receptors on the mast cells....

Saxon: No, I am relying on the fact that it expresses the ones that are unoccupied. Because we know for other molecules, like GE2, that you always can get it on. You may not get on 20% of them, but can get on 5%. There are always some that are unoccupied. So this does not require getting 98%, just “get me in there and get me expressed”.

Frew: Have you thought about using the same platform to actually attack the mast cell itself and hook on some other interesting things in order to try and paralyze them in the context of short-term symptomatology?

Saxon: We have done it, but in a totally different way. I do not mean to blow my own horn but I will. We have a molecule, GE2, that binds on the mast cells in a normal and negative way and shuts them off. And one dose of that stops lethal asthma in rhesus monkeys, for four weeks and that is dominant negative. That is where all you need is get a few molecules on the surface to shut the cell off. Not with this platform, however, this platform could get the gene in. The other thing I forgot to add is it also triggers TLR9 because the DNA in the plasmid triggers TLR9 within the antigen-presenting cell. Whether that is good or bad I do not know. I mean, you could see we are really at the beginning and that is why it is going to be fun because we may end up not doing anything in allergy but we may end up curing some other diseases. But we are aiming for allergy because we thought Peanut was a real good place to go. We have all the pieces in place, we have all the criminals in place.