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A DNA minigene vaccine encoding Legumain epitopes suppresses cancer growth

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Abstract

Here two DNA minigene vaccines against the protein Legumain over-expressed by cancer cells were constructed to suppress growth and metastasis of cancer. The two epitope-based minigenes, each encoding three different epitopes of Legumain, separated by a spacer sequence, were targeted to the endogenous Ag presenting pathway for MHC class I-restricted $CD8^+$ T-cell to mediate tumor cell killing via an ER targeting signal. This Legumain based minigene vaccine was delivered orally by a bacterial carrier system of doubly attenuated *S. typhimurium (aroA*$^-$ and *dam*$^-$*)* and immunized mice were lethally challenged with breast and lung carcinoma cells. The specifically induced immune response partially protected mice from growth of primary subcutaneous tumors and suppressed dissemination of pulmonary metastases.

Keywords

DNA vaccine, minigene, Antigen (Ag), MHC class I, Legumain, Spacer, ER, CTL/CTL-assay

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Sammanfattning

Vaccinering är en accepterad metod för att förebygga sjukdomar genom aktivering av immunförsvaret mot en främmande organism. Cancer är en sjukdom men är inte kroppsfrämmande. En ny framträdande teknologi är genetisk immunisering såsom DNA-vacciner. Ett DNA-vaccin kodar för proteiner och genom att i hög grad inducera ett kroppseget protein för naiva cytolytiska T lymfocyter (CTL:er), s.k. ”mördarceller”, kan dessa aktiveras mot kroppsegna proteiner som tumören överuttrycker.

Legumain är ett stressinducerat aspargin–enzym som är mycket aktivt och involverat i cancers spridning. I normala fall uttrycks inte Legumain, förutom i mycket låga nivåer i lever och njure. Här syntetiserades ett DNA-minigenvaccin. Nyttjandet av en minigen d.v.s. en DNA-sträng som kodar för subenheter av proteinet, möjliggör identifiering av specificiteten i immunsvaret respektive eventuell toxicitet i form av autoimmunitet.

Vaccinet administrerades oralt med en muterad variant av bakterien *Salmonella typhimurium (aroA*$^-$ och *dam*$^-$*)*. Därmed levererades plasmid DNAt direkt till sekundära lymforgan som Peyers patches. Möss immuniserades med tre vaccinationer, därefter injicerades två olika cancermodeller: en bröstcancer subkutant och en lungcancer intravenöst. DNA-vaccinet visade sig i båda modellerna ge en skyddande anti-cancerogen effekt. För att undersöka om ett specifikt Legumain-inducerat immunsvar hade bildats gjordes ett CTL-experiment, vilket indikerade att så var fallet.

Examensarbete 20p i Molekylär bioteknikprogrammet

Uppsala universitet juni 2005

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1. Introduction

Vaccinations have for a long time been accepted to be one of the most proven effective weapons to fight diseases and represent a great success in the improvement of public health. Vaccination equals immunization e.g. introduction of a protein of a foreign organism in our body and induces, in turn, an immune response against that specific protein. Vaccines can be designed to protect against infection. Genetic immunization i.e. DNA vaccines, are part of a rapidly developing technology which offers new approaches in designing treatment of autoimmunity, allergies and cancer (2). To fight cancer today one uses established treatments like surgery, irradiation and chemotherapy. Although, these methods can be effective, they often have severe side effects and in too many cases do not save human life. Tumor invasion and metastasis are the major causes of fatal human cancer and are linked to 90% of cancer related deaths (3). One approach that attempts to block tumor growth and metastasis is the use of DNA vaccines encoding self-proteins that are over-expressed during cancer development. One way to assess the efficacy of such treatments in inhibiting tumor growth is by targeting cells, which over-express these proteins to actively evoke immunity. Importantly, in contrast to "normal" vaccinations, these proteins are not foreign to our body. DNA vaccines are comprised of plasmid DNA encoding self-antigen which can trigger protein production directly in transfected cells of the target organism (2). Thus, these DNA vaccines have the possibility of direct entry of the antigen into the major histocompatibility complex (MHC) pathway, which present peptides, derived from proteins, to the immune system (4). The name of the DNA constructs here are $H2D^b$ and $H2K^b/H2D^d$ and $H2K^d$, this being also the name of the mouse MHC locus, the uppercase letters b and d indicate the type of allele of the MHC (1). The evolution of DNA vaccine-applications is based upon a variety of reports of successful vaccinations with plasmid DNA, applying either injection of naked DNA or delivery systems of attenuated microorganisms carrying such plasmids as used here. Thus far, DNA vaccines mainly serve to complement other cancer treatments and many of the questions of the efficacy of peptide vaccines need to be answered by clinical trials (5).

Several advantages of genetic immunization over conventional vaccination have been observed. One advantage using DNA vaccine in this field compared to conventional vaccines, is that the DNA can be detected for a considerable period of time and thus serve as a depot of antigen and may be efficacious in inducing long-lived, tumor mediated, T-memory cells (6,7,8). Although the precise mechanisms involved in the induction of an immune response following DNA immunization have not been fully determined, there is a fairly good understanding of how antigens are processed, presented and recognized by cells of the immune system (2).

1.1 The aim of the project

The protein Legumain was previously identified and was shown to be involved in cancer progression. Also, there have been attempts to develop a new cancer drug based on this enzyme (3). It is evident that, this protein seems to be a perfect target for the creation of a DNA vaccine to prevent cancer. The demonstration of the most and efficacious DNA vaccines requires the characterization of cytotoxic T lymphocytes (CTL's), induced by it, specificity and determination of immune dominant peptide/epitopes. These epitopes which generate the immune-response against cancer

cells also can give rise to possible harmful side effects such as autoimmunity mediated by these specific CTL's. However, careful deliniation of these epitopes makes it possible to elucidate and optimize the underlying immunological mechanisms, in terms of generating an optimal anti-tumor activity and strong cytotoxicity. In this thesis, the primary aim was to construct Legumain epitope based DNA minigene vaccines, more specifically, two minigenes each enclosing a string of three different Legumain epitopes. This approach enables us to study the mechanisms of the constructed epitopes and eventually to outline in more detail the specific elicitation the immune response to Legumain DNA vaccination. Since this project is still in its early phase, it will focus on the creation of these vaccine constructs and test these Legumain epitopes in mouse models, *in vivo,* to assess whether they contribute to any anti-cancer effects.

2. Background

2.1 Prerequisites

The idea of genetic vaccines came from the surprising observation that injection of naked plasmid DNA into muscle or skin of the host resulted in transfection of the muscle cells and lead to expression of proteins encoded by the DNA (9). Since transcription is driven by eukaryotic control elements the bacteria cannot synthesize the proteins of the plasmid DNA-antigen (6), which trigger protective responses of B and T-cell stimulation (2). From the beginning, specific CTL's in cancer patients provided evidence for an ongoing, though insufficient, immune response against these tumors (1,5). The idea is to evoke an immune response by activating CTL's as efficient killer cells, which can be effective therapeutic agents against malignant diseases. Most antigens recognized by CTL's are short peptides, 8-10 residues (amino acids) long that are bound to MHC class I molecules. These peptides have been produced by the degradation of proteins that are endogenously produced inside the cell (1). It has been postulated that tumor immunity is weak or ineffective because the tumor antigens (Ag's) are self-antigens, which are recognized by low affinity T-cells. For CTL recognition of these tumor antigens to occur, tolerance to these antigens either must occur or must be overcome *in vivo* or *in vitro* (7). Thus, it is necessary to break peripheral T-cell tolerance for a self-Ag by inducing its over-expression making this Ag immunogenic. Thus, likelihood of success in cancer immunotherapy is determined by the efficiency of the therapy in inducing an immune response against the tumor rejection of the Ag(s). A major focus of tumor immunologists therefore is the identification of the subset of MHC-associated peptides that are selectively expressed on tumor cells and that act as epitopes for tumor-specific CTL's.

Identification of several CTL epitopes derived from normal native gene products provide evidence that a component of tumor self-antigen is autoimmunity and other side effects like cross-reactivity with normal tissue (5). Multiple factors contributing to a elicitation of a CTL response are: the efficiency of production (e.g. proteolysis and concentration) of the peptide, transport to the endoplasmic reticulum (ER), peptide affinity to the MHC complex and the frequency of potentially reactive "avidity" T cells in the repertoire (10,11).

2.2 Experimental trials

DNA vaccines have been very successful in rodents and work in mice and rats have shown very promising results (12). In prophylactive murine tumors models, a naïve animal can be protected by vaccination against a subsequent lethal tumor cell challenge and can therapeutically eradicate two weeks established tumors (13). Today, DNA vaccines are in clinical trials and are distributed as a prime-boost vaccine. However, results of human immunotherapy have not been as successful as those done in murine tumor models. In general, tumor Ag's appear to be less immunogenic in large animals (12). Unlike experimental murine cancer, human cancer is better established; potential barriers against equivalent success in humans are the gradual growth of tumors over years and the difficulty of immunizing against tumor self-antigens to which a considerable degree of tolerance exists (5). Probably due to these strictures, the results of tumor treatment in early stage I cancer patients have been relatively good but proved less successful in advanced stage III/IV patients (14). One should not forget that practical problems arise when the drugs, in this case DNA vaccines, are tested in human clinical trials where optimal dose may not be the maximum tolerated dose and where it is difficult to define the major dose-related toxicity. Evaluation of novel tumor vaccines depends therefore on the evaluation of those aspects of the immune response that are believed to be affected by the vaccine and to evaluate cross-reactivities and toxicities. If these occur one should have in mind that they could also depend on other factors such as the immunologic milieu into which the vaccine is administered (5).

