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Engineering DNA Vaccines that Include Plant Virus Coat Proteins

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Introduction

There is growing interest among researchers in the various strategies for vaccination against cancer. One promising approach is the use of naked plasmid DNA for antigen delivery (Tang *et al.*, 1992). An advantage of DNA vaccination is the ability to induce humoral, as well as cellular immune responses to control tumours. DNA vaccines can be made easily using standard DNA recombination technology, thus allowing many candidate antigens to be tested rapidly.

A DNA vaccine combines two functional units; one encodes the antigen of interest, and the other acts as an adjuvant. A cassette capable of driving gene expression in eukaryotic cells is inserted into a plasmid. This cassette usually includes a CMV promoter/enhancer and a terminator/polyadenylation signal (*Figure 5.1*). Upon delivery of the bacterial plasmid DNA into muscle or skin, the gene is expressed, producing the antigen directly in transfected cells. The adjuvant effect comes from immunostimulatory DNA sequences (ISSs), i.e. unmethylated CpG motifs within particular flanking sequences (Krieg *et al.*, 1995). These motifs are more frequent in bacterial than in eukaryotic DNA, and act as so-called 'danger' signals of bacterial invasion. ISS are recognized by the cells of the immune system, such as dendritic cells, B cells, and natural killer cells (review: Krieg, 2002). This leads to release of inflammatory cytokines, such as IFN-γ, IL-12 and IL-18, chemokine secretion, and upregulation of co-stimulatory molecules on APCs. The exact sequences and frequency of ISS within a plasmid DNA backbone vary for different species. TLR9, a member of the TLR-receptor family, has been identified as a pattern recognition

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Abbreviations: APC, antigen-presenting cell; BCR, B cell receptor; CMV, cytomegalovirus; CP, coat protein; DC, dendritic cells; DR, death receptor; FrC, fragment C; Id, idiotypic determinants; IFN, interferon; Ig, immunoglobulin; IL, interleukin; MAb, monoclonal antibody; MHC, main histocompatibility complex; PVX, potato virus X; TA, tumour antigen; TCR, T cell receptor; Th, T helper; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TWEAK, TNF-like weak inducer of apoptosis.

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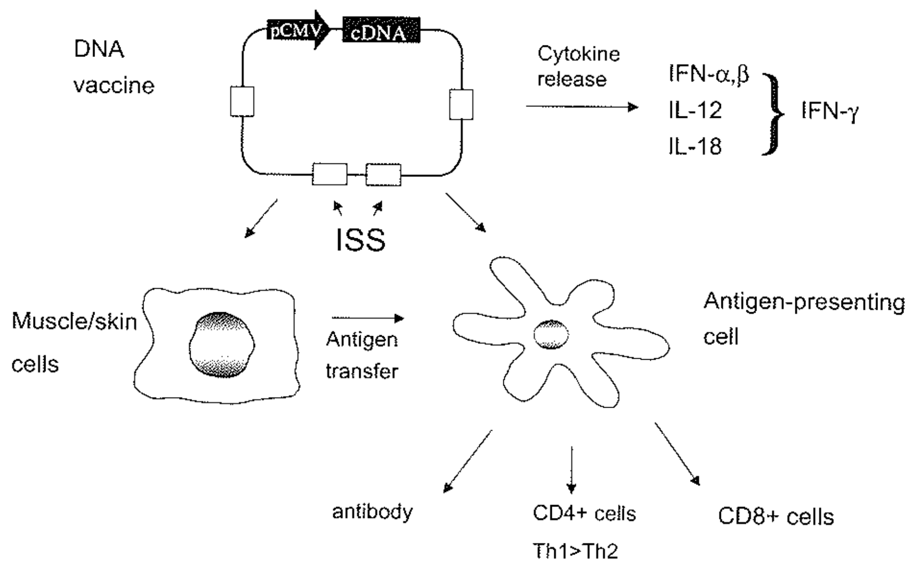

Figure 5.1. Induction of the immune response by DNA vaccines. ISSs containing CpG motifs in the plasmid backbone stimulate the production of pro-inflammatory cytokines by cells of the innate immune system through interaction with TLR9. Transfection of muscle/skin and dendritic cells leads to antigen expression, with a subsequent activation of CD4+ and CD8+ T cells and antibody.

receptor for unmethylated CpG motifs (Hemmi *et al.*, 2000). Activation of TLR-9 leads to induction of an NF-κB signal transduction pathway (Hemmi *et al.*, 2000).

