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Particulate carrier systems for mucosal DNA vaccine delivery

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Why mucosal?

``Improvements that make vaccine delivery easier and safer, decrease dependency on the cold chain or reduce number of immunization interventions needed, could have a significant impact…``

Friede & Aguado, ADDR 57 (2005) 325-331
Initiative for Vaccine Research , WHO

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'Ideal' vaccine: the SAFE concept

Stable under high temperature and freezing conditions

Affordable, allowing large scale vaccination campaigns in developing countries

Fast: single-shot (pulsatile release?) increasing compliance, coverage of certain age groups (i.e. adolescents)

Easy application (nasal, topical, oral, pulmonary), avoiding parenteral administration and risk of infection

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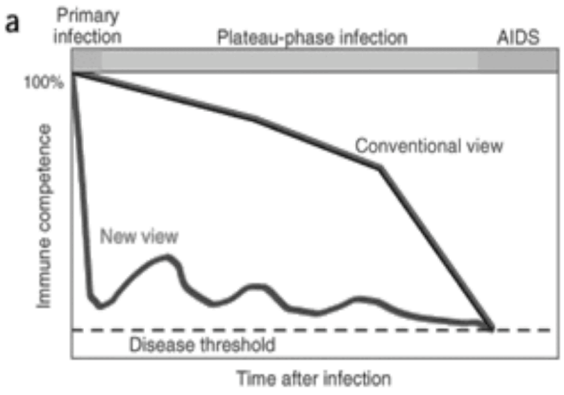
Disease burden in developing countries caused by unsafe injections

	Number	Percentage
Hep B	21.7 M	33%
Hep C	2 M	42%
HIV*	96 k	2%

*worldwide

WHO/BHT/DCT/01.3, pp. 1-7

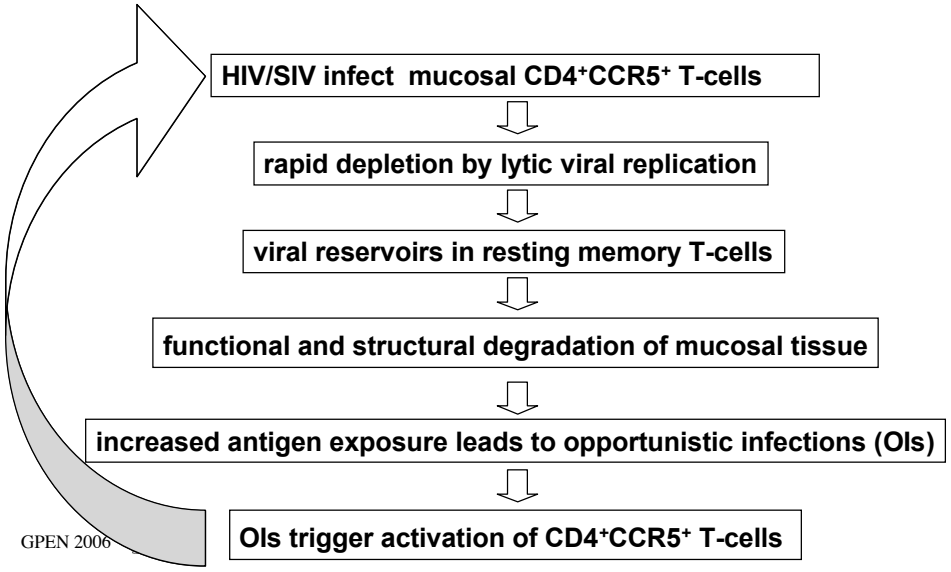
HIV infection changing paradigm: a 'tale of two infections'



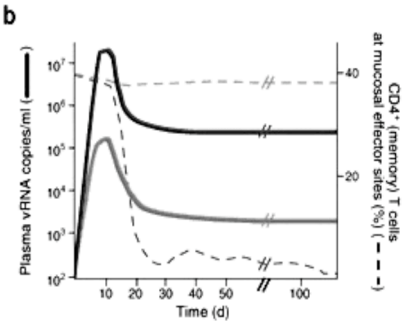
Picker & Watkins, Nat Immunol 6 (2005) 430

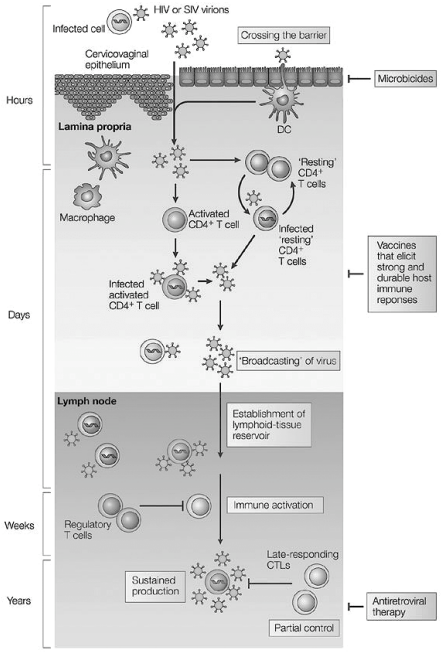
Mucosal surfaces are the port-of-entry for infectious diseases

Role of Mucosal T-cells in HIV:



1st [mucosal] line of defense: Present and Future





Picker & Watkins, Nat Immunol 6 (2005) 430
Haase Nat Rev Immunol 5 (2005) 783

''There has been minimal global effort for clinical trial assessment of vaccine approaches that have the potential to protect at mucosal surfaces during early events…''

''…strategies are needed that could elicit mucosal immune responses in addition to systemic immune responses…''

EU Strategic Position on HIV Vaccine Development, Vaccine 2005, in press

Pulmonary Immunity

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Reed Life Science News updated daily!
By Elizabeth Tolchin

FDA Panel Recommends Inhaled Insulin 9/12/05

A U.S. Food and Drug Administration (FDA) advisory committee voted 7-2 to recommend the approval of Pfizer's, New York, Exubera, the long-anticipated inhaled insulin product for treatment of Type 1 and Type 2 diabetes. If approved, Exubera would be the first non-injectable insulin available in the United States and the first inhaled insulin product on the market.