2.3 DNA vaccine/Minigene

In contrast to protein vaccines consisting of killed tumor cells or recombinant proteins, DNA vaccines can stimulate both humoral and cell mediated immune responses i.e., B and T-cell mediated responses (2). Compared to protein vaccines DNA vaccines are easy and rapidly constructed and have a greater chemical stability than RNA vaccines that are degradation sensitive and require more elaborate and expensive experimental methods. Although virus delivered DNA vaccines have the advantage to be strongly expressed by target cells, there exists less of a risk of development of insertional mutagenesis (4). Due to the fact that DNA vaccines are easy and cheap to produce and have almost no side effects, they can be given to patients at risk e.g. persons with a family history of breast cancer.

DNA vaccinations with onco-genes still carry with them the risk of transformation in transfected cells, which receive and express them. However, onco-gene peptide based vaccines have no transformation risk (13). Normal host genes encode cancer antigens. The same or different type of cancers in different individuals, can express the same or different antigen(s) where cancer escapes our immune system by down-regulation of MHC antigens or loss of immunogenic epitopes (13). Therefore, one advantage over single epitope-based vaccines, in designing minigene vaccines is the possibility to create a cocktail of combined epitopes from different proteins in one vaccine, encoding these immunogenic peptides. Thus, an antigen or a series of antigens expressed by one type of cancer in different individuals can be put into one construct, where some shared antigens are required. These polypeptides comprising multiple epitopes from native heterologus proteins as subunits of the minigenes vaccines have an advantage compared over entire genes, since such genes have a limited capacity when combined with vectors (10).

Identification of the antigens recognized by human T-cells makes it possible to deliver the same Ag's in large quantities, thereby augmenting the immune response to a specific protein/epitope, by excluding other non-activating epitopes to be presented (15). A minigene provides also the opportunity to evaluate immunologic toxicity in terms of specific autoimmunity and the ability to assess immunologic responses in terms of known specific epitopes (5).

2.4 Legumain

Legumain is an asparaginyl endopeptidase conserved from plants to humans. Human Legumain shares 83% homology with murine Legumain (16). It functions as a stress-responsive enzyme i.e. a serine protease induced on the cell surface by stress, such as heat shock, drug treatment and hypoxia. The unique serine protease Legumain is over-expressed *in vivo* on most solid tumors tested especially on neoplastic cells and tumor associated macrophages (TAM's) in the tumor microenvironment (TME) of solid tumors and endothelial cells in the tumor vasculature. Corresponding cultured tumor cell lines tested did not express Legumain *in vitro* (3). Furthermore Legumain was expressed at low or undetectable levels in most normal tissue, suggesting that these tissues would act as poor target for Legumain specific CTL-mediated lysis. In liver and kidney cells Legumain is expressed as a lysosomal protease (16). This agrees well with Legumain functioning in the TME.

Legumain is active in acidic conditions like those in the TME; it has been associated with lysosome-autophagy in tumors since cells self-digest to meet metabolic demands. In cancer cells with migratory and invasive properties, it has been demonstrated that Legumain is concentrated in membrane-associated vesicles at the invading areas of tumor cells and on cell surfaces were it co-localizes with integrins (fig.1). There even exists a correlation between some cysteine peptidases and activation of the enzymes zymogen and progelatinaseA, i.e. mediators of extracellular matrix degradation that are helping to invade other tissue giving the tumor a more progressive phenotype (3).

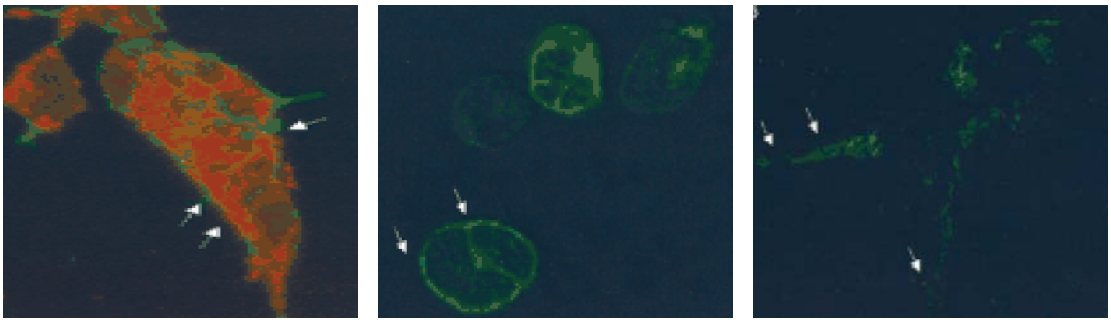

Fig. 1) Cellular distribution of Legumain and activity (Legumain detected in green) pictures used with permission from Liu *et al*., 2003.

TAM's are found in the stroma of many solid tumors were they are recruited by specific gradients of chemokines produced by tumors to promote tumor progression and to increase both metastasis and tumor angiogenesis. The over-expression of Legumain's in TAM's implies an increased tumor growth and metastasis. Since TAM's, which are recruited by chemokines into the TME, have an immuno-suppressive function and are promoting angiogenesis by synthesizing angiogenic regulators and proteases. They are also involved in enhancing tumor progression allowing tumor cells to escape into the circulation. The main task of TAM's is to

function as providers for the tumor's survival (17). Thus, this novel approach in killing TAM's could result in suppression of angiogenesis and induction of tumor cell apoptosis. Advantages in targeting the neovasculature in contrast to tumors cells are genetic stability, lack of down regulation of MHC class I and II antigens and decrease in the tumor blood supply (7). All these arguments suggest that Legumain can serve as an effective target for a DNA-based vaccine.

2.5 Pathway of a minigene - design of a construct

Normally CTL epitopes like viral and tumor-associated Ag's are synthesized within the cell, processed in the cytoplasm/proteasome into peptides, which are transported by transporter associated with antigen processing (TAP) (fig.2.E) molecules into the endoplasmic reticulum (ER) (18). Previous findings demonstrated that cells which are TAP deficient are still capable of presenting peptide-antigens through the MHC class I pathway where, such peptides must have entered the ER by another route. In TAP-deficient cells Ag processing in the ER contributes to the generation of antigenic peptides where the response was even enhanced (18). It was stated that DNA vaccines induce a potent "killer" $CD8^+$-restricted CTL- response in mice, encoding a whole protein down to a single minimal CTL peptide. In this case the antigens are targeted to the cytoplasm, nucleus or ER (fig.2) (19). Minigene with peptides corresponding to MHC class I antigen restricted CTL epitopes derived from tumor-associated antigens were identified and were shown to contribute to tumor cell killing, demonstrating that minigene targeting to the ER works (15,19). Soluble exogenous proteins like bacteria associated antigens are taken up the APC's, processed in low pH endosomal compartments within the cell, and presented by MHC class II molecules to the CD4+ T-helper and B-cells of the immune system (the other part of the dashed line in fig.2).This pathway is not targeted by the minigenes used in this project.

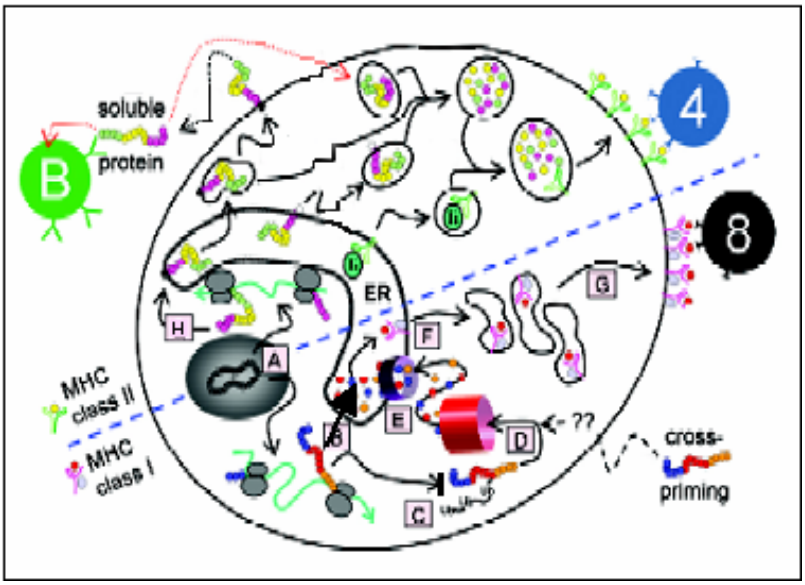

Fig. 2) Different MHC class I/II presenting pathways, in this case the bold black arrow indicates the pathway targeted by the minigene here. A) DNA transcribed in the nucleus B) subsequently translated in the cytosol and in the ER D) Proteasome and E) TAP-transporters are avoided here. F) Transported to the golgi-apparatus. G) Further to the cell surface, here the peptides are ready for the $CD8^+$ T-cell repertoire. The figure is a modification from Leifert *et al*. 2004.

2.5.1 Targeting the ER

There are several research reports concerning tumors progression after minigene-DNA vaccinations, with the first reported successfully DNA vaccination being a minigene targeted to the ER (12). Specific targeting of epitope-based DNA vaccines to several cellular compartments was shown to increase protection against lethal

cancer cell challenge (20). There is experimental evidence that a given antigen can be highly immunogenic in one form and poorly or non immunogenic in another. In these cases the antigens needed to be targeted to the ER in order to become immunogenic/protective (2). TAP independent Ag processing contributes to antigen presentation and is enhanced by a signal peptide targeted to the ER (20). Ag presentation can also be independent of the proteasome according to Schwarts *et al.,* 2000 and nontoxic concentrations of proteasome inhibitors do not effect MHC class I expression (21). Surface expression of $H2D^k$ is even increased by the proteasome inhibitor lactacystine (22). CTL priming is thought to require higher concentration of MHC class I peptides-complexes than normal recognition and limited peptide generation may result at a cell surface density below the threshold required for induction of a protective CTL-response (12). ER-targeting may be able to increase the epitope/MHC class I ratio on the APCs cell surfaces up to the threshold to activate naïve T-cells (10). The production efficiency of MHC class I peptide complexes for minigenes injected into the cytosol were 1 in 50 vs. 1 in 4000 for whole protein antigens (22).

