THE CANARYPOX VIRUS ALVAC AS A VECTOR IN CANCER GENE THERAPY

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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The Canarypox Virus ALVAC as a Vector in Cancer Gene Therapy

By Abhijit Ghose

A Thesis submitted in conformity with the requirements for the degree of Master of Science, Graduate Department of Institute of Medical Science, University of Toronto, 1999.

Abstract

The immunogenicity of recombinant canarypox (ALVAC) viral vectors within whole cell tumor vaccines was evaluated using the early T-cell thymic Lymphoma STF10. STF10 cells were modified with the recombinant ALVAC vectors and injected into syngeneic mice. Control mice receiving unmodified cells developed tumors, while mice injected with STF10/ALVAC, STF10/ALVAC B7-1 or STF10/ALVAC B7-1 or STF10/ALVAC B7-1/ALVAC IL-12 completely rejected their tumors. Rechallenge at day 55 showed that none of these groups were significantly protected. However, modified regimens incorporating two vaccines induced a protective effect in all vaccinated groups. Notably, the parental ALVAC virus was equivalent to all other recombinant ALVAC viruses in conferring antitumor immunity. Tumorigenicity experiments in nude mice revealed that the effector mechanism mediating rejection of tumor cells bearing ALVAC vectors is multifactorial. Finally, in vitro experiments revealed that cytotoxic T-cells specific for parental STF10 cells could be generated as a result of in vivo immunization with STF10/ALVAC vaccines.
Acknowledgments

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Figure 1: Genomic Organization of recombinant ALVAC viruses

The ALVAC virus vector consists of a linear double stranded DNA molecule measuring about 325 Kbp. The multiple cloning sites C5 and C6 have been inserted as shown above. The ALVAC B7-1 construct consists of a human B7-1 cDNA driven by the vaccinia H6 promoter inserted into the C6 MCS. The ALVAC IL-12 construct consists of two p35 subunit cDNA sequences each driven by one E3L vaccinia promoter, and a p40 subunit cDNA driven by the entomopox 42K promoter. The p35 and p40 subunit constructs are inserted into the C5 and C6 loci, respectively.

Figure 2: Heterogeneity in ALVAC Viral Transduction

To determine the tropism exhibited by the Canarypox virus ALVAC, cell lines of different lineages and stages were assayed for the ability to successfully take up the virus and express the virally encoded protein B7-1. Clear histograms represent cells that were specifically stained by anti-human B7-1 antibody, while shaded histograms represent staining via an isotype matched non-specific antibody. Results indicate that recombinant B7-1 is expressed only in select tissues represented by NFS-70, B16, ST, as well as two
ST subclones STF10 and STG4. A20, K46J, C1498 and P815 are completely negative for ALVAC-encoded products.

**Figure 3: Surface Marker Phenotype of STF10**

STF10 cells were stained with antibodies specific for the surface-expressed proteins indicated, and then assayed by cell cytometry as outlined in Materials and Methods. Clear histograms represent specific staining for the marker indicated, while shaded histograms represent irrelevant staining of STF10 via a non-specific isotype matched antibody. STF10 cells are CD44+, CD25-, HSA+, CD4 low, CD8 low, and CD3-.

**Figure 4: RT-PCR analysis of ST-F10, and STF10 infected with ALVAC, for TdT, RAG-1, RAG-2, and HPRT.**

ST-F10 yields the expected size PCR product for RAG-1, RAG-2, as well as TdT. The positive control for PCR amplification of TdT consisted of a genomic sample from mouse tail-DNA. Positive controls for amplification of RAG-1, RAG-2 and HPRT consisted of various cDNA preps of the mouse Pro-B Cell line NFS-70.

**Figure 5: Growth Characteristics of STF10 Cells after infection via ALVAC vectors**

STF10 cells were infected with ALVAC B7-1 as outlined in Materials and Methods, and then replated in culture media at a concentration of 20,000 cells/mL. Growth was
monitored over a period of 4 days, and was compared to the growth rate of a STF10 culture that was plated at the same concentration, but was uninfected with ALVAC B7-1. Results indicate that infection of cells via recombinant ALVAC vectors does not alter the growth characteristics of the STF10 cells as the rate of expansion of the two cultures is almost identical.