Exubera, a joint-development program between Sanofi-Aventis, Bridgewater, N.J., and Pfizer, is inhaled through the mouth into the lungs prior to eating, using a proprietary inhalation device and powdered insulin formulation developed by Nektar Therapeutic, San Carlos, Calif.

"By inhalation, the drug is delivered down into the deep lung tissue where it is easily absorbed into the bloodstream," says John Patton, PhD, co-founder and chief scientific officer, Nektar. "The lung is the only port of entry that is naturally permeable. The molecule can go across the membrane without any enhancers or anything that might damage the natural cell layer."

The drug, they say, closely mimics the normal physiological insulin response to meals by quickly being absorbed into the bloodstream to reduce meal-related spikes in glucose levels in people with diabetes.

Because of concerns about the drug's long-term pulmonary safety, filings for regulatory approval in Europe and the U.S. have been put back several times to allow for more safety data. The concerns were whether Exubera will compromise lung capacity or damage lung tissue in long-term use. In clinical trials, the frequency and nature of adverse events were similar in the Exubera and control groups, Pfizer says.

Other inhaled insulin products are in development from Novo Nordisk, Princeton, N.J., and Eli Lilly, Indianapolis, Ind., but Novo has stated that it is working to have its product on the market by 2010.

By Elizabeth Tolchin

Bronchial Associated Lymphoid Tissue (BALT):

- **BALT is *not* a constitutive structure of the healthy adult lung.**
- **Induced by high antigen load, infection, inflammation.**
- **Sampling from lumen by epithelial cells, not through lymph system.**
- **Formed <u>independently</u> of lymphotoxin α (Lt α), inducer of 2° lymphoid organs in embryogenesis and modulator of immune response.**

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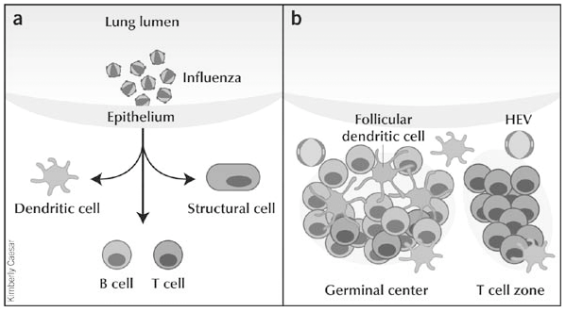
Respiratory immunity in the absence of lymphoid structures: iBALT

- Lymphotoxin (LT) $\alpha^{-/-}$ lack lymph nodes and PP, show disrupted spleen and NALT
- LTα KO mice form lymphoid structures *de novo* in the lung on influenza challenge
- Formation suggested to be mediated by epithelial cells, affecting Mø, DC, T-cells, etc.
- ''iBALT'' structures are capable of staging adaptive immune response on 2° infection



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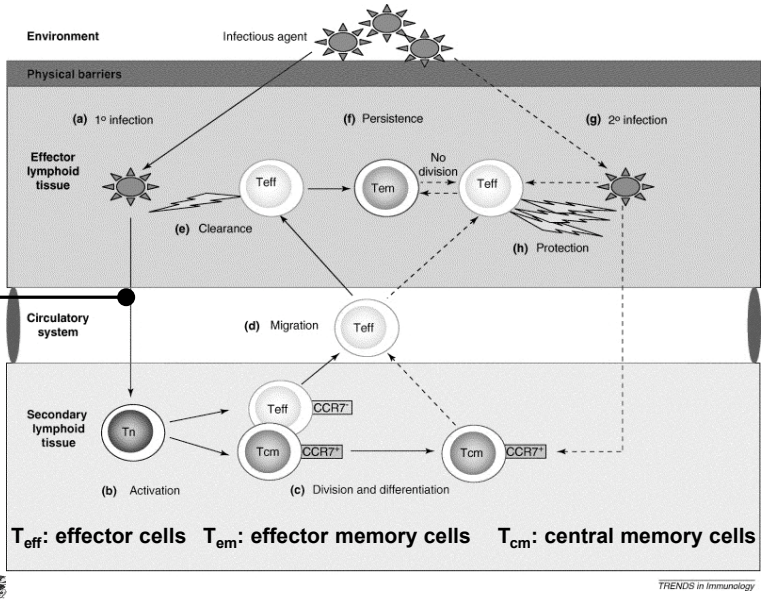
Corbett & Kraehenbuhl, Nat Med 10 (2004) 904
Moyron-Quiroz, Nat Med 10 (2004) 927

Effector Lymphoid Tissue (ELT)



DC migration & presentation

T_{eff}: effector cells T_{em}: effector memory cells T_{cm}: central memory cells

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van Panhuys, Trends Immunol 26 (2005) 242

ELT paradigm:

- Defines and includes pool of T_{em}/T_{eff} cells *outside* 2° lymphoid tissue.
- Formation is the result of stable retention of T-cells post AG stimulation.
- T_{eff} and T_{em} cells stably localized at port-of-pathogen-entry for fast reaction to 2° infection.
- Not limited to mucosal tissues, includes all organs exposed to pathogens.
- Not encapsulated, no anatomically or histologically defined structures.

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Questions:

- Which cells, mediators, receptors play important role in ELT formation?
- How is selective recruitment, retention, long-term survival and replenishment of T_{em}/T_{eff} cells regulated?
- Orchestration of immune response between ELT and 2° lymphoid tissue on 2° infection?
- Optimal vaccine/mucosal delivery system? Adjuvant? Targeting?