This information supports the design of a minigene construct with an ER leader sequence that targets proteins directly to the ER after being translated. These native antigens we use are weak as compared to others strong foreign antigens such as viral antigens, OVA, and myco antigens (7,12). We propose to use an ER leader sequence from the adenovirus glycoprotein, E3/19K and the maintenance by an ER retention signal, which was shown to significantly enhance the immunogenicity of immunorecessive antigens (11,20). This approach favors proteins being processed by the proteasome and circumvents TAP transporters. Preferentially, this provides a more efficient way for peptide presentation, where the peptides are protected from cytosolic degradation, especially since the cytosol contains a number of endopeptidases and aminopeptidases that are known to degrade petides (23). This information support further experiments and the design of our contructs should diminish the number of compartments where peptides are being processed and minimize chances of these peptides being digested by peptidases.

2.5.2 Importance of spacers

Flanking residues/spacers are important to distinguish epitopes and poly-epitopes. In fact it was documented that residues localized directly adjacent to CTL epitopes dramatically influence Ag presentation efficiency (10) and that epitopes can be optimized by linking them with appropriate spacer residues (24). The precise mechanism(s) and specificities involved in Ag presentation are still insufficiently established to predict the influence of flanking residues (10). Peptide-minigenes with ER targeting sequence were shown to be antigenic and immunogenic if the peptides had short flanking sequences. Velders *et al.,* 2001 concluded that addition of spacers between the epitopes was crucial for the epitope-induced tumor protection. Tandem construct with a triple alanine (A), AAA, spacer in TAP deficient cells yielded a specific CTL-response MHC class I pathway (15). Aromatic amino acids, like tyrosine (Y) and amino acid, with a small aliphatic side chain like A, supported efficient CTL recognition, via preferential protosomal cleavage sites; however, the extent of proteasome involvement in generating the peptides was not determined and alternative contribution of proteases in the cytoplasm or the ER could not be ruled out (10). AAY spacers have been very successful in Ag presentation, e.g. gene gun-delivered poly epitopes string vaccine with AAY spacer, increased the possibility of

the right epitope to be presented and also increased the protection against tumors as compared to constructs made without spacers (13).

Trimming in the ER exists by the aminopeptidase's ERAP1 and in the Trans-golgi region by the protease furin, which recognize basic and hydrophobic sequences, respectively (18,25). However, different results contradict each other since according to Snyder *et al.*, 2003 exclusive presentation of C-terminally located peptides and almost complete failure of N-terminally peptides catalyzed by aminopeptidases removes the N-terminally residues while leaving the C-terminally peptide intact due to protection of the MHC class I complex. Importantly, the ER targeting signal has to be placed at the N-terminal and not the C-terminal, otherwise there is hardly any presentation (20,25). The other approach is that both N and C-terminal trimming exist in the ER, since an ER targeted 170 residues fragment could be processed and provide the restricted epitope to TAP negative cells. This would require both N and C terminal trimming (22). Algorithms were designed to predict antigenic sequences, recognized by CTL:s, for different allelic MHC molecules. They predict the peptides to be presented regardless of their position in the protein from which they are derived by processing. If this was not the case, it would matters where in the protein the peptides are being located if they would be selected for presentation (24). Creation of a minigene with spacers that distinguish the epitopes by acting as protease cleavage sites could circumvent this problem as well since all the desirable epitopes will be brought forth and presented by the cell. These objectives point to the importance of flanking residues/spacers.

2.6 Salmonella *as a DNA vaccine-vector delivering system*

One of the most important aspects of a successful DNA vaccination strategy is the route of delivery, thus the application technique used to target plasmid DNA to host cells is crucial for antigen expression and priming of the immune response. Gene transfer from bacteria to mammalian cells was first observed in vitro already more than 20 years ago. It was shown that plasmid DNA is efficiently transferred from dying Salmonella in mammalian host cells where it could not be the bacteria that controlled the transcription of the plasmid since it is a eukaryotic/viral promoter (26).

A series of application routes for naked plasmid DNA were investigated over the past 10 years. These included: needle injection, gene-gun gold particles and jet-injection i.e. needle free injection topical exposure to mucosal sites. Like most conventional vaccines they are administered peripherally, consequently varying in their ability to elicit an immunogenic response (26). Naked DNA immunizations are not really optimal since they are hard to steer towards obtaining a desirable immune response (6). The efficiency of transfection via syringe injection is probably low because of the requirement for cellular uptake of DNA (19). Although it appears possible to induce a substantial CTL response, relatively high doses of DNA are required (26). Many vaccines need to be co-administered with adjuvant proteins, using attenuated *Salmonella typhimurium* to deliver the DNA vaccine, which in itself produces immunomodulatory factors leading to these self-antigens being presented in an immunogenic context (15). More over *Salmonella* is well suited for this purpose because of extensive knowledge of their genetics and physiology. There is a large documentation of the availability of safe *Salmonella* strains and recombinant plasmids of *E.coli* can be directly introduced into *Salmonella* without manipulation and the natural route of entry. This is of benefit since it uses mucosal epithelium targeting as a

mode of entry into the host from the gut lumen via the M cells of Peyer's patches. In addition, this natural route of administration delivers the DNA to cell types that have especially evolved in inducing immune responses. These antigen-presenting cells (APC's) eventually migrate into lymph nodes and spleen, thus allowing targeting of the vaccines to inductive sites of the immune system (fig.3). It is generally assumed that targeting of the plasmid DNA to APC's can improve immune responses (6,26).

After crossing the intestinal mucosal barrier mainly via M cells (i.e. specialized epithelial cells that collect antigen and pass it into mucosal-associated lymphoid tissue) a large fraction of the bacterial carrier are taken up by APC's, macrophages or other phagocytical dendritic cells (DC's), either in local, lymphoid organs like Peyer's patches in the small intestine or after systemic spread in spleen, liver and lymph nodes. DC's are the most efficient and specialized of APC's who with their dendrites can open tight junctions between epithelial cells. Upon monitoring the environment they can detect pathogens in the intestinal lumen, trigger B and T-cells activation by potent presentation of antigenic epitopes via MHC class I and II pathways (26).

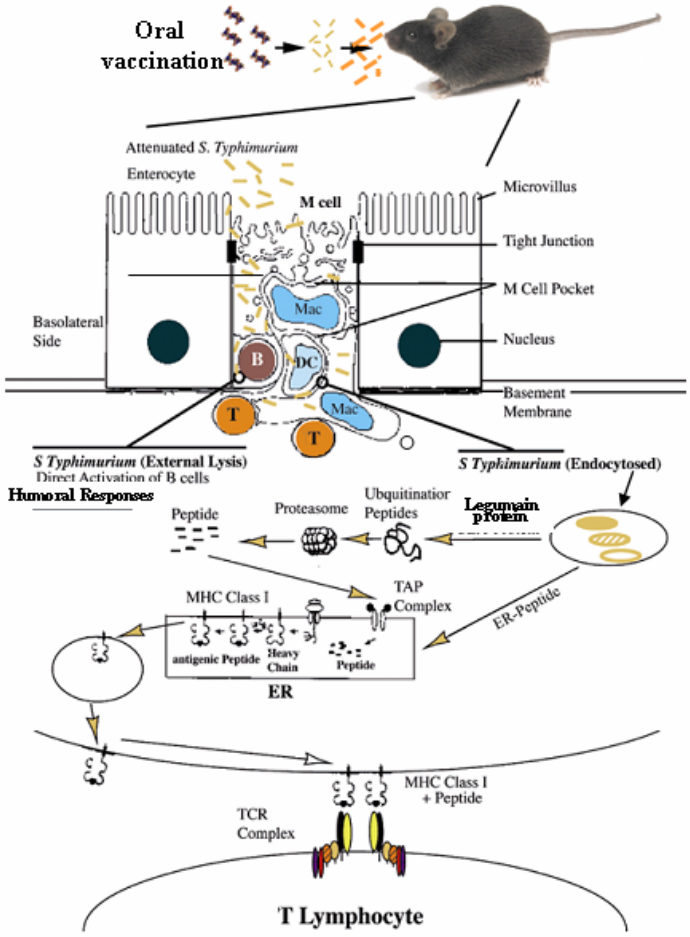

Fig. 3) Flowchart showing schematically the events in the host of the orally distributed Legumain DNA vaccine. Courtesy of Dr. R. Xiang.

After ingestion, bacteria transfer plasmid DNA into the cytosol of DC's, which in turn, is expressed by the cell. After endocytosis and a few rounds of division the attenuated intracellular bacteria die due to their mutations. During lysis of the bacteria in the phagolysosome, the plasmid DNA is released and transferred, either via a specific transport system or by endosomal leakage, into the cytosol and the nucleus of the infected cells (more detailed is depicted in (fig.4). Eventually the encoded "genes" are expressed and presented by major histocompatibility complex (MHC) class I peptide complexes to T-cell receptors (TCR's) of naïve T-cells by the host's APC's (6,7,26). Specific cytotoxic T-cells are induced by these activated APC's that lyse antigen-expressing cells. Free antigen or dying cells are taken up by other APC's, which in turn stimulate T-helper cells that are responsible for the induction of an antibody response. Intrinsic adjuvant properties like those of bacterial endotoxin, cell wall components and DNA, unmethylated CpG motifs of the vector all contribute to

strengthen the T-helper cell response (2,9). The T-helper cell response induced with this type of genetic immunizations seems strongly biased toward the Th1 type, which supports the development of cellular immune responses. In many vaccination strategies, especially in cancer treatment, this is the most desirable type of immune response (6,12). This is not unexpected since bacteria usually induce an inflammatory type of response. It is suggested that DNA vaccines, encoding minimal epitopes can induce $CD8^+$ T-cell responses independently of $CD4^+$ helper T-cells (27). In this case the response gets mediated, "T-help", by direct or indirect activation of APC's by the bacterially derived plasmid (6).