Plasmid DNA is usually delivered by intramuscular injection, or an intradermal injection using a gene gun. Myocytes or keratinocytes are transfected, and these cells then act as a major source of antigen. It is possible that APC can also be transfected directly (Condon *et al.*, 1996). In addition, APC can acquire soluble antigen, or take up cell debris, which have occurred as a result of apoptotic or necrotic death of transfected cells in the site of injection (Fu *et al.*, 1997). These APC migrate to the draining lymph nodes, where they prime naïve T cells (Bot *et al.*, 2000). All arms of the immune response can be induced, including antibody, CD4+ T cells and cytotoxic CD8+ T cells. Intramuscular injection generally induces a Th1 type of immune response, which is associated with activation of cellular immunity, whereas the gene gun method favours a Th2 type response with induction of humoral responses (Feltquate *et al.*, 1997). However, the nature of the antigen can also influence the type of helper response induced during DNA vaccination.

For DNA vaccination against cancer, our strategy is to use genes that code for TA. We have focused on Id, a specific TA expressed by B cell malignancies. Ids are encoded by variable regions of heavy and light chains of Ig genes, and are unique for each B cell. Rearrangement of V, D, and J gene segments of the heavy chain, and V and J segments of the light chain during B cell differentiation leads to the expression of an immunoglobulin of a single specificity. During antigen-induced maturation of

a B cell, further diversity of Ig is achieved by somatic hypermutation. Ids provide a clear tumour-specific target for immune attack against B cell tumours. Vaccination with purified idiotypic Ig protein induces specific protective antibody-mediated immunity in a number of mouse lymphoma models (Campbell *et al.*, 1987; George *et al.*, 1987). This principle of idiotypic vaccination is also applicable to patients, as demonstrated by the results of recent clinical trials (Hsu *et al.*, 1997). However, a wide clinical application of Id protein vaccines is hindered by the need to prepare protein vaccines for each patient individually. DNA vaccination offers a more convenient alternative approach.

In a number of models of infectious diseases, a simple DNA delivery of pathogen-derived antigen alone can induce potent immune responses. However, for B cell tumours, DNA vaccination with Id alone leads to the induction of poor and erratic immune responses. This is because Ids are essentially self-antigens, and the immune system either ignores them or has been tolerized during prolonged exposure. One principle we have established is that genetic fusion of Id to a highly immunogenic molecule induces significant anti-Id immune responses by providing cognate T cell help. Our initial choice for a fusion partner was the non-toxic FrC of tetanus toxin, a component of a prophylactic vaccine against tetanus (Spellerberg *et al.*, 1997). A DNA vaccine encoding the Id–FrC fusion antigen induced a dramatic promotion of anti-Id response, which resulted in protection against tumour challenge in several tumour models (King *et al.*, 1998 and unpublished data). This fusion vaccine design is now in clinical trial for patients with a number of B cell malignancies. However, vaccines that include FrC will have to operate in patients with pre-existing immunity due to vaccinations against tetanus. This could be deleterious, due to clearance of the expressed antigen. Modelling this situation in mice by pre-vaccination with tetanus toxoid showed that protection can still be induced. However, slowing of the anti-Id antibody responses occurred (Forconi *et al.*, 2002). This limitation would not apply to antigens to which the immune system has not been exposed either by vaccination or by infection (primary antigens).

We turned our attention to the group of carrier molecules, CPs of plant viruses. Our initial consideration was that it would be unlikely that the human population would have pre-existing immunity to these immunogenic molecules, and therefore antigens fused to them would be presented in the setting of a primary immune response to carrier molecules. Importantly, plant viral CPs have been used to promote antibody responses to linked B cell epitopes in a number of models of infectious diseases (Turpen *et al.*, 1995; Dalsgaard *et al.*, 1996). The ability of such proteins to self-assemble into viral-like particles in the absence of viral RNA made them the molecules of choice for multiple presentation of defined B cell epitopes on the surface of large particles (review: Porta and Lomonossoff, 1998). This type of chimeric molecule induces potent immune responses, even without the addition of an adjuvant (McInerney *et al.*, 1999). The majority of the studies using plant viral CPs as carrier molecules concentrated on inserting short pathogen-derived epitopes, rather than using whole antigen sequences. For B cell malignancies, our strategy has been to use the full length variable fragments of heavy and light chain gene sequences assembled as a single chain (scFv), rather than defined short idiotopes (Hawkins *et al.*, 1994). This induces broader immunity, and eliminates the need for idiotope identification. One particular CP from PVX especially caught our attention because a few studies