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Pulmonary vaccination: Tuberculosis

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Pulmonary delivery of a TB vaccine

Advantages

Immunity at primary infection site

Mucosal and systemic immunity

Reduced need for medical staff

Non-invasive

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Tuberculosis

- **2.2 million deaths per year**
- **2 billion infected**
- **8 million new cases per year**
- **10-15 individuals annually infected by single untreated patient**
- **BCG is <u>not</u> a satisfactory vaccine**
- **No vaccine available for HIV patients more exposed to active TB**
- **Drug regimens are complicated, poor compliance, development of resistant strains**
- **MDR-TB rising, therapy is expensive**

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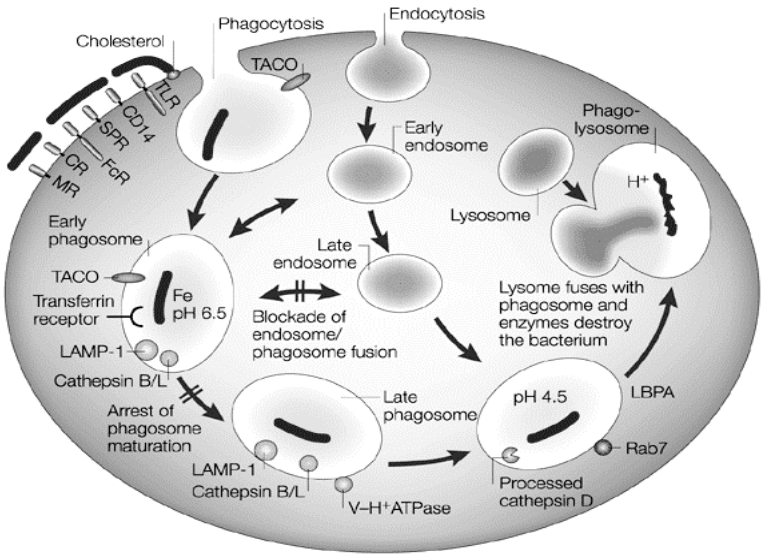
M. tuberculosis, HIV have an intracellular lifestyle



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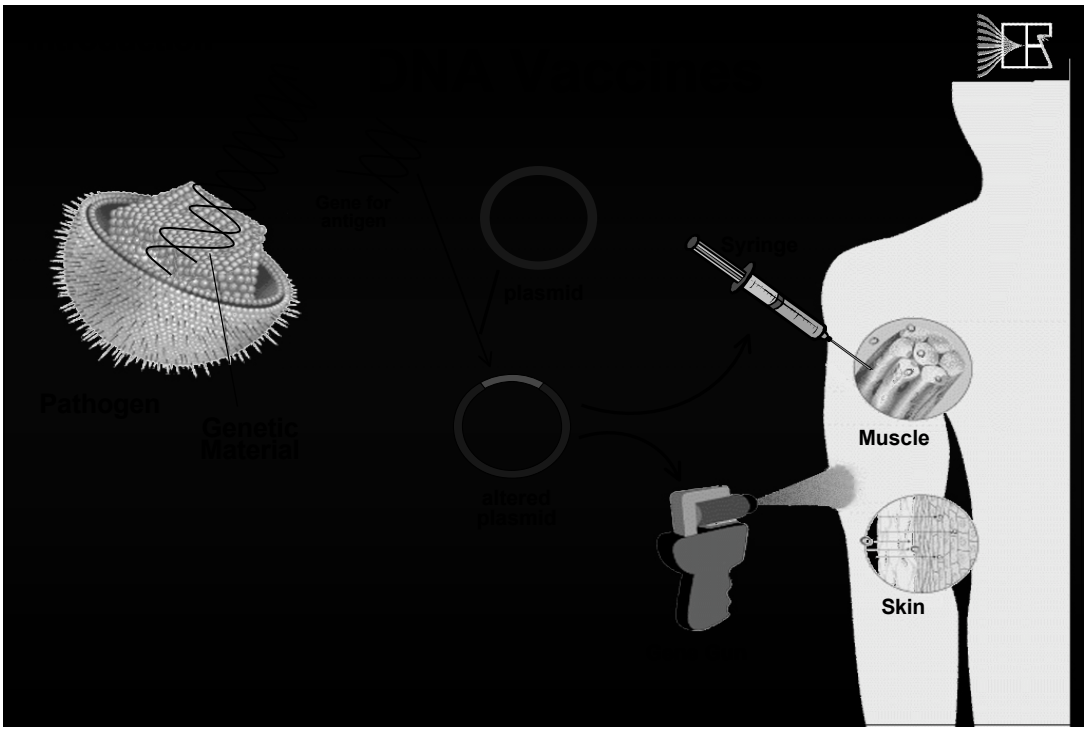
Nature Reviews | Immunology



Example: the M. *tuberculosis* genome

- **4.411 Mbp, 90.8% protein coding genes**
- **Genes with attributed functions: 2,441, unknown: 606**
- **Specific open reading frames (ORF) absent from *M. bovis*: 129.**
- **Absent ORF represent information for potential anti-gens to be integrated in novel pDNA vaccines against tuberculosis.**

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DNA Vaccines for Tuberculosis

- **Ag85 complex (Ag85A, B and C) induces humoral and cell-mediated immunity, protects against *M. tuberculosis* challenge (Ag85A most efficient), encodes fibronectin binding protein. *Huygen et al., Nature Med. 2, 893-898, 1996.***
- **hsp65 induces specific cellular and humoral responses, protects against *M. tuberculosis* challenge, encodes a 65 kDa heat shock protein (hsp). *Tascon et al., Nature Med. 2, 888-892, 1996.***
- **ESAT-6 induces T cell response and IFN-γ secretion. *Olsen et al., Infect. Immun. 69, 2773-2778, 2001.***
- **Other plasmids encoding proteins related to different stages of *M. tuberculosis* development.**

Optimisation of DNA vaccines - increasing cellular/humoral responses by:

• immunostimulatory sequences neighbouring CpG motifs:

pupuCGpypy (pu: A,G; py: T, C)

• integration of genetic information for cytokines:

-> Th1 cytokines (IL-12, IFN-γ) to stimulate cytotoxic T-cell (CTL) response

-> Th2 cytokines (IL-4, -5, -10) to stimulate humoral response

DNA vaccines: formulation parameters

- **DNA vaccine parameters:**
  polyepitope, size, enzyme stability
- **Nature pathogen/disease:**
  viral/bacterial, route of entry, progression of disease
- **Desired immune response:**
  Humoral, CTL, Th1/Th2
- **Delivery system:**
  Administration route, targeting, delivery device

DNA vaccines: administration routes alternative to injection

1) mucosal: oral, nasal, vaginal, rectal, pulmonary

- **interaction with local immunoactive tissues, e.g. Peyer's patches**
- **induction of both, local and systemic immune response (i.e., IgA and IgG)**
- **cross-talk between mucosal tissues (Mucosal Associated Lymphoid Tissues, MALT)**
- **strong involvement of dendritic cells (DC), especially in the lung**

2) Gene gun

- **intradermal injection of DNA vaccine coated gold particles**
- **stronger Th2 bias than i.m. injection**