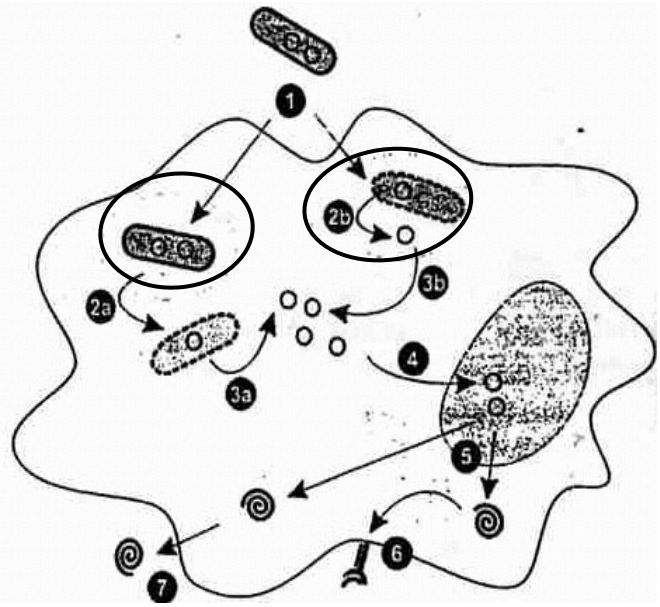

Fig. 4) Invasion of the host cell by bacterial carrier strain, *Salmonella* can induce its own uptake into phagocytes. *Salmonella* carrier strain are lysed 2a) in the cytoplasm due to phagosomal escape, or 2b) within the phagolysosome, resulting in intraphagosomal release of plasmid, DNA free in phagosome 3a,b) transfer or leakage of plasmid DNA into the cytosol 4) Finally the plasmid DNA once released from the carrier has to enter the nuclear compartments for transcription 5) Expression of plasmid-encoded antigens 6) processing of the antigen and presentation of epitopes to T cells 7) alternatively posttranslation of modified antigens engulfed and presented by other APC's.This picture is modified from Schoen *et al*. 2004.

Here we used strain RE88 of doubly attenuated *S. typhimurium* with the two mutations *dam*$^-$ and *aroA*$^-$: *dam*$^-$, DNA adenine methylase mutants have been demonstrated (28) to be highly attenuated but still can be useful as live vaccines. They cause any nonspecific immune suppression. *aroA*$^-$, carboxyvinyltransferase, inhibits the synthesis of aromatic amino acids. Both mutations cause the bacteria to die after a few passages. This delivery system has the capability to evoke most of the important effector cells necessary for an immune response induced by a DNA vaccine. Thus, it is concluded that tolerance against tumor-associated antigens can be broken with *Salmonella typhimurium* as carrier for these DNA vaccines (7).

3. Experimental Procedures

3.1 Creating a Legumain epitope based minigene vaccine

The Legumain epitopes were selected *in silico* based upon predicted binding motifs and antigenic intrinsic properties of the epitopes/peptides using the peptide prediction programs DNASTAR, www.dnastar.com and BIMAS NIH http://bimas.cit.nih.gov/molbio/hlabind.three. The two minigenes $H_2D^{b/d}$ and $H_2K^{b/d}$ cDNA sequences containing: three epitopes, spacer sequences and flanking restriction sites on both sides were designed. Two overlapping primer pairs was constructed based on the designed cDNA sequence. PCR was performed using the overlapping primer pairs (table 1) as template (*Thermal Ace™ DNA Polymerase Kit*, Invitrogen)

Table 1) Overlapping primers for PCR, synthesize both of the constructs.

Primers extended with restriction cleavage site:
Forward: *Bss*H II-H2D^{d} 5'-GCG CGC ACT CCT CAG GAC CAC GAG ACC ACG TAT TCA TAG CAG CAT ACC TAC CAC CAG TAA CAC ACC TAG CA-3'
Reverse: H2D^{d}-*Xho*I 5'-CTC GAG TAG GTA TGT TCC TCG TTC GTC GTA GTA TGC TGC TAG GTC TAG GTG TGT TAC TGG TGG-3'
Forward: *Bss*H II-H2K^{d} 5'-GCG CGC ACT CCC GAT ACC TAT ACG TAC TAG CAA ACC TAG CAG CAT ACA TGT ACC AAA AAA TGG TAT TCT AC-3'
Reverse: H2K^{d}-*Xho*I 5'-CTC GAG TAC YGA GTA CCA GTC TCC TAG GTA TGT GTA TGC TGC TAT GTA GAA TAC CAT TTT TTG GTA C-3'

The PCR product was analyzed on a gel to verify the size of the produced fragments. The PCR product was recovered from the gel (Gel purification Kit, QIAGEN). The next step was cloning the small inserts with blunt end ligation into a TOPO vector (*TOPO Blunt Kit*, Invitrogen) according to instructions from the manufacturers. The TOPO vector containing the inserts was transformed into chemically competent *E. coli* cells, *strain DH5α,* (Subcloning into plasmid vectors, Invitrogen). Successfully transformed clones were selected by on positive ampicillin-containing plates. This was followed by plasmid DNA-extraction (QIAquik MiniprepKit, QIAGEN). The purified plasmid DNA was run on a 2 % *Nusieve Agarose* gel using 50bp ladder, 120 V in 45 min to confirm insert and transformation. The vectors were sent for sequencing to *Retrogen Inc*, to verify that they contain the correct insert and that no mutations had occurred. Thereafter, the constructs were digested for one hour with the restriction enzymes *Bss*H II and *Xho* I in buffer two from *Newengland BioLabs* and ligated with T4 ligase for 15 min at 37°C into the pCMV/myc/ER vector (Invitrogen) which carries an ER-leader sequence and an ER retention signal for targeting purposes (fig.5, appendix 1). This plasmid carries a strong promoter from the human cytolomegavirus (CMV) with the immediate early enhancer IE-EP, a multiple cloning site (appendix 1) for insertion of the gene of interest and an appropriate transcription terminator segment. The vector is also equipped with an ampicillin (and neomycin) resistance marker for selection.

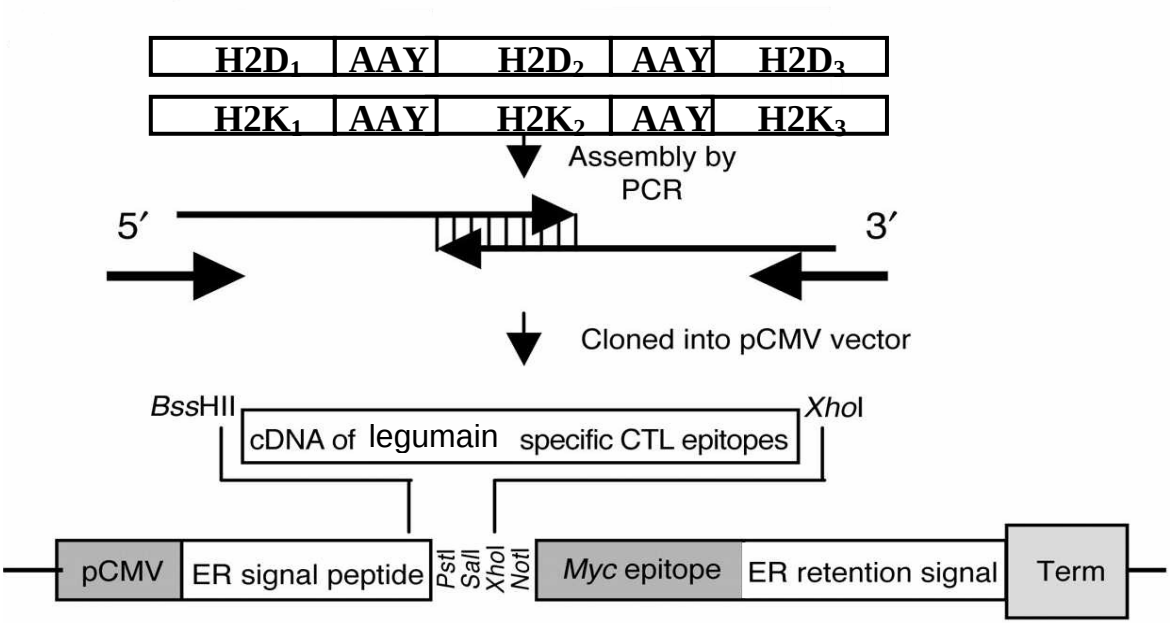

Fig. 5) Schematic map of vector constructs, epitopes encoding Legumain were assembled by PCR, thereafter cloned into pCMV/myc/ER expression vector by using *BssH II* and *Xho I* restriction sites. The picture is modified from Zhou *et al*., 2004.

3.1.1 The minigene - construct

The following expression vectors were constructed: P_{CMV}/ER-H-2D$^{b/d}$, P_{CMV}/ER-H-2K$^{b/d}$ and the empty vector as a control. Table 2 shows the selected peptides and their properties chosen by the peptide computer programs BIMAS NIH and DNA STAR. The first one is based upon MHC class I allele restricted anchor residues, score equals affinity thus, halftime that the peptide is bound to the MHC complex, respectively the later mentioned program, predicts the antigen's intrinsic properties with an antigenic score ranging from (-1,7) - 1,7. The constructs are also equipped with AAY spacer, ER leader sequence and ER retention signal (fig.5) E3/19K from the human adenovirus.