Figure 6: Effect of ALVAC viral Infection on MHC class I expression via STF10 cells

STF10 cells were infected with ALVAC B7-1 as outlined in Materials and Methods, and assayed for the expression of MHC I at timepoints corresponding to 6 hours into viral infection, and 24 hours following viral infection. Results indicate that there is no downregulation of classical MHC class I (H-2K<sup>d</sup> and H-2D<sup>d</sup>) as the geometric mean fluorescence of infected cells is 17 at 6 hours, and 19 at 24 hours, compared to uninfected cells which display a geometric mean fluorescence of 14 at 6 hours and 15 at 24 hours.

Figure 7: Stability of ALVAC Encoded Products Over Time

Because the ALVAC viral vector is non-replicative in mammalian tissues, the expression of ALVAC-encoded products will be transient as the population of cells divide and ALVAC proteins approach their half-life in situ. The SCID Thymoma cell line ST was used as an indicator of ALVAC product stability: SCID Thymoma bulk culture was transduced with ALVAC-B7-1 and half the culture was irradiated to stop it from dividing
further, while the other half was kept in log phase. Both cultures were assayed for ALVAC-encoded B7-1 expression at discrete timepoints. Results indicate that in a non dividing culture, ALVAC-encoded products can be detected by immunostaining up to four days (> 96 hours) post infection, whereas for a rapidly dividing culture, expression is optimal for about two days (48 hours), after which expression levels start declining, and is lost completely at four days.

Figure 8: Survival of Mice Post STF10 Tumor Variant Vaccination

STF10 tumor cells were infected with a) No virus b) ALVAC c) ALVAC-B7-1 d) ALVAC-IL-12 or e) ALVAC-B7-1 and ALVAC-IL-12, and injected subcutaneously into mice (using 5 mice per vaccine). Mice receiving cells alone succumbed to tumor, whereas mice that received cells containing either parental or recombinant ALVAC vectors all rejected their tumors at a frequency of 100%.

Figure 9: Lack of Systemic Protection against Wild Type Challenge After a Single Vaccination

Subsequent to vaccination of mice with STF10 transduced with a) No Virus b) ALVAC c) ALVAC-B7-1 d) ALVAC-IL-12 or e) ALVAC-B7-1 and ALVAC-IL-12, all surviving mice were challenged at day 55 with a subcutaneous injection of wild type (unmodified) STF10 cells on the opposite (left) lateral flank. Previously unvaccinated (naive) mice were also injected with wild type SF10 cells as a positive control for
tumorigenicity. Survival of immunized mice is not statistically improved for any group denoted above (p > 0.05), even though there appears to be a delay in tumor formation in mice previously immunized with STF10/ALVAC-IL-12.

**Figure 10: Tumorigenicity of STF10 Variants in Nude Mice**

STF10 cells were infected with parental or recombinant ALVAC viruses as described in Materials and Methods, and injected subcutaneously into nude mice. All animals receiving cells alone succumbed to tumor in both experiments (A & B). Animals receiving STF10 cells containing either parental or recombinant ALVAC vectors displayed tumor rejection at statistically significant (p < 0.05) frequencies, relative to mice receiving cells alone, in both independent experiments.

**Figure 11: Survival of Mice Post Wild Type STF10 Challenge Following Vaccine Boost**

Mice were primed and boosted with cellular vaccines of a) STF10 / ALVAC b) STF10 / ALVAC-B71 c) STF10 / ALVAC-IL-12 d) STF10/ALVAC-B7-1 and STF10 / ALVAC-IL-12. In addition, one group of mice was only primed with STF10 / ALVAC-IL-12, but left unboosted. Vaccinations and boosts were administered subcutaneously, on the right flank, with the boost taking place 35 days after the initial vaccination. 21 days post the boost, mice were challenged subcutaneously on the left flank with wild type STF10 cells and assayed for survival. Naive controls that had not been previously vaccinated all
developed tumors, while all other vaccinated groups displayed improved survival relative to naive mice (p < 0.05 for all groups relative to naive mice).