indicated that long polypeptides could be fused to the N terminus of this CP while maintaining the structural integrity of both fusion partners (Santa Cruz *et al.*, 1996; Smolenska *et al.*, 1998). As one aim was to identify a fusion partner with no pre-existing antibody in humans, we tested the sera of normal individuals for antibodies to PVXCP. We found that all serum samples tested were positive for anti-FrC antibodies, but were negative for antibodies to PVXCP (unpublished data). A number of studies of protein vaccination reported that linkage of B cell epitopes to CP of plant viruses including PVXCP induced potent antibody responses, preferentially of IgG2a isotype in mice (Brennan *et al.*, 1999; Marusic *et al.*, 2001). This indicates that the nature of these carrier molecules tends to direct the immune response towards the Th1 type. Therefore, we hypothesized that a DNA fusion vaccine that includes PVXCP would likely direct the response against attached antigens towards the Th1 type. Thus, PVXCP was chosen both to explore the responses induced by DNA fusion vaccines in the absence of pre-existing immunity, and to determine the effect of an alternate carrier molecule on the nature of immune response.

Assembly and characterization of Id-fusion vaccines

Individual Id DNA vaccines can be assembled as scFv by linking variable fragments of heavy and light chain genes together by PCR using family-specific V and J segment primers. The fused V_H–V_L molecule also incorporates a flexible linker sequence between the V_H and V_L to allow Ids to fold properly (*Figure 5.2*). The assembled scFv molecule can be joined to the PVXCP sequence by PCR, or by ligating the two genes together (*Figure 5.2*). We have also incorporated either a four amino acid (GPGP) or seven amino acid linker (AAAGPGP) between scFv and PVXCP. The latter contains a *Not*I restriction site to facilitate fusion gene assembly. We repeatedly found that the usage of either the four or seven amino acid linker allows the chimeric molecules to retain structural integrity. All our DNA vaccines contain an Ig leader sequence to direct the synthesized protein to the endoplasmic reticulum. Assembled fusion genes are cloned into the pcDNA3 plasmid using *Hin*dIII and *Xba*I restriction sites. Expression is tested by transcription/translation in rabbit reticulocyte lysates and transfection into Cos-1 cells. Cos-1 cell supernatants are analysed by ELISA using anti-PVXCP polyclonal antibody. We also found that chimeric PVXCP fusion proteins can be sedimented by ultracentrifugation. This is due to the ability of scFv–PVXCP fusion proteins to self-aggregate. The pellets can be further analysed by Western blot analysis using polyclonal anti-PVXCP antiserum, and electron microscopy using immunotrapping with anti-PVX IgG. Such an *in vitro* analysis prior to testing a vaccine *in vivo* gives a good indication of the size of the chimeric product, levels of secretion, and the state of aggregation. scFv–PVXCP chimeric proteins are generally secreted into supernatants at high levels. However, we found that the fusion of small fragments, like a leader sequence to PVXCP, reduced secretion from Cos-1 cells when compared to the long scFv fragments attached to PVXCP. For immunization, DNA vaccines are prepared using a Qiagen giga prep plasmid kit.

Promotion of CD4+ mediated protection against lymphoma and myeloma

The measure of a successful vaccine design against a tumour is the ability to induce