Table 2) Generation of Legumain epitopes for DNA minigenes (- = undefined)

H-2	Epitope	Position	Score	Antigenic Index	Rank
H2D$^{b/d}$1	SGPRDHVFI	137	9/240	1.7	8/1
H2D$^{b/d}$2	LPPVTHLDL	238	7/20	1.7	7/3
H2D$^{b/d}$3	YDEERGTYL	223	-/6	1.7	-/16
H2K$^{b/d}$1	RYLYVLANL	405	1/4800	0.4	17/1
H2K$^{b/d}$2	MYQKMVFYI	180	1/2400	0.8	-/2
H2K$^{b/d}$3	TYLGDWYSV	229	-/1200	1.7	-/5

3.2 Transformation of S. typhimurium *with DNA plasmid*

Freshly prepared double attenuated *S. typhimurium (aroA*$^{-}$ and *dam*$^{-}$*)*, strain RE88 at midlog growth phase were transformed with the DNA vaccine plasmids H2D$^{b/d}$, H2K$^{b/d}$ and empty vector (pCMV/*myc*/ER) by electroporation prepared plasmid DNA 2µg (cut and ligated checked on 2% gel) on ice in a 0.1-cm cuvette and electroporated 2,0 kV, 25µF and 100 Ω. Even here clones were selected by the plasmid's positive selection marker, ampicillin and after that, resistant colonies harboring the three different DNA at –80°C. The DNA vaccines constructs were now ready to be tested. *Salmonella* were washed three times in 1×PBS (phosphate buffered saline) spin at

3000 rpm 25 min 4° C instantly before vaccination of the mice. In order to assess the anti-cancer effects of the DNA vaccine we exposed vaccination.

3.3 Experiments on animals - conduction of murine work

We performed vaccination experiments in mice to examine the extent of anti-tumor effects achieved by the oral DNA minigene vaccine encoding epitopes of Legumain. To this end, two different strains of mice were immunized orally with double attenuated *S. typhimurium (aroA*$^{-}$ and *dam*$^{-}$*)* 1×10^{8} CFU (colony formation unit) of the RE88 strain carrying the plasmid DNA, encoding either the empty vector or the minigenes $H2D^{b/d}$ and $H2K^{b/d}$. Vaccinations were executed, three times at 1-week intervals giving prophylactic settings. The Legumain, minigene vaccines were administered by gavage in 100µl of 7.5% sodium bicarbonate to neutralize the acid pH in the stomach of the mouse. This was followed by tumor cell challenges in two different tumor models, D2F2 breast cancer and D121-lu lung cancer. All cell lines were shown to express Legumain (appendix 2). Cancer cell line were grown a few days before challenging *in vitro* at *37*°C and a level of 7 % CO_2 in their respective cell media (appendix 3). The inbred mouse strains, BALB/c and C57BL/6J were provided by the animal facility of The Scripps Research Institute. Animal experiments were performed according to the National Institutes of Health Guide Care and Use of Experimental Animals approved by the Scripps Animal Care Committee.

3.3.1 Prophylactic model of breast carcinoma in BALB/c mice

Induction of breast carcinoma was done by a subcutaneous needle injection of 2×10^{5} D2F2 cells (non metastatic cell line) one week after the last of the three immunizations. Balb/c mice, challenged by the D2F2 cells, were monitored for tumor growth, determining their volume, by measuring the size, length and width of the tumors a few times each week. The mice were sacrificed using an overdose of the anesthetic halothane in a jar. After 27 days, the subcutaneous tumors were harvested, weighed and checked for reduced growth when compared to the empty vector-vaccinated control mice. The experiment was terminated after 48 days and the experimental losses were four mice due to death from faulty gavage.

3.3.2 Prophylactic model of lung carcinoma in C57BL/6 mice

Experimental lung metastases were induced by intravenous injection (i.v.) injection of 1×10^{5} D121-lu cells one week after the last three immunizations. After 27 days, C57 BL/6 mice were sacrificed, with the same procedure as with the BALB/c mice above, and their lungs harvested by cutting them out with sterile utilities. The lungs were weighed and directly put in Bouin's fixative (picric acid 0.9%, formaldehyde 9% and acetic acid 5%, Sigma). Following this, the amount of tumor foci per lung could be determined. The experiment was terminated 48 days after the first immunization.

3.4 Cytotoxicity (CTL)-Assay

To examine the existence of a Legumain specific CTL- response, spleens were harvested from BALB/c mice which were immunized three times with the $H2K^{d}$. CTL-assays were performed with a 4T1 breast cancer tissue cells and a macrophage cell line, RAW 264,7 cells, (stimulated with LPS, see below, to express Legumain) used as target cells. To use 4T1 as target cells we had to inject non-immunized mice s.c. with a lethal dose of 4T1 1×10^{4} cancer cells. In this 4 hour cytotoxicity assay target cells are briefly labeled with ^{51}Cr, washed, then mixed with effector CTL cells at appropriate effector-to-target (E:T) ratios for a certain period of time here for

approximately four hours. The amount of ^{51}Cr released into the supernatant by killed target cells is quantified. Comparison with ^{51}Cr release of controls, positive-effector with target cells treated with 2% Triton X-100 (lysing agent) and negative-target cells only with medium; to measure maximum respectively background release. The corrected percent lysis is calculated for each concentration of effector cells.

4T1 tissue and RAW macrophages were used as target cells. Cells from 4T1 tissue from spontaneous murine cancer metastases were harvested since *in vitro* cultured 4T1 cells do not express Legumain. Single-cell suspension was made of the cancer lumps by treatment with collagenaseI, 125µ/ml, incubated for 1.5 hours at 37ºC. RAW macrophages cultured *in vitro* stimulated with the endotoxin LPS (lipopolysaccharide) for expression of Legumain (unpublished Luo Y) was also used. Cytotoxicity was measured and calculated by a standard chromium –51 (^{51} Cr) release assay. Splenocytes harvested 9 days after challenge with 4T1 5×10^3 cells s.c. was followed by subsequent tissue culture for 5 days in T-cell media with penicillin-streptomycin at 1% (Gibco) of which the first 24 hours was supplemented with T-STIM, Con A and Il-2, thereafter the cells were moved to T-cell media with IL-2 but without T-STIM ConA. The last 24 hours the splenocytes were primed *in vitro* by stimulation of irradiated (1:1000 Gy) 4T1 tissue cells at 37ºC for 45 min DMEM Target cells, 1×10^4, were labeled with ^{51}Cr for 1,5 hours at room temperature and incubated with effector cells at three E:T cell ratios: 25:1, 50:1 and 100:1 at 37ºC for 4 hours. Effector cells starting at number $0{,}25 \times 10^6$, $0{,}5 \times 10^6$ up to 1×10^6.

Determination of CTL activity: the percentage of specific target cell lysis was calculated by counting ^{51}Cr in a γ scintillation counter, 1 min/ sample in triplicates, then using the mean cpm (counts per minute) for each replicate of wells, according to the formula:

$$\text{Correct \% lysis} = 100 \times \frac{\text{Test-sample } ^{51}\text{Cr released} - \text{control } ^{51}\text{Cr released}}{\underset{\text{(Positive control)}}{\text{Maximum } ^{51}\text{Cr released}} - \underset{\text{(Negative control)}}{\text{control } ^{51}\text{Cr released}}}$$

4. Results

4.1 Protein expression

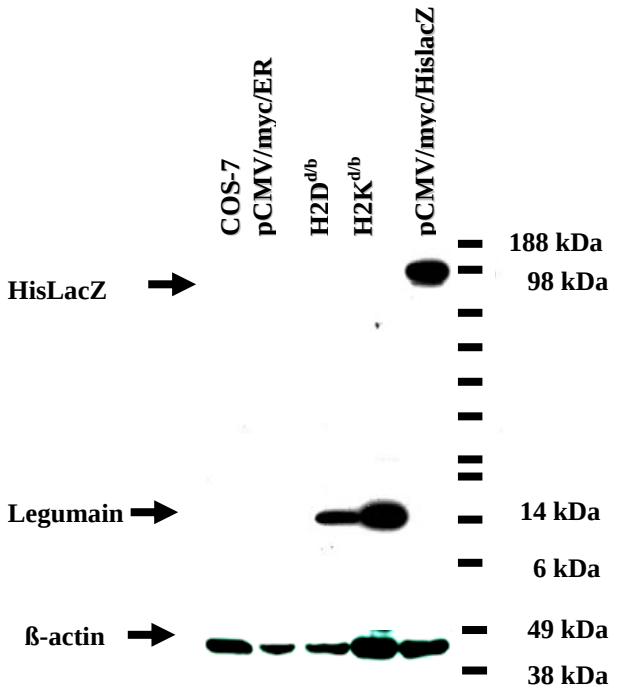

Fig. 6) Protein expression was demonstrated by Western Blotting. The proteins are detected by the myc epitope.

Two minigenes were constructed each of which encodes three different epitopes, peptides, of Legumain (Table 2) distinguished by different enzymatic cleavage sites. All peptides were assured to be in frame with the *myc* epitope to be able to detect by myc expression by western (fig.6). For protein expression of $H2D^{b/d}$ and $H2K^{b/d}$ COS-7 cells were transfected according to the manufacturer's instruction LIPOFECTAMIN ™ 2000 from Life Technologies Reagent. The COS-7 cells were lysed in lysis buffer containing of 50nM TRIS pH 8.0, 150mM NaCl, 1% Triton X-100, 5 mM complete and 1-2 mM PMSF (Phenylmethylsulfonyl fluorid) inhibitor of proteases. Proteins concentrations were measured by a BCA protein assay *Kit* (PIERCE). Peptides expression was demonstrated by western blotting using NuPAGE reagents and instructions from Invitrogen with a10 well 4-12% Bis-Tris gels. Before loading the gel, the samples were boiled at 10 min at 70°C, wells loaded with 14.6µg of protein for each sample and a seeblueplus ladder. Since the string-peptides are a very small protein, ca 16 kDa, MES running buffer was used. The membranes stained with ponseau to make sure the transfer went successfully. To prevent unspecific binding of the antibodies blocking solution was used; 5% milk buffer, dry milk in TBS-TWEEN (0.2% tween). Monoclonal primary mouse-anti-myc antibody (Ab) Invitrogen and a polyclonal secondary goat-anti-mouse, HRP-labeled Ab, from Bio-Rad were used. Washing buffer used was TBS-TWEEN. COS-7 alone and pCMV/*myc*/ER (empty vector) are negative controls and pCMV/myc/HisLAcZ is a positive control.