**Figure 12: Specific Activity of Splenocytes from STF10/ALVAC-IL-12 immunized mice**

Mice were immunized with STF10/ALVAC-IL-12 and spleens were harvested on days 13, 17, 27 and 35. Splenocytes were stimulated with STF10/ALVAC and specific killing against STF10/ALVAC-IL-12 (A & B) or wild type STF10 (C & D) cells was evaluated. Cytolytic activity was observed on day 27 against STF10/ALVAC-IL-12 cells (B) and not STF10 cells (D). No cytolytic activity against either type of cells was observed on day 13 (not shown), day 17 (A & C) and day 35 (not shown).

**Figure 13: Effects of Differential Stimulation on Splenocyte Reactivity against STF10**

Mice were immunized with STF10/ALVAC and then boosted with the same vaccine. 21 days following the boost, splenocytes from an immunized mouse and a naïve control were harvested and stimulated with STF10 (A & B) or STF10/ALVAC (C). Results indicate that cytolytic precursors specific for STF10 are indeed present in the splenocyte culture (A), in addition to those specific for STF10/ALVAC (B); however, their detection
is dependent on stimulation with wild type STF10. Stimulation via STF10/ALVAC does not yield efficient cytotoxicity against wild type STF10, relative to naive controls (C).
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>β-gal</td>
<td>beta-galactosidase</td>
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<td>bp</td>
<td>base pair</td>
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<tr>
<td>CEA</td>
<td>Carcinoembryonic Antigen</td>
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<td>CEF</td>
<td>Chicken Embryo Fibroblast</td>
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<td>cGy</td>
<td>centigray</td>
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<td>CHO</td>
<td>Chinese Hamster Ovary</td>
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<td>CLMF</td>
<td>Cytotoxic T-lymphocyte Maturation Factor</td>
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<td>CM</td>
<td>Culture Media</td>
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<td>CON-A</td>
<td>Concanavalin-A</td>
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<td>CTL</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DTH</td>
<td>Delayed Type Hypersensitivity</td>
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<tr>
<td>DTT</td>
<td>Dithioxytreitol</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>GP</td>
<td>Glycoprotein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
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<tr>
<td>HPRT</td>
<td>Hypoxanthine Phosphoribosyl Transferase</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IRES</td>
<td>Internal Ribosome Entry Site</td>
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<td>Kb</td>
<td>Kilobase</td>
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<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis Virus</td>
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<tr>
<td>LTR</td>
<td>Long Terminal Repeat</td>
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<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
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<tr>
<td>MCS</td>
<td>Multiple Cloning Site</td>
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<tr>
<td>Me</td>
<td>Mercaptoethanol</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>µCi</td>
<td>microcurie</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
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<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
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<tr>
<td>NKSF</td>
<td>Natural Killer Stimulatory Factor</td>
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<tr>
<td>NTP</td>
<td>Nucleoside Triphosphate</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
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<tr>
<td>Pfu</td>
<td>Plaque Forming Unit</td>
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<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
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<tr>
<td>PLP</td>
<td>Proteolipid</td>
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<tr>
<td>RAG</td>
<td>Recombination Activating Gene</td>
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<tr>
<td>R-EAE</td>
<td>Relapsing-Remitting Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>RG</td>
<td>Rabies Glycoprotein</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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xiv
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>rVV</td>
<td>recombinant Vaccinia Virus</td>
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<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
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<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl Transferase</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler’s Murine Encephalomyelitis Virus</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>VSV</td>
<td>Vesicular Stomatitis Virus</td>
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Introduction

Advances in our understanding of antigen presentation and effector activation have provided the basis for new and exciting strategies for the therapy of cancer. It is widely believed that spontaneous tumors arise due to an acquired ability of cancer cells to evade immune surveillance, a process whereby immune effectors specifically recognize and eliminate tumor cells from the body (1).

Normally tumor cells express various protein epitopes (tumor rejection antigens) on their cell surface via their MHC class I and class II molecules, which can be specifically recognized by T-cells (2). In the course of a progressive cancer, the interaction between the cancer cell and the T-cell, which should cause specific activation of the T-cell, does not occur. This could be induced by several factors including immunological ignorance of the tumor cell, down-regulation of MHC molecules expressing the tumor antigen (3), down-regulation of costimulatory or adhesion molecules, loss of tumor antigen due to genetic instability (4), and expression of immunosuppressive cytokines (5).