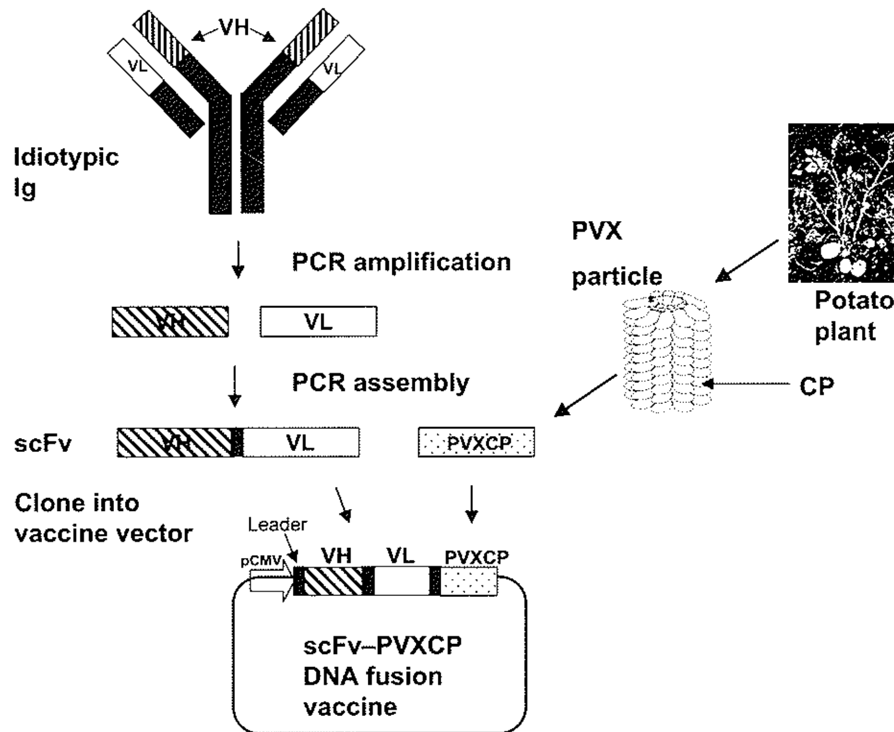

Figure 5.2. Construction of scFv–PVXCP fusion vaccines. Assembled as scFv, V_H and V_L genes derived from a tumour are cloned into a DNA vaccine with previously inserted PVXCP gene.

protective responses against tumour challenge. Initially, we evaluated the performance of the scFv–PVXCP DNA fusion vaccine design in two murine models of B cell malignances, the A31 lymphoma and the 5T33 myeloma (Savelyeva *et al.*, 2001). The A31 lymphoma carries an idiotypic IgM on the cell surface and is MHC class II positive. Vaccination of mice with the scFvA31–PVXCP DNA vaccine induced protection against challenge with the A31 tumour, with no effect of either PVXCP alone or scFvA31 alone (*Figure 5.3a*). The protection was accompanied by induction of low levels of antibody response to idiotypic IgM (typically only half of the mice responded), whereas anti-PVXCP antibody responses were high (*Figure 5.3b*). Anti-Id antibody was of an IgG2a isotype, indicating induction of a Th1 response. Since previously published data demonstrated that antibody was the key effector mechanism in protection in a number of Ig-surface positive lymphoma models, the lack of correlation of anti-Id antibody induction and protection was surprising. This suggested that other protective mechanisms could be induced in this model. In fact, depletion of CD4+ T cell subsets completely abrogated protection, indicating the importance of CD4+ T cells in protection against the A31 lymphoma (*Figure 5.3c*). Passive transfer of the immune sera at the levels induced in vaccinated mice did not confer long-term protection, though a low level of protection was seen, indicating that the role for antibody in protection was possible but not adequate (unpublished data).