4.2 Protection of tumor progression of D2F2 breast carcinoma

With large fluctuations standard deviations (S.D.'s), an anti-tumor affect was observed in the BALB/c mice with the s.c. distributed tumor, cell line D2F2. $H2K^d$ showed a statistically significance *=P<0.05 according to normal distribution the, Student's T-test but not the $H2D^d$ (fig.7). The volumes were estimated by calculating the formula: $((W^2 \times L)/2)=V$, were W=weight, L= length and V=volume.

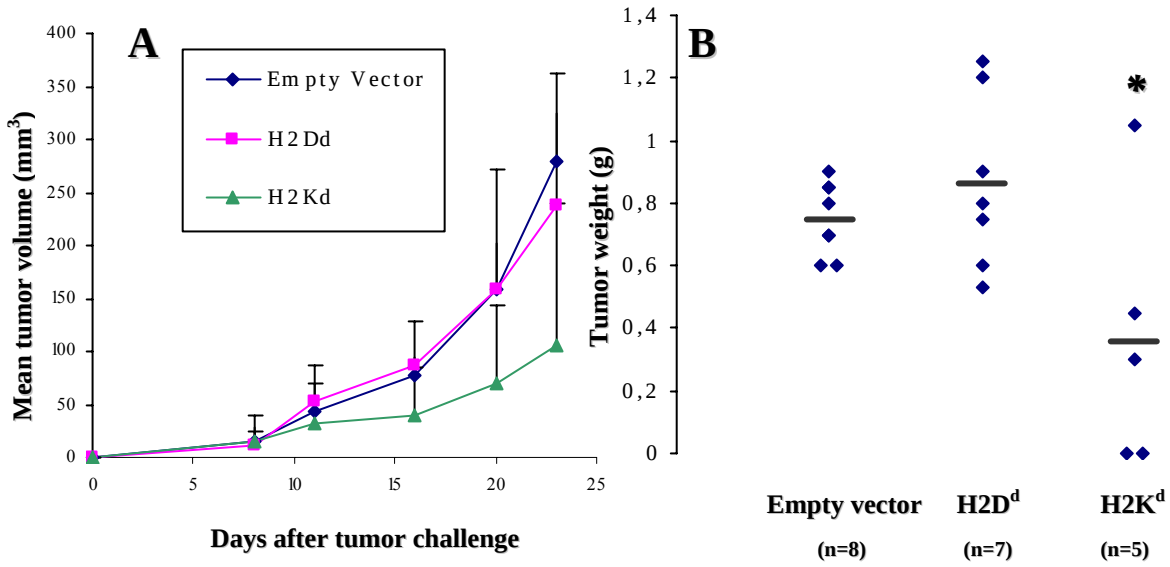

Fig. 7) **A**) Average volume for D2F2 cancer growth in BALB/c, number of mice in each group: blue n=8, pink n=7 and green n=8 **B**) distributed D2F2 tumor weight, horizontal bars shows the average.

4.3 Suppression of experimental pulmonary metastases of D121 lung carcinoma

In the prophylactive MHC I, non-allele, restricted, model with C57BL/6J mice challenge with D121-lu, both constructs $H2D^b$ and $H2K^b$ showed a diminishing tumor growth and for $H2K^b$ even a 50% complete tumor protection (fig. 8). Differences in lung weights between mice treated with the DNA vaccine compared with the control groups were statistically significant. The statistic significance according to T-Test was *=P<0,05 and **=P<0,005.

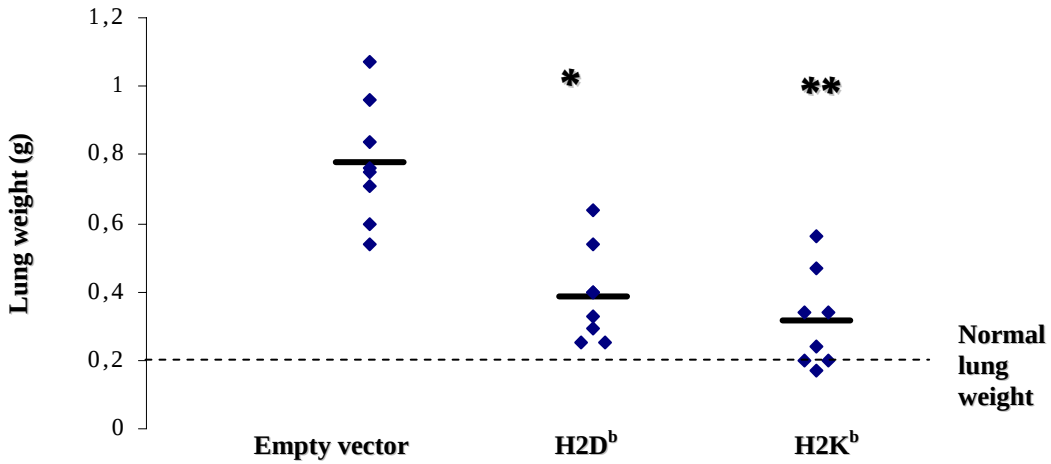

Fig. 8) Distributed lung weight of C57BL/6J, each group of mice (n=8) challenge with D121 tumor cells. Even here the horizontal bars show the average for each group.

4.3.1 Metastatic score

The metastasis score i.e. the percent of lung surface covered by fused metastasis foci were estimated in the lungs harvested from the C57BL/6J mice, as follows: 0=none 1 = <25% 2 = 25-50% 3 = >50% of lung coverage. A significant decrease in the number of lung experimental metastases was found as indicated by the metastasis scores (fig.9), in comparison with control mice, using the Mann-Whitney U test (non-normal distribution) for non-parametric data, here *=P<0,05 and **=P<0,005 as same as above.

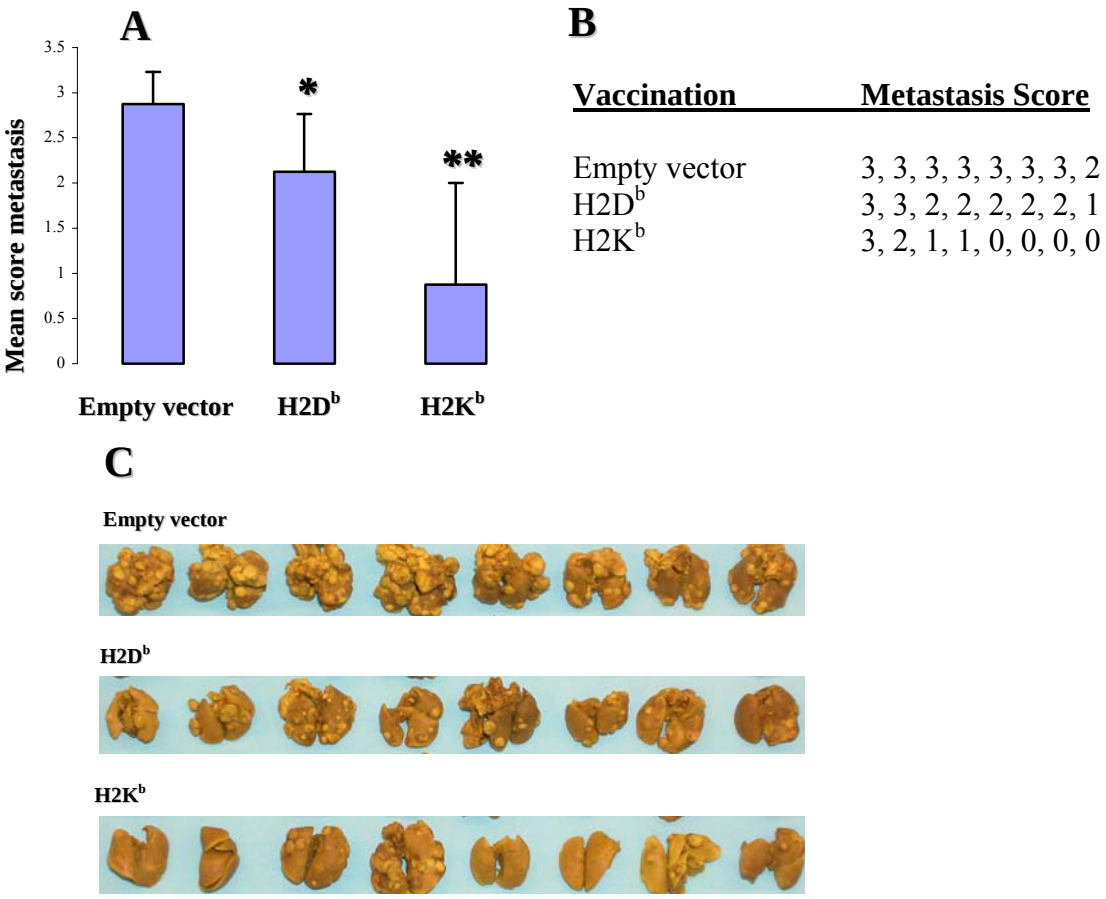

Fig. 9) **A**) The average number of tumor foci covering the lung surface. **B**) The individual metastasis scores for each mouse of the same group of mice as above. **C**) Photograph of the fixed lungs from C57BL/6J mice challenged with D121-lu tumor cells.

4.4 Results of CTL-assays

Cytotoxicity assays were performed with the minigene $H2K^d$ that had produced the strongest anti-cancer results and also showed a Legumain immune specificity in the majority of activated CTL's (fig.10). RAW 264.7 cells stressed *in vitro* had a relatively high occurrence of cell lysis. The constructs with the Legumain minigene $H2K^d$ showed a significant increase in cell lysis compared with the empty vector control according to Student's T-test even used here. The low % lysis of the target cells, 4T1-tumor tissue, was caused by an insufficient cell amount yielding relatively large S.D.'s (not shown).