Gene gun approach:

- **DNA coated particles are injected into the cells: improvement of uptake by Langerhans' cells**
- **less priming by CpG motifs through TIR interaction**
- **lower expression of CD, MHC**
- **resulting in Th2 bias**

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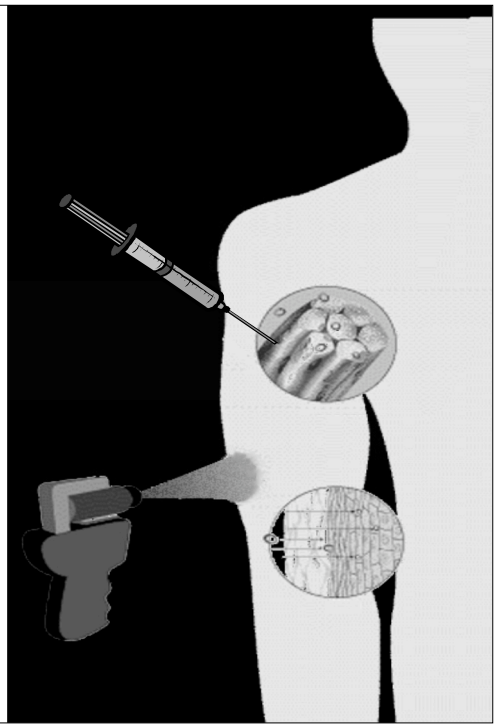
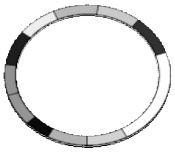


Concept

New DNA construct
Class I specific epitopes



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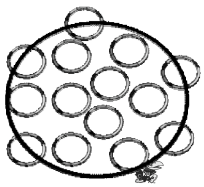
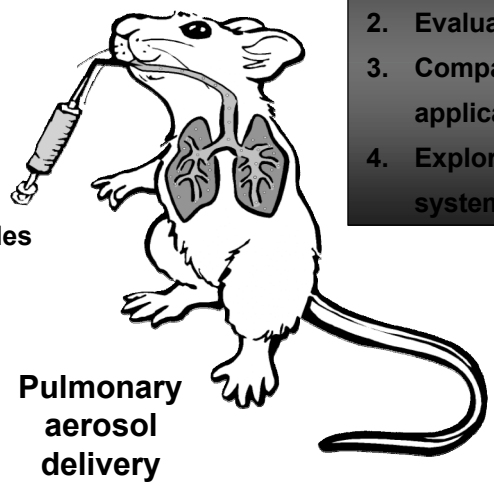
Chitosan nanoparticles



Class I transgenic mouse model



Pulmonary aerosol delivery

Aims:

1. **In vitro testing Calu-3, DC**
2. **Evaluate T-cell response**
3. **Compare i.m. to pulmonary application**
4. **Explore the effect of carrier system**

Polymer-based DNA vaccine delivery systems

- **condensation of DNA by electrostatic interactions**
- **reduction in size, zetapotential**
- **protection against enzymatic degradation, DNase I/II**
- **endolysosomal escape**
- **stability, shelf-life**
- **toxicity**

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Chitosan nanoparticles

- **Chitosan n.p. were proven to be efficient carriers for oral delivery of DNA vaccine against peanut allergy (Leong et al.)**
- **Chitosan-DNA complexes (nano-size) showed good pulmonary transfection in-vivo (Köping-Höggård et al.)**
- **Chitosan-DNA complexes (nano-size) were shown to be safe and efficient gene delivery systems in epithelial cells (Thanou et al.)**

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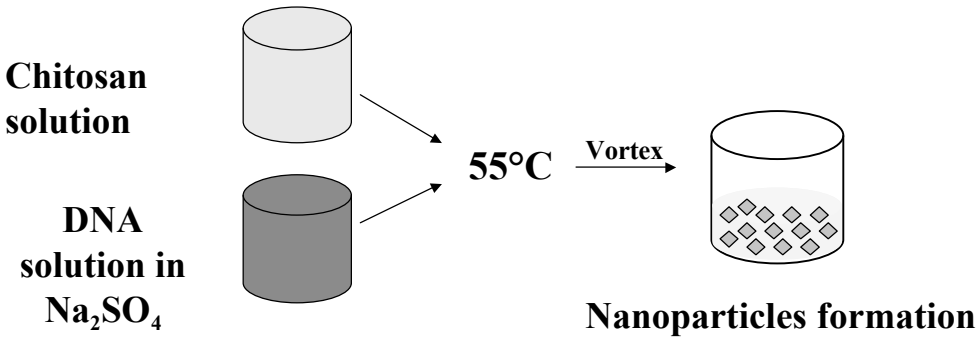
Preparation of chitosan nanoparticles



Characterization of size, zetapotential, DNase protection, DNA loading and release

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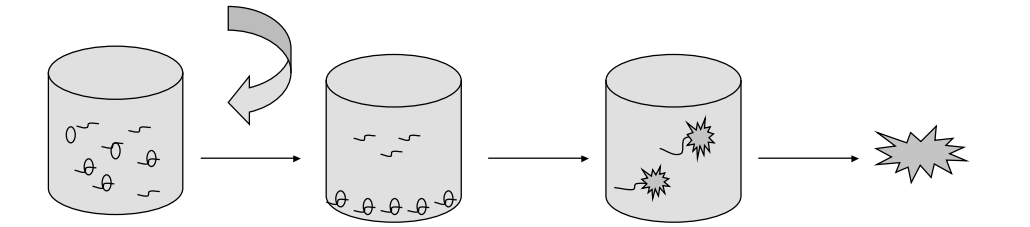
Loading Efficiency



$$\text{Loading Efficiency (LE)} = \frac{(\text{total DNA} - \text{free DNA})}{\text{total DNA}} \times 100\ \%$$

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Characteristics of chitoplexes

- Size of chitoplexes: 200 - 400 nm
- Charge at pH 5.5: 20 - 27 mV
  - strongly dependent on pH
  - positive charge good for cell attachment and uptake
- Loading Efficiency: > 95%
  - efficient procedure; no material loss

→ Size, zetapotential and LE independent of (N/P) ratio
→ Strong charge interactions