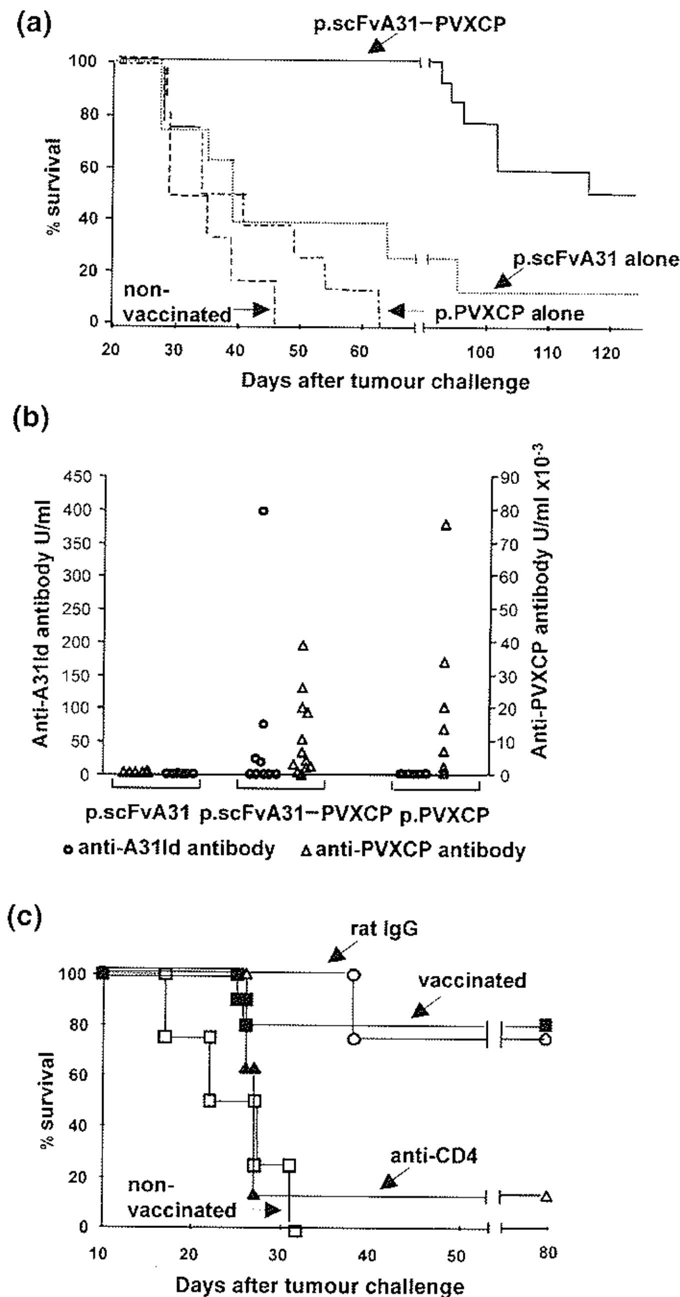

Figure 5.3. Performance of the scFv–PVXCP DNA fusion vaccine in the A31 lymphoma model. (a) Induction of protective immunity: mice were vaccinated with 50 μg of the p.scFvA31–PVXCP fusion vaccine, or p.scFvA31 alone, or p.PVXCP alone three times at days 0, 21 and 42. At day 63, mice were challenged with the A31 tumour, non-vaccinated mice were included as controls. (b) Antibody responses to A31 Id and PVXCP at day 35 as measured by ELISA. (c) Protection against challenge with the A31 tumour is dependent on CD4+ T cells. Mice vaccinated with the p.scFvA31–PVXCP fusion vaccine received MAbs against CD4+ T cells or rat IgG as a control before and after tumour challenge.

We also demonstrated that the effector mechanism induced by the scFv–PVXCP DNA vaccine against lymphoma was different from that induced by the scFv–FrC DNA vaccine where antibody was the key mediator of protection (Savelyeva *et al.*, 2001). The important lesson learnt from these experiments was that the protective effector mechanism induced by idiotypic fusion vaccine was influenced by the nature of the carrier molecule fused to the Id.

A similar scFv–PVXCP DNA vaccine design was tested in the 5T33 myeloma model that resembles human myeloma in that it secretes an idiotypic IgG paraprotein, and is surface Ig and MHC class II negative. Since the tumour cells, in contrast to lymphoma, do not express Id on the cell surface, we did not expect antibody to play a role in protection in this model. In fact, using Id protein vaccination, we confirmed previously that antibodies do not protect against the 5T33 myeloma. Vaccination with the scFv5T33–PVXCP DNA format, however, induced significant protection against challenge with the myeloma (*Figure 5.4a*). Although the antibody is not important for protection, it provides a useful indicator of the immune response. Generation of antigen-specific IgG in particular is indicative of induction of antigen-specific T cells, and IgG isotypes are markers of which type of helper cell has been induced. The 5T33–PVXCP vaccine induced high levels of anti-5T33 Id and PVXCP antibodies (*Figure 5.4b*). The antibodies were preferentially of the IgG2a isotype, consistent with induction of the Th1 type of response. Again, as for lymphoma, we looked for cellular effector mechanisms by depletion of CD4+/CD8+ T cell subsets. These results demonstrated that protection in this model was mediated by CD4+ T cells (*Figure 5.4c*). Furthermore, we have shown that for induction of both antibody and protective CD4+ T cells, fusion of scFv and PVXCP was required, because a mixture of the two plasmids encoding each individual component induced neither anti-Id antibody nor CD4+ T cell-mediated protection.

Mechanism of DNA fusion vaccine operation

Promotion of specific anti-Id immunity by vaccination with scFv–PVXCP DNA vaccines is likely to operate through several mechanisms. First, PVXCP, similar to FrC, provides cognate T cell help for the idiotype-specific B and T cells. In the case of B cells, CD4+ T cells specific for PVXCP provide help for idiotype-specific B cells in a hapten-carrier manner (*Figure 5.5*). This leads to production of Id-specific IgG in both models. For induction of Id-specific antibody, activation of PVXCP-specific CD4+ T cells is sufficient, and Id-specific CD4+ T cells are not required. However, these cells are absolutely critical for protection in the A31 lymphoma and the 5T33 myeloma models. They appear as a result of T cell help received from PVXCP-specific CD4+ T cells. Recently, Gerloni and co-workers have demonstrated a novel helper role for CD4+ T cells, i.e. CD4+ T cells specific for a dominant epitope provide help to CD4+ T cells that recognize an immunologically silent epitope (Gerloni *et al.*, 2000). This is the so-called 'linked T cell help for helpers' mechanism. It involves action of dominant epitope-specific CD4+ cells not directly on CD4+ T cells specific for the silent determinant, but through dendritic cells, in a fashion similar to that of activation of CD8+ cells with the help of CD4+ T cells (*Figure 5.5*). The help results in upregulation of co-stimulatory molecules on the cognate dendritic cell. The Th–Th co-operation requires that both dominant and silent epitopes are presented by the