Thus, many biological therapies against cancer are aimed toward reactivation of these tumor antigen specific T-cells. One such approach in active immunotherapy involves the modification of an MHC expressing cell line by transfection of a gene encoding a costimulatory molecule (6). This costimulatory molecule provides the second activating signal to the T-cell, the first signal comprising of activation via the T-cell receptor as it recognizes a cognate MHC-peptide complex on the antigen presenting cell (7). Once a
A cytotoxic T-cell is activated it can specifically recognize and kill other cells with or without receiving the costimulatory signal.

An alternative strategy is to introduce into the tumor cell a gene encoding a cytokine (8). Cytokines can have a wide array of targets and effects, and generally they serve either to activate immune effectors like T-cells, NK cells and macrophages directly, or recruit professional antigen presenting cells (APC). These APC take up tumor antigens, migrate to the lymph node and then activate T-cells more potently due to a greater number of costimulatory molecules present on their surface (9).

**Models in Antitumor Immunity – Recombinant Costimulatory Molecule Expression**

In order for T-cells to be activated against an antigenic epitope they must receive two signals from the antigen presenting cell (10): signal 1, arising from cognate interactions between the MHC-peptide complex on the APC and a complementary T-cell receptor on the T-cell, and signal 2, arising from a costimulatory molecule on the APC and a complementary receptor on the T-cell. Various cell surface receptors have been shown to deliver costimulatory signals to the T-cells including B7-1 and B7-2, 41BB-Ligand, CD40-Ligand, ICAM 1, 2, and 3, and LFA 3 (11).

Expression of costimulatory molecules has also been used in active immunotherapy models, whereby tumor cells have been genetically modified to express genes which
provide this second activating signal, and manipulate the immune system to mount a response against the tumor cells.

**B7-1**

In a landmark paper, Chen *et al.* (6) showed that transfection of a murine melanoma K1735-M2 with an antigen E7 and the costimulatory molecule B7-1 induced complete tumor rejection in 100% of mice, after an initial phase of tumor growth. This rejection was dependent on the presence of the antigen E7, and was abrogated in nude mice, showing that the *in vivo* response was T-cell mediated. More specifically, depletion experiments showed that CD8+ cytotoxic T-cells were responsible for tumor rejection and CD4+ T-helper cells could be removed without changing the antitumor response. Furthermore, wild type tumors could be rejected with concomitant injection of B7-1 expressing tumors on the opposite flank, and 4 day established lung metastases could be cured in 40% of mice with injection of B7-1 expressing tumor cells.

Townsend and Allison (12) performed a similar experiment where they showed that K1735 cells were rejected at a frequency of 90% if expressing recombinant B7- and that this was an effective vaccine in mice, as 90% of the survivors were protected upon rechallenge. Once again CD8+ T-cells were responsible.

Subsequently, however, Chen *et al.* (13) found that the inherent immunogenicity of the tumor cell may be a factor in B7-1 mediated antitumor immunity. They assayed four immunogenic lines RMA, EL-4, P815 and E6B2, and four non immunogenic lines MCA
101, MCA 102, Ag104 (sarcomas) and B16 melanoma and found that in the immunogenic tumors, recombinant B7-1 expression induced 100% rejection, while in all the non-immunogenic tumors, B7-1 expression had no effect. Furthermore, in vitro analysis showed that the only two immunogenic tumors tested, EL-4 and P815, induced a CTL response from immunized mice, while the non-immunogenic tumors MCA 102 and B16 did not.

B7-2

A second B7 molecule, designated B7-2 was cloned in 1993 by Freeman et al. (14), based on its ability to induce T-cells to produce IL-2 and proliferate, and the abrogation of this activity by CTLA4-Ig but not anti-B7-1 antibody. Subsequently, B7-2 was also tried in several active immunotherapy models. La Motte et al. (15) showed that P815 mastocytoma cells expressing B7-2 were just as effective as B7-1 expressing cells in inducing an antitumoral response, and B7-1 and B7-2 tumor variants were equally effective in retarding the growth of established tumors. Yang et al. (16) also demonstrated that P815 cells expressing B7-2 were immunogenic, and protected mice against wild type challenge through specific recruitment of CD8+ T-cells only.