Enzymatic assays

Is DNA in chitoplexes protected against nucleic acid degradation by chitosan?
→ Incubation with DNase I

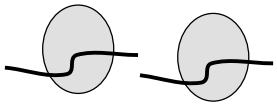
When the chitosan in chitoplexes is degraded by enzymes, is the DNA released in intact form?
→ Incubation with chitosanase

Incubation with DNase I (1)

Free DNA

Incubation with DNase, 37ºC

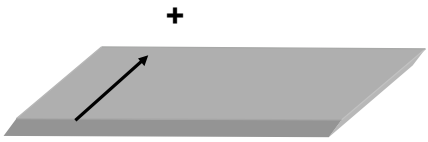
Fragmented DNA



chitoplexes

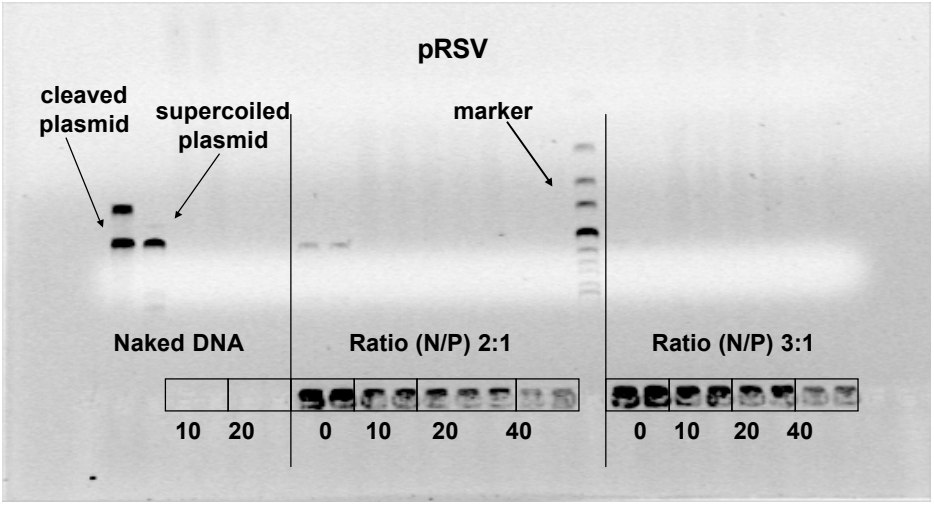
Intact DNA ?

Analysis by agarose gel electrophoresis

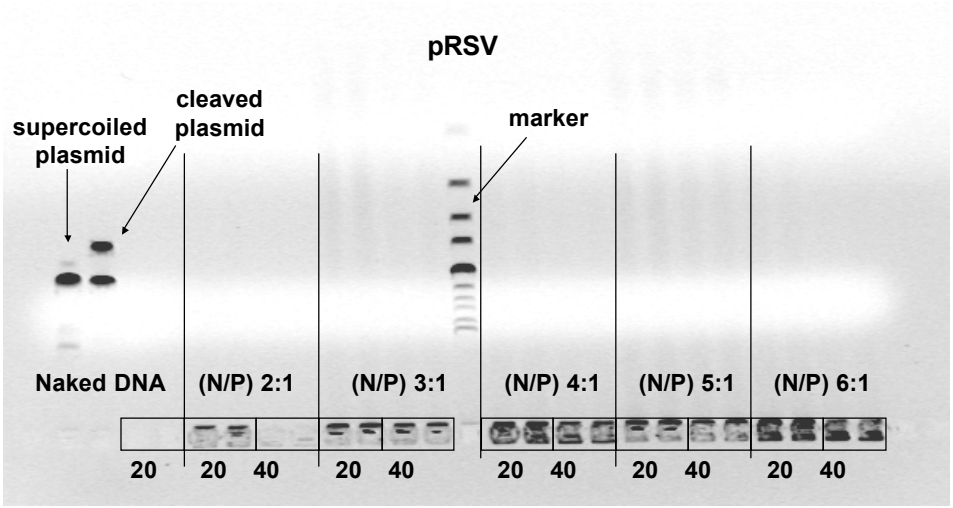


55 min, 100 V

Incubation with DNase I (2)



Incubation with DNase I (3)

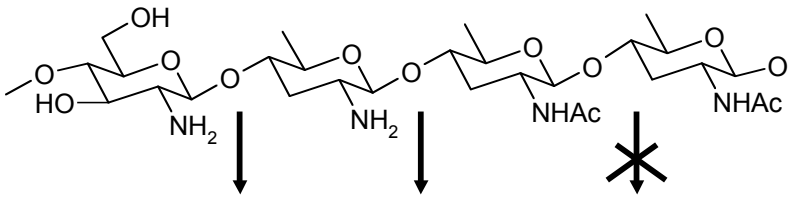


Conclusion: Incubation with DNase I

- Compared with naked DNA, the DNA in chitoplexes is protected against nucleic acid degradation by chitosan
- The more chitosan, the more protection?
  - Ratio (N/P) 2:1 is less protected
  - no significant differences at ratios between (N/P) 3:1 and 6:1

Incubation with chitosanase (1)

- **Chitosanase present in micro-organisms and plants**
- **used chitosanase: from *Streptomyces griseus***



degradation products: oligochitosan 2 - 6

Stop solution: 1M KOH

In humans: degradation by lysozyme

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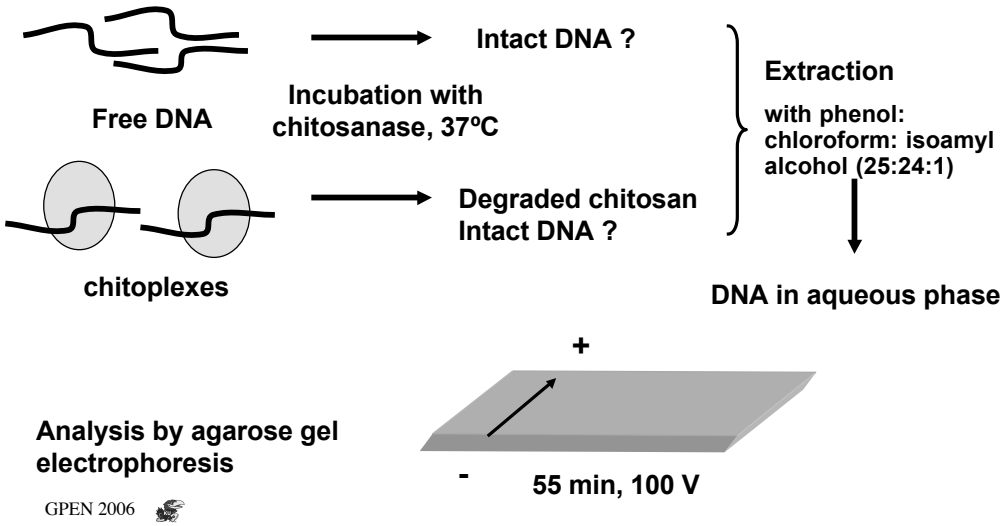
Incubation with chitosanase (2)



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Conclusions: chitosanase assay

Chitoplexes are partly degraded by chitosanase.