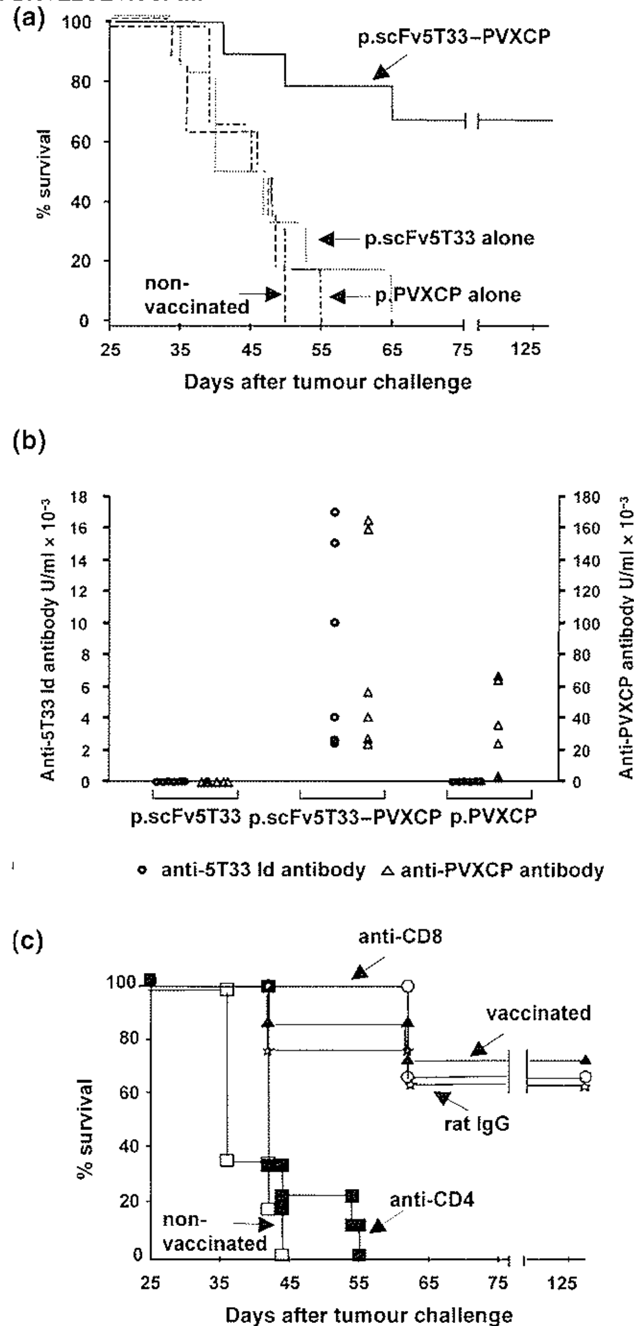

Figure 5.4. Performance of the scFv–PVXCP DNA fusion vaccine in the 5T33 myeloma model. (a) Induction protective immunity: mice were vaccinated with 50 μg of the p.scFv5T33–PVXCP fusion vaccine, or p.scFv5T33 alone, or p.PVXCP alone three times at days 0, 21 and 42. At day 63 mice were challenged with the 5T33 tumour, non-vaccinated mice were included as controls. (b) Antibody responses to 5T33 Id and PVXCP at day 63 as measured by ELISA. (c) Protection against challenge with the 5T33 tumour is dependent on CD4+ T cells. Mice vaccinated with the p.scFv5T33–PVXCP fusion vaccine received MAbs against CD4+ or CD8+ T cells or rat IgG as a control before and after tumour challenge.