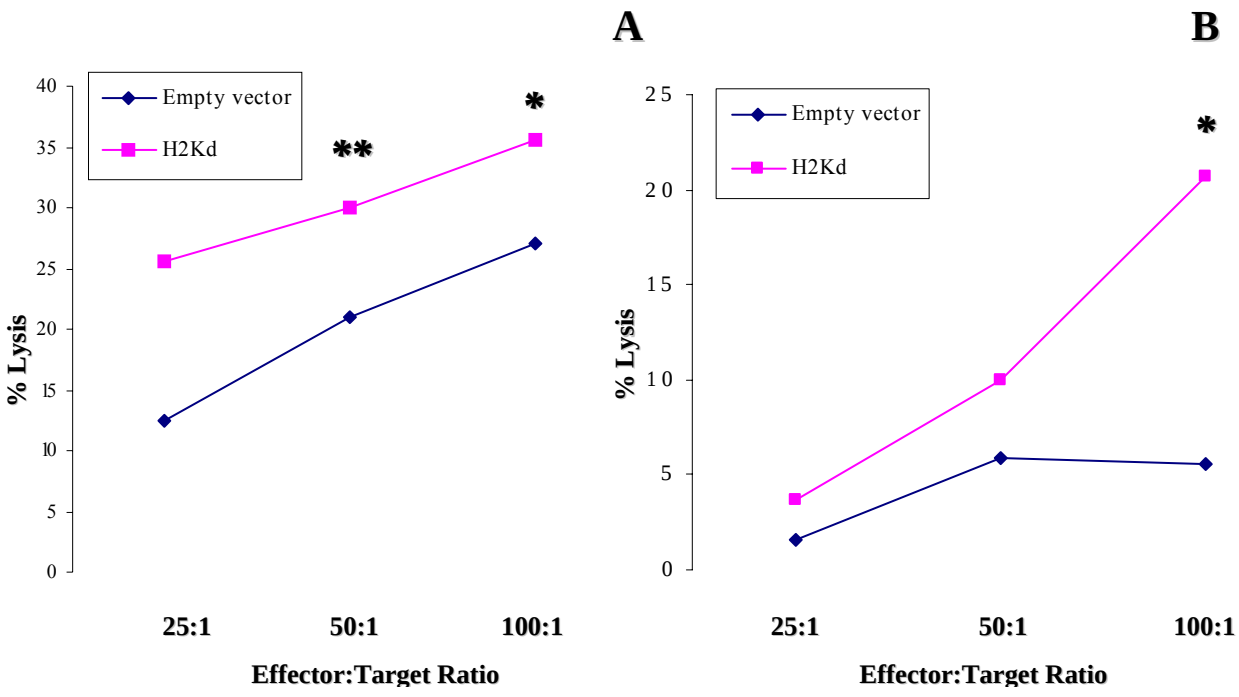

Fig. 10) Cytotoxicity assays were performed with **A**) Legumain-loaded RAW 264.7 cells and **B**) Legumain-loaded 4T1 tissue cells. The graphs show the % Legumain-specific lysis.

5. Discussion

This project is still in its early phase and one can only draw limited conclusions from the experiments described. Here, by targeting the minigene vaccine to the endogenous MHC class I pathway, we aimed to, elucidate specific $CD8^+$ CTL's by induction of a cellular immune response. However, it is questionable from our results whether $CD4^+$ T-cells were also required for secondary expansion of memory $CD8^+$ T-lymphocytes. In fact it is a long-standing paradox in cellular immunology that there is a conditional requirement for $CD4^+$ T-helper in priming of $CD8^+$ T-cells (27).

In the breast cancer mouse model we observed a significant anti-cancer effect of one of the minigene constructs, $H2K^d$. Since there was a wide range in distribution among individual mice there were relatively large S.D.'s. Although these mice are inbreed, they are still animals differing in behavior and size and experimental mishandling may account for these results. The fact that one of the constructs showed a better anti-cancer protection can be explained by the fact of how the protein Legumain normally is processed in cells within the host and by the type of epitopes that are actually preferentially expressed.

The lung cancer mouse model was not the most optimal one, as predicted peptides were not always in accordance with the experimental data; however, we still had a good chance of succeeding, even though one parameter was not being optimized. Even though a low binding score was discovered later in the experiment, we still wanted to test this model because it was metastatic. Hence we continued with the work. One also has to consider that these peptide prediction programs only give prediction and their algorithms do not always work optimally. There is of course the possibility that those peptides selected will not be T-cell-specific. Other peptide prediction programs such as SYFPEITHI (250105) and ProPed-I (100405) showed a better allele-binding score of our selected peptides. To assure that the peptides really bind to MHC class I antigens, *in vitro* affinity studies should still be performed. However, a successful minigene does not only depend on one factor since flanking residues and concentration also contributes to its overall presentation and antigenicity.

We attempted to assess the specificity of T-cells induced by our minigene encoding Legumain epitopes with a 4 hour cytotoxicity assay, using splenocytes of immunized animals and controls against LPS stimulated RAW 267.4 macrophage cells (appendix 3) and 4T1 murine breast tumor tissue target cells. Unfortunately, our assay conditions were not optimal due to the lack of appropriate target cells expressing Legumain *in vitro,* even though such cells express this stress antigen *in vivo*, in tumor-bearing mice. Additionally, we lacked experience in optimizing the RAW macrophages cell line as a target cell in the cytotoxicity assay. We obtained some cell lysis for the RAW 267.4 cell line, which is difficult to attain, in a 4 hour assay, in these kinds of experiments. The empty vector group also demonstrated a gradual increase in lysis, which has been sometimes observed in other experiments. This non-specific cell lysis can be partly explained by the fact that stimulated macrophages may be better recognized than regular cancer cells by a more diverse group of CTL's, i.e. non specific primed CTL's. Even so, the increase in CTL lysis was clearly distinguishable. When the asset of target cells are not the problem, an increase in lysis by the empty vector splenocyte group could occur, especially since all the cells had

been primed previously for 24 hours with Legumain expressing 4T1 tissue cells. Nevertheless we could demonstrate a low but clearly detectable cell lysis ratio and as predicted, the empty vector group did not show any immune specificity. In conclusion, both assays with RAW and tumor tissue target cells showed a specific immune response. To obtain a better result in the future, a comparison with a non-Legumain expressing cell line needs to be done. This would confirm an even greater immune specificity for Legumain. In spite of difficulties with the CTL assay, we could still established proof of concept that these Legumain based minigene vaccines can indeed suppress tumor growth as well as the formation of experimental metastases.

Once we gain a better future understanding of the relationships between tumor immunity, autoimmunity and tolerance it should be possible to overcome failures of tumor immunity. The successful immunization against cancer may then become a reality and methods for quantifying the status of the immune response and for its augmentation can than be developed. During the next decade, a better understanding of the immune response against solid human tumors is certain to emerge.

6. Future experiments

6.1 Creation of a secretion plasmid co-expressing the chemokines CCL19 and CXCL13

The delivery of antigenic information to different compartments of the specific immune system can be further enhanced and modulated when combined with co-delivering immune-stimulating molecules i.e. chemokines or co-stimulatory molecules, which enhance the immunogenicity of antigen, encoded by DNA vaccines (29). One aspect of these related tumor vaccines under investigation are the choice of an optimal adjuvant. Vaccination with a string of naked DNA epitopes has been found to be far less effective than virus-mediated introduction of the same string of epitopes (13). A novel approach is to use a vector that co-expresses the chemokines CCL19 and CXCL13. These are known to be principal regulators of lymphocytes and dendritic cell migration in the immune system homeostasis, infection and inflammation. They guide the cells to the appropriate microenvironment working as key signals that allows lymphocytes to transmigrate from the blood stream into lymphoid tissue at the site of high endothelial venules (HEV) (fig.11). The mediation of recruitment and clustering of the critical immune effector cells involved in lymphoid organogenesis occurs in secondary lymphoid organs such as Peyer's patches in the small intestine. By directing lymphocytes and DC's to this distinct area allows for promoted of priming of naïve T-cells, thereby improving the anti-tumor vaccine efficacy (30).

There seems to be a steady state condition of naïve T cells and DC's trafficking to secondary lymphoid organs (SLO); however, there exists evidence for factors, which negatively or positively regulate T cell and HEV interactions. Homeostatic chemokines, such as CCL19 and CXCL13 are constitutively expressed within the SLO's by stromal cells and DC's within the T cell zone and respectively the B cell rich zone. These chemokines regulate lymphocytes and DC's homing to these organs. By using a trancytosed transport with intravescular vesicles to the luminal surface of HEV they are presented to leukocytes and DC's in the blood stream where after an alteration in the establishment, multi-step adhesion cascades occur. CCL19 is the ligand for the receptor CCR7 that is highly expressed on naïve T-cells, central T memory cells and at a low level on B cells. There exist a transient increase in receptor expression but following activation when naïve T cells differentiate into effector cells a down regulation of the CCR7 receptor expression is induced. This is followed by increased up regulation of maturation of DC's. CXCL13 is a ligand for the receptor CXCR5 which is expressed on mature recirculating B-cells, a small subset of $CD4^+$, $CD8^+$ and skin- derived migratory DC's. CXCL13 is essentially responsible for guiding B-cells and T-cells that mediate "T-cell help" which allows B-cells to produce antibodies (30).