Only free DNA, no chitoplexes, are extracted.

After extraction some free DNA stays at loading position.

Free DNA is released and partially fragmented.

Fragmentation is due to the stop solution (1M KOH).

After enzymatic degradation of chitoplexes, DNA is intactly released.

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DNA vaccines: advantages

Immunogenicity	**induces humoral and cellular immune responses** **low effective dosage in animal models**
Safety	**unable to revert into virulence,** **no toxic treatment needed as in live vaccines**
Engineering	**vectors easy to manipulate, fast testing** **combinatorial approaches easily adapted**
Manufacture	**low costs, reproducible large-scale production**
Stability	**temperature-stable than conventional vaccines** **long shelf-life**
Mobility	**easy storage and transport, no cold chain**

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DNA vaccines: Challenges

- **adequate animal models**
- **extention of plasmid survival: better immune response?**
- **will prolongation of antigen synthesis elicit autoimmune responses?**
- **interindividual differences in immune responses?**
- **dendritic cell targeting**
- **selection of antigens -> genomics approach (inverse vaccinology)**
- **prime/boost regimens and adjuvants**
- **….**

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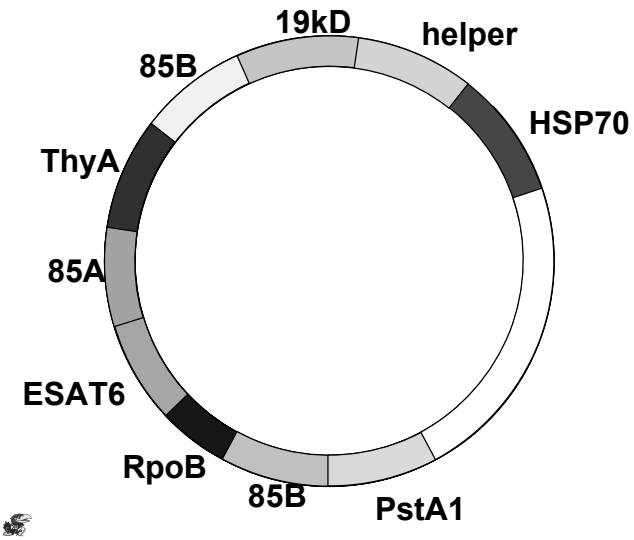
The DNA plasmid



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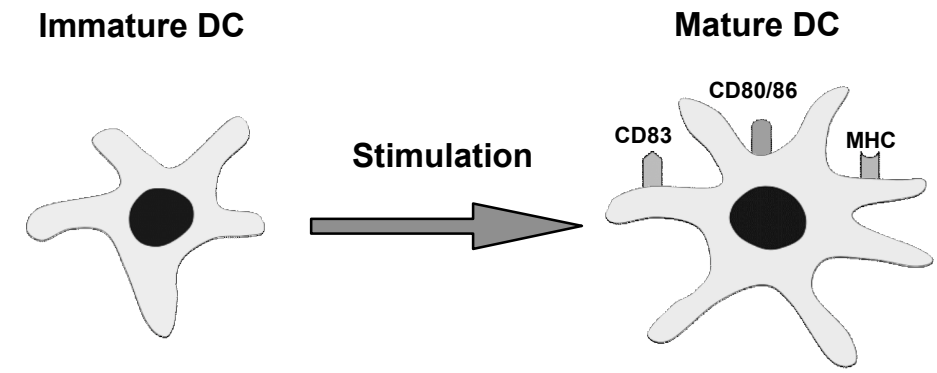
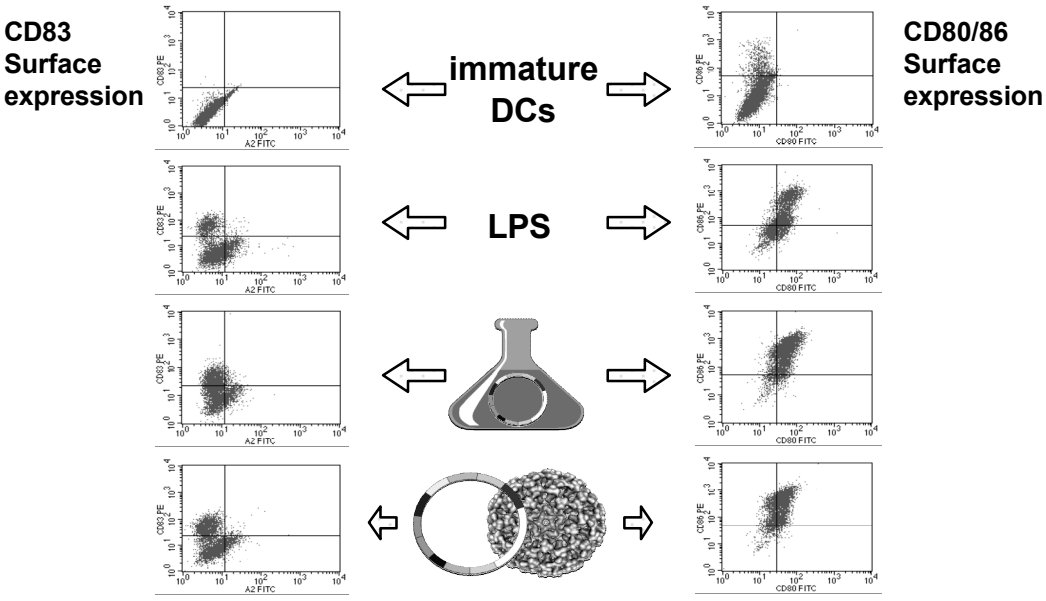
In vitro testing