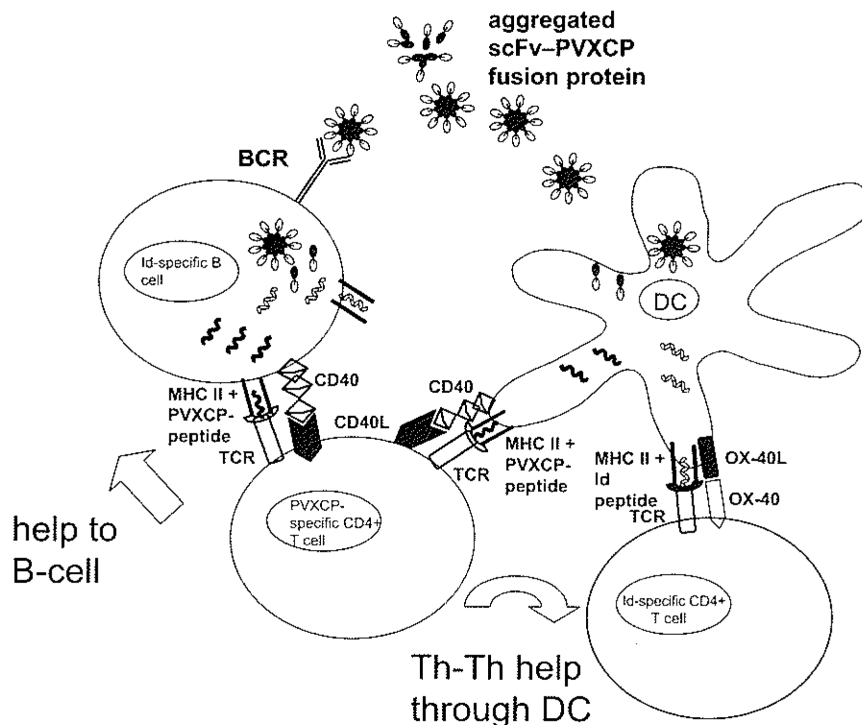

Figure 5.5. Enhancement of immunity to Id by fusion to PVXCP. Primed by DC that have taken up scFv–PVXCP protein, CD4+ T cells specific for PVXCP derived peptide interact with Id-specific B cells that internalized the fusion protein through BCR specific for Id. Such interaction together with CD40/CD40L ligation leads to production of anti-Id antibody. Specific for PVXCP, CD4+ T cells also activate DC by increasing expression of co-stimulatory molecules. This licenses DC to then interact and activate Id-specific CD4+ T cells. Aggregation of Id–PVXCP protein enhances recognition by DC.

same APC. This is in agreement with our findings that for induction of protective CD4+ T cells, fusion of Id and PVXCP is required. The second mechanism is attributed to a special feature of the coat protein to self-aggregate. PVXCP-fused chimeras self-assemble into multimeric molecules (Savelyeva *et al.*, 2001). Such fusion molecules closely resemble the structures formed by coat proteins of potexviruses, i.e. two layer discs, and stacks of discs. It appears that DC may better recognize such molecules as they are likely to resemble pathogens. This may act on the level of enhanced uptake of the antigen by DC, as PVXCP does not directly change the maturation status of DC (unpublished data).

How do CD4+ T cells kill tumours?

The p.scFv–PVXCP vaccine format induces potent protective Id-specific CD4+ T cell-mediated responses against the A31 lymphoma and the 5T33 myeloma. These tumours are not only distinct in the form of the Id antigen expressed, being surface-expressed for the lymphoma and secreted for the myeloma. There is also a difference in terms of immune recognition by antitumour Id-specific CD4+ T cells, since the

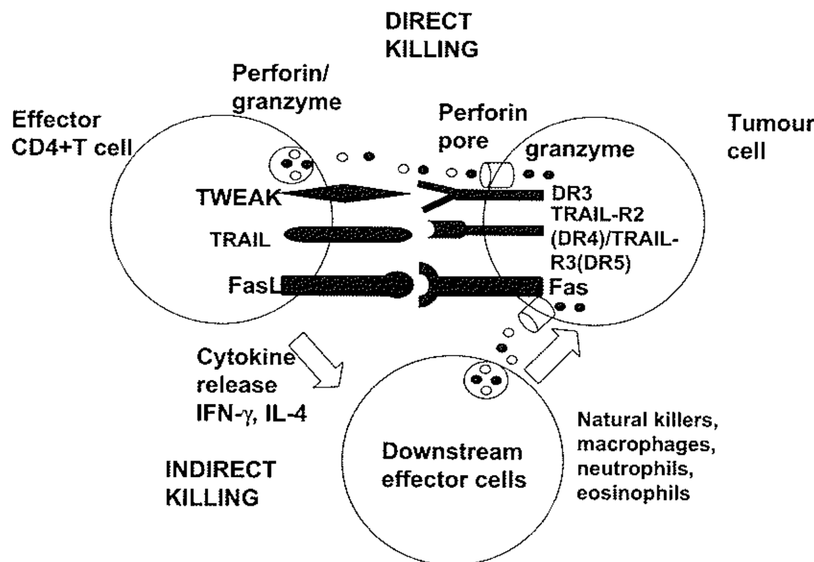

Figure 5.6. The role of CD4+ T cells in antitumour immunity. CD4+ T cells kill tumours acting directly through interaction of ligands; FasL, TRAIL, and TWEAK with death receptors (Fas, TRAIL-R2/R3, DR3), or by perforin/granzyme-mediated lysis. CD4+ T cells can also act indirectly through cytokine release (IFN-γ and IL-4) and engagement of downstream effector cells, such as natural killers, macrophages, neutrophils, and eosinophils.