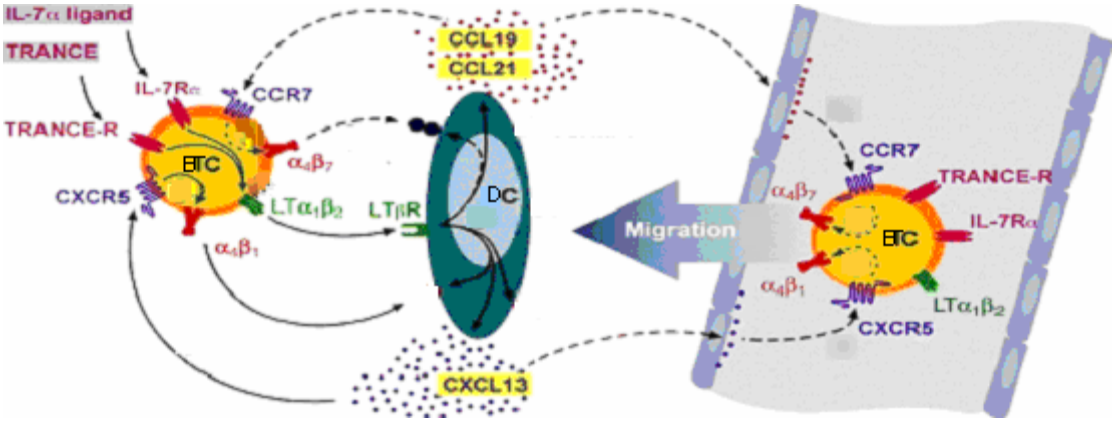

Fig. 11) Model for the role of CCL19 and CXCL13 in the lymph nodes e.g. Peyer's patches. CCL19 and CXCL13 expressed here by DC:s activated naïve B,T-cells (BTC:s) by reinforcing the interaction of DC:s and BTC:s and promoting the expression of adhesion molecules leading to naïve B,T migration across the HEV. The picture is modified from Müller *et al.*, 2003.

6.2 Construction of the fusion Latency-associated protein (LAP)

Creation of a fusion protein will increase the chemokines halflife and extend their biologic activities by prolonging their release. The engineered fusion protein called Latency-associated protein (LAP) of TGF-β (transforming growth factor) is catenated with enzymatic cleavage sites of matrix metalloproteinase (MMP). LAP acts as a protective "shell" until removed by cleavage at the MMP sites in areas of inflammation and tissue remodeling, thereby releasing the active chemokine and giving the chemokine more target specific properties. Co-expression of chemokines enhances the immune response and offers the possibility of modulating the induction of this response into a desired direction (6,31).

6.3 Generation of expression plasmid of legumain for efficient delivery to the conventional MHC class II pathway

Some researchers believe that to fight cancer both the cellular-and humoral immune responses are required (22). It would be optimal to further investigate that possibility to target the minigene to the secretory pathway so that an already induced specific $CD8^+$ T-cell response can be combined with a potent $CD4^+$ mediated response. This could be achieved by expressing the Legumain epitopes as secreted antigens by targeting them within the cell to such relevant compartments as the lysosome and endosome (fig.2,H). Therefore, another DNA vector will be constructed with Legumain epitopes expressed by the MHC class II presenting pathway and distributed together with the plasmid encoding CXCL13 to also augment the humoral immune response.

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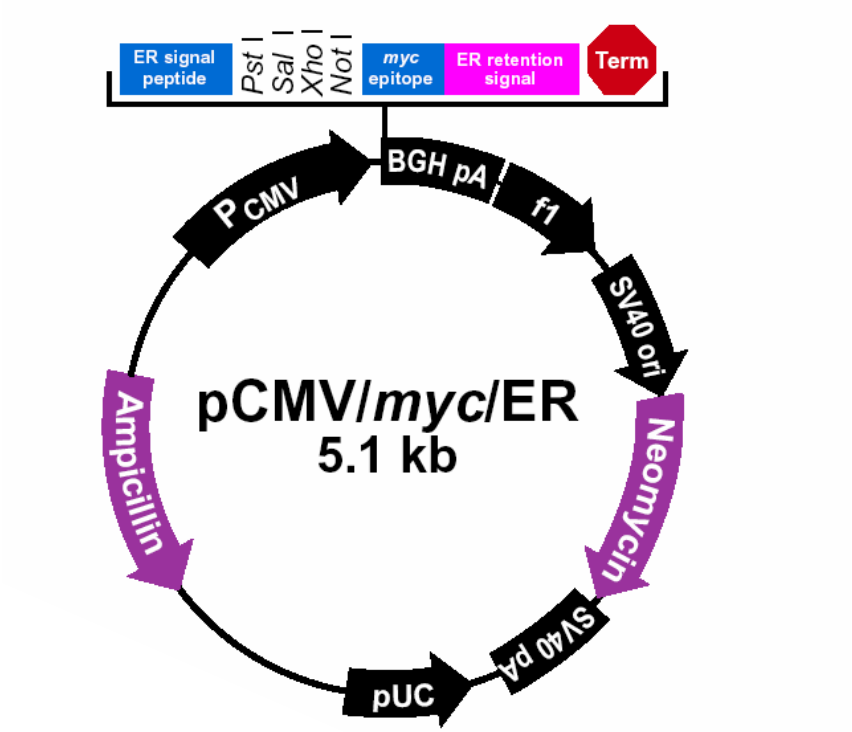
9. Appendix
1: Expression vector from Invitrogen®

Map

The figure below summarizes the features of pCMV/myc/ER. The complete nucleotide sequence for pCMV/*myc*/ER is available for downloading from the World Wide Web site, www.invitrogen.com (country: United States).



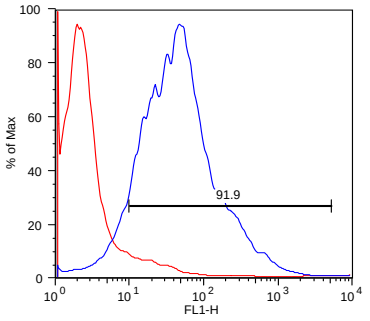
Comments for pCMV/*myc*/ER
5066 nucleotides
CMV immediate-early promoter: bases 12-627
pCMV priming site: bases 544-564
ER Signal sequence (exon 1): bases 656-700
Intron: bases 701-782
ER Signal sequence (exon 2): bases 783-794
Multiple cloning site: bases 804-838
myc epitope: bases 840-869
ER retention signal sequence: bases 882-899
BGH Reverse priming site: bases 923-940
BGH polyadenylation sequence: bases 922-1136
f1 origin: bases 1199-1612
SV40 promoter/origin: bases 1677-1988
Neomycin (G418) resistance gene (ORF): bases 1996-2790
SV40 polyadenylation sequence: bases 2806-3045
pUC origin: bases 3230-3903 (opposite strand)
Ampicillin resistance gene (ORF): 4048-4908 (opposite strand)

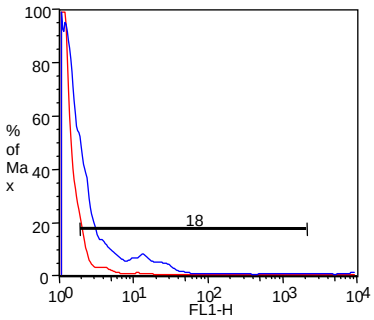
2: Legumain expression

FACS analysis of Legumain expression on cultured tumor cells and their derived tumor tissues

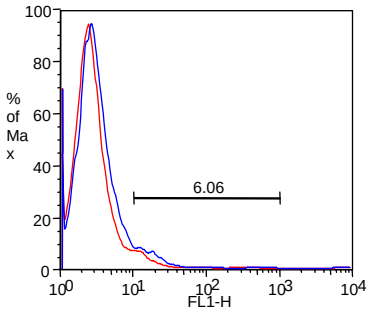
4T1 **D2F2**

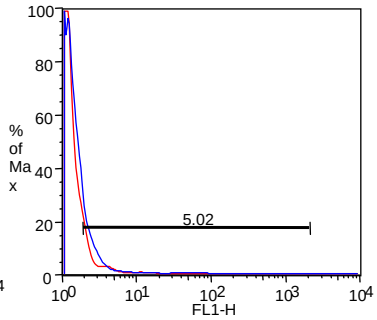
Tumor tissue



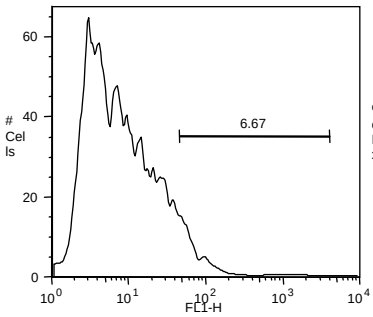


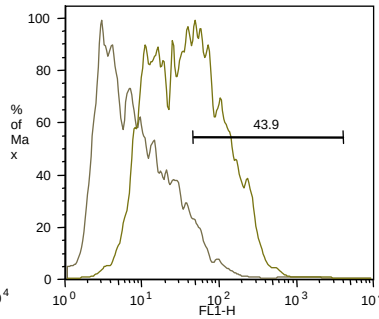
Tumor cell





Legumain expression on RAW cells after stimulated by 10ug/ml LPS





No FACS data was available for D121-lu, instead expression of Legumain has been demonstrated by Western Blot (unpublished by Luo Y).

3: Cell media

4T1 cell media:

Modified RPMI 1640 from **ATCC** Cat# 30-2001
10% FCS
1% Sodium Bicarbonate (of a 7.5% solution)
CellGro Cat# 25-035-CI

D2F2 cell media:

DMEM Gibco Cat# 10313-021
10% FCS
1% *GLUTAMAX* (new, more stable version of Glutamine)
Gibco Cat# 35050-061
1% NEAA (Non-Essential Amino Acids, 10mM)
Gibco Cat# 11140-050
1% Sodium Pyruvate - 100mM solution Gibco Cat# 11360-070
1% Beta-MercaptoEthanol - 55mM in D-PBS
Gibco Cat# 21985-023
5% Sodium BiCarbonate (of a 7.5% solution)
CellGro Cat# 25-035-CI

D121-lu cell media:

DMEM Gibco Cat# 10313-021
10% FCS
1% *GLUTAMAX* (new, more stable version of Glutamine)
Gibco Cat# 35050-061
1% NEAA (Non-Essential Amino Acids, 10mM)
Gibco Cat# 11140-050
1% Sodium Pyruvate - 100mM solution Gibco Cat# 11360-070

RAW cell media:

RPMI 1640 Gibco Cat# 21870-076
10% FCS
1% *GLUTAMAX* (new, more stable version of Glutamine)
Gibco Cat# 35050-061

The trypsin we used was a 0.05% Trypsin-EDTA solution from Gibco, Cat# 25300-054