Dendritic cells



Dendritic cells maturation



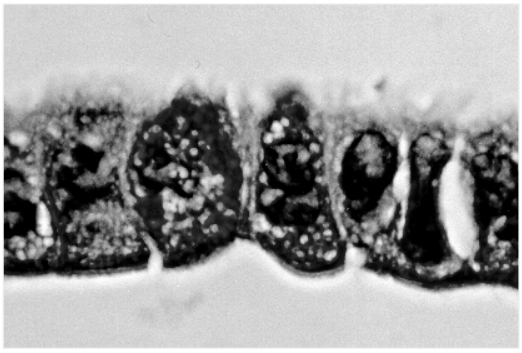
Comparison of cell culture models of the airway epithelium

	in vivo	PTC#	RTC	Calu-3	16HBE14o-
Tight junctions	+	+	+	+	+
P_{app} mannitol	5-10	1.5-3.5	1.2-2.8	0.5-1.0#	3.1
Cilia	+	+	+	+	+
Mucus	+	+	+	+	-
CFTR expression	+	?	?	+	+
P-gp expression	+	+	?	+#	?
Cell yield/trachea	-	$6x10^7$	$2.5x10^7$	-	-

RTC: rabbit tracheal epithelial cells, Calu-3: human submucosal gland cell line, 16HBE14o-: human bronchial cell line, P_{app}: apparent permeability (10^{-7}cm s^{-1}), CFTR: Cystic Fibrosis Transmembrane Regulator protein, P-gp: P-glycoprotein

 # = own data, all other data taken from current literature.

Calu-3 cells: mucus staining Periodic Schiff's, Alcian Blue

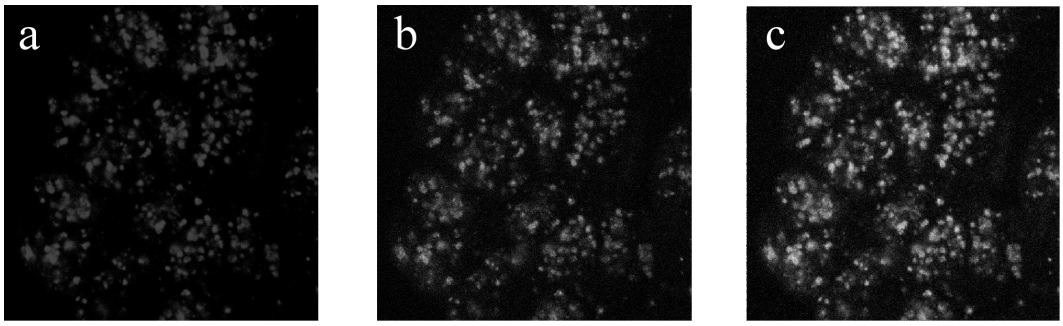


Calu-3 cells, grown at air interface, 100X

- Calu-3 express human *MUC*1, *MUC*4, *MUC*5 and *MUC*5B genes
- Calu-3 secrete proteoglycans and sulfated mucins
- Calu-3 apical surface fluid exerts anti-bacterial activity
- Calu-3 are used for investigation of mucus as a barrier to gene delivery

Meaney et al., Cell culture models of biological barriers, Harwood 2002

Uptake by human bronchial epithelial cells (Calu-3) *in vitro*

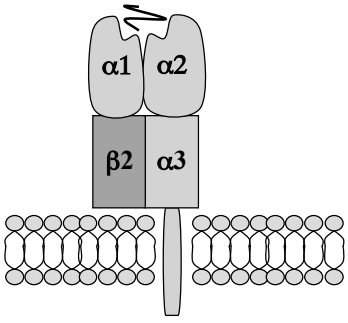


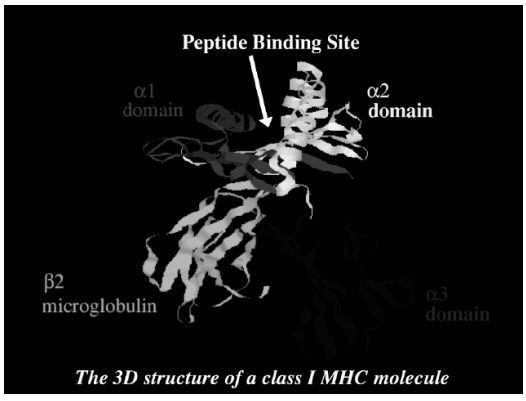
LAMP-1 **rhodamine-DNA** **superimposition**

Bivas-Benita et al., *Eur. J. Pharm. Biopharm.* 58 (2004) 1-6

In vivo testing

MHC class I

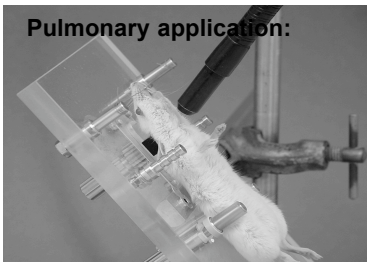




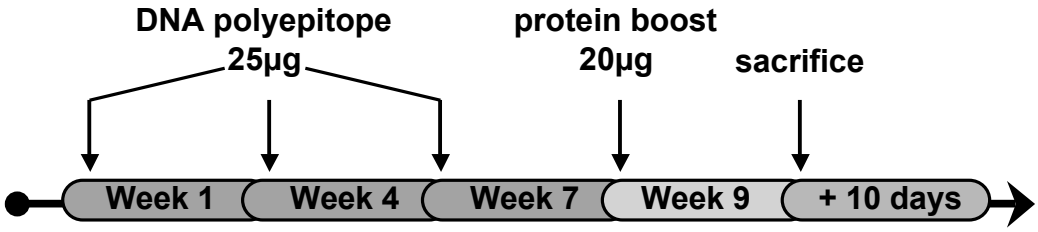
The 3D structure of a class I MHC molecule