lymphoma is MHC class II positive, whereas the myeloma is negative. The importance of anti-Id CD4+ T cells in the effector phase of the immune response indicates that they play a direct, rather than helper, role in mediating antitumour immunity. We have not fully understood the mechanisms of tumour destruction that are induced by vaccination with scFv–PVXCP DNA vaccines. However, there are several mechanisms that can potentially be used by these CD4+ cells for tumour attack (*Figure 5.6*). The most common mechanism that can be used for either MHC class II positive or negative tumour is direct killing by Th1 CD4+ T cells via interaction of FasL (CD95L) on activated CD4+ T cells, and Fas (CD95) on the target cell (Ju *et al.*, 1994). For Fas-negative targets and for targets that are Fas-positive but non-responsive to Fas signals, which is often the case for tumours, CD4+ T cells can lyse target cells through two other pathways. These involve other members of TNF-receptor ligand family, namely TRAIL, the ligand for Apo2 (TRAIL-R2 and R3, also known as DR4 and DR5 respectively) (Thomas and Hersey, 1998; Dorothee *et al.*, 2002), and TWEAK with Apo3 (DR3) as a receptor (Kaplan *et al.*, 2000). Recent evidence also indicates that CD4+ T cells also use perforin/granzyme-mediated cytotoxity against MHC class II positive tumours (Dorothee *et al.*, 2002).

Indirect antitumour immunity can be induced by activated CD4+ T cells through production of cytokines, with IFN-γ (Th1 cells) and IL-4 (Th2 cells) playing a key role in engaging the downstream effector cells, such as natural killers, granulocytes, and macrophages (Hung *et al.*, 1998; review: Pardoll and Topalian, 1998). A broader role in antitumour immunity has been implicated for IFN-γ. This includes a direct

cytotoxic effect on tumour cells, upregulation of MHC class I and Fas expression, and inhibition of angiogenesis (Spets *et al.*, 1998; Mumberg *et al.*, 1999; Qin and Blankenstein, 2000; Beatty and Paterson, 2001).

Concluding remarks and future prospects

There are several approaches used for activating tumour immunity by DNA vaccines that utilize TA. They include fusion or co-delivery of cytokines (Syrengelas *et al.*, 1996), chemokines (Biragyn *et al.*, 1999) or co-stimulatory molecules (Conry *et al.*, 1996), and some other approaches (review: Zhu *et al.*, 2001). The approach we have adopted is to fuse our TA of interest, Id, to highly immunogenic sequences derived from either a human pathogen, like FrC, or a plant pathogen, like PVXCP. The principle of this is that CD4+ T cells specific for a pathogenic sequence provide cognate help for Id-specific CD4+ B and T cells. The ability of PVXCP to aggregate contributes further to immunogenicity of the scFv–PVXCP vaccine design, and the lack of pre-existing immunity is an additional advantage of this design when applied to patients. An important lesson is that immune responses to tumours can be manipulated by attaching different immuno-enhancing sequences to TA. Another fusion vaccine design recently developed in our laboratory further supports these findings. This design utilizes a truncated FrC molecule which activates cytotoxic CD8+ to a defined MHC class I epitope derived from TAs (Rice *et al.*, 2001, 2002). Furthermore, our findings emphasize a much broader role for CD4+ T cells in antitumour immunity than simply providing help for B cells and T cells, i.e. direct involvement in tumour destruction either by a direct or indirect mechanism. The scFv–PVXCP fusion design provides the opportunity to activate CD4+ mediated immunity, which can act alone or potentially synergistically with vaccines activating other arms of the immune response.

In the future, incorporation of an alpha viral replicon, an RNA polymerase that can amplify the antigen-encoding RNA, may allow further improvement of performance of DNA fusion vaccines that include immuno-enhancing sequences fused to Id (review: Polo *et al.*, 2000). Another promising approach is a heterologous prime/boost regime. In this case, chimeric PVX viral particles that display Id on their surface can be used as a booster vaccine after priming with the scFv–PVXCP DNA vaccine.

To address the problem arising from the technical demand of individual Id vaccine assembly, universal antigens, which can be included into a generic fusion 'off the shelf' vaccine against B cell tumours, need to be identified. With the success of DNA micro-array technology, proteomics and sequencing of the human genome, more of this type of TA will be revealed.

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