Experimental groups

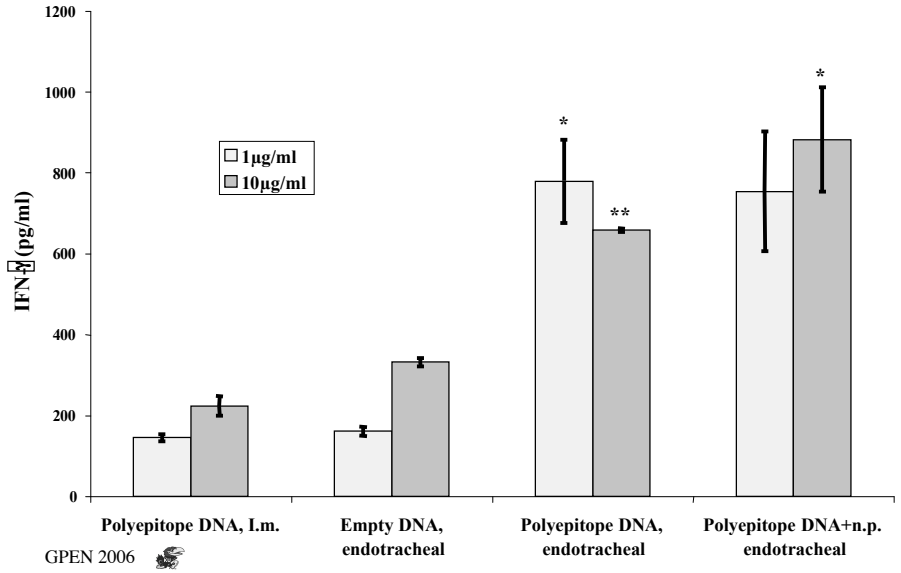
Group	DNA plasmid	Application	Formulation
I	Polyepitope	Intra-muscular	Solution
II	empty	Endotracheal	Chitosan n.p.
III	Polyepitope	Endotracheal	Solution
IV	Polyepitope	Endotracheal	Chitosan n.p.



Immunization Regimen



IFN-γ (*M. tuberculosis* sonicate)



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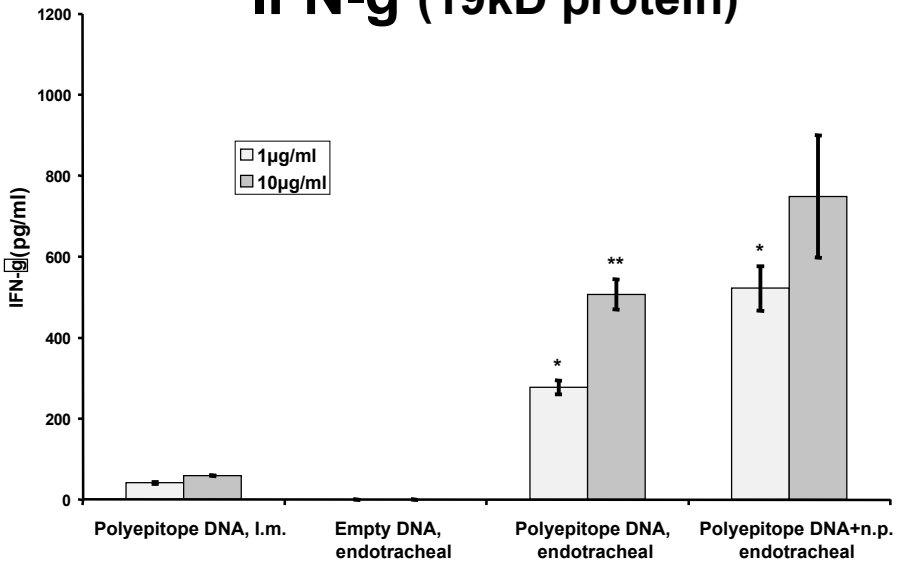
IFN-g (19kD protein)



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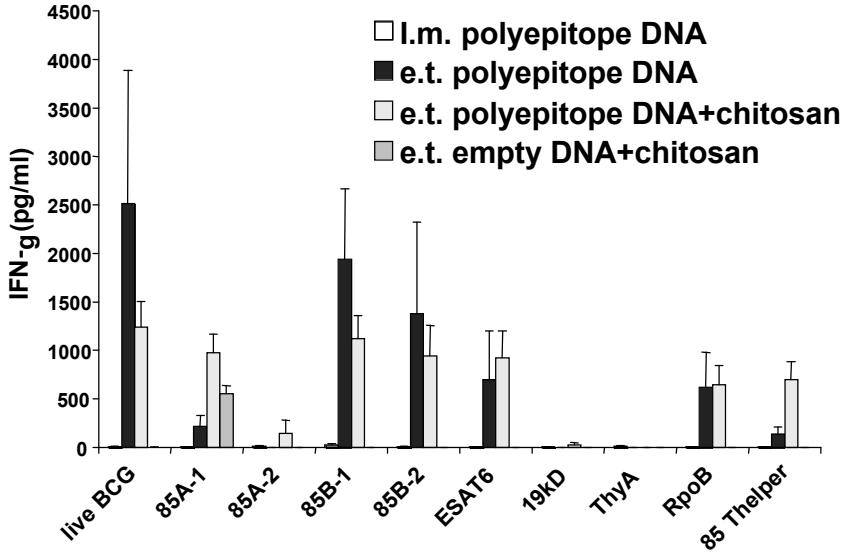


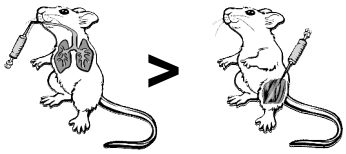
GPEN 2006

Bivas-Benita et al., *submitted*

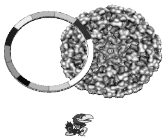
Conclusions

Maturation of DCs in culture

Induced in-vivo T cell responses toward M. tuberculosis sonicate.



The pulmonary (e.t.) immunization had a significant advantage over i.m. administration.



Chitosan n.p. enhanced IFN-g production in comparison to the DNA solution

Conclusions:

Mucosal surfaces of the lung are suitable for eliciting local and systemic immune response.

Problems of parenterally applied vaccines are avoided (patient compliance, risk of infection, infrastructure).

DNA-vaccine offer advantages over subunit vaccines (combination of antigenic structures and adjuvants, stability).

Optimization of both vaccines and carrier systems for mucosal application, especially if applied pulmonary, is necessary.

GPEN 2006