ICAM 1

Initial active immunotherapy studies with ICAM 1 showed that ICAM 1 gene transfection into the murine fibrosarcoma MCA 105 significantly slowed tumor growth (17, 18). Subsequent work by Uzendoski et al. (19) revealed that with MC38 tumor cells, expression of ICAM 1 stimulated concanavalin A-activated T-cells to secrete TNF-
α. As well, a specific alloreactive cytotoxic T-cell reaction was observed against MC38 cells that were expressing recombinant ICAM 1. In vivo MC38 cells that were expressing ICAM 1 were rejected at a frequency of 100% and furthermore, all surviving mice were protected from further tumor challenge, even when the dose of the challenge was twice that of the immunogen.

*4-1BBL*

4-1BB ligand (4-1BBL) is a type II surface glycoprotein which belongs to the tumor necrosis factor (TNF) family, and is expressed on antigen presenting cells such as activated B-cells, macrophages and splenic dendritic cells (20, 21). It is a costimulatory molecule which can activate T-cells independently of CD28-engagement by either B7-1 or B7-2, but can also synergize with the latter two molecules, especially at low levels of CD3 activation (22). *In vivo* studies have shown that like B7-1, expression of 4-1BBL on tumor cells can effectively activate T-cells against the respective tumor, and in the long run, confer antitumor immunity. Guinn et al. (23) demonstrated that while A20 cells expressing B7-1 were 100% tumorigenic, and those expressing B7-2 were more immunogenic, A20 cells expressing 4-BBL in conjunction with B7-2 were completely rejected in syngeneic mice. Furthermore, challenge of surviving mice with wild type A20 revealed protective effects in 90-100% of mice, depending on the clone that was used as the immunogen. Similarly, Melero et al. (24) showed that P815 cells expressing 4-1BBL were rejected in >90% of mice, and all surviving mice were protected against systemic challenge. Antibody depletion experiments showed that only CD8+ T-cells were required for tumor rejection when tumor cells themselves were expressing 4-1BBL,
and both CD4+ and CD8+ T-cells were needed if wild type tumors were already established (24, 25).

Models in Antitumor Immunity -- Recombinant Cytokine Expression

Cytokines are key modulators of host immune and inflammatory responses. The transfer and expression of cytokine expressing genes into tumor cells has yielded a novel strategy to augment antitumor reactivity in various murine models. These gene modified tumors have been shown to induce a rejection of the tumor variants themselves, and in many cases, induce systemic immunity against the parental tumor cells.

Toward the goal of reducing tumorigenicity via stimulation of local immune and inflammatory reactions, a number of cytokine genes have been used to genetically modify tumor cells (26, 27). These include IL-1 (28), IL-2 (29, 30), IL-4 (31, 32), IFN-γ (33-35), IL-6 (36, 37), IL-7 (38, 39), IL-12 (40, 41), TNF-α (42, 43), and GM-CSF (8). The pleiotropic effect of cytokines on the various arms of the innate and adaptive immune system poses a problem in establishing a definite mechanism by which tumor cells are rejected. Because different cytokines ultimately affect different downstream cell types, different models of antitumor immunity have evolved.

IL-2

A variety of tumor models utilizing expression of IL-2 have been tested, including a colon carcinoma CT26 and a melanoma B16 (29), a fibrosarcoma CMS-5 (30), a
mastocytoma (44), a mammary adenocarcinoma TSA (45), a fibrosarcoma MCA 102 (46) as well as bladder and lung carcinomas (47, 48).

Predominantly, the tumor rejection or onset delay that is observed upon injection of tumor cells expressing IL-2 is mediated by cytotoxic T-cells, though in some cases, neutrophils and NK cells have been implicated (45, 46). A protective immunity toward parental challenge was also found in a number of these cases. In a model of an established tumor, Connor et al. (47) grew a 7 day bladder carcinoma MBT-2 tumor in mice, and observed a decreased tumor size with concomitant injection of radiation killed IL-2 secreting cells.

IL-4

Unlike studies with IL-2, tumor rejection models utilizing IL-4 secreting cells have shown a number of different effector mechanisms to be responsible, depending on the particular tumor line studied. Using a renal carcinoma RENCA, it was shown that local rejection was actually T-cell independent and mediated by eosinophils; however, systemic immunity toward wild type challenge was conferred and this was mediated only through CD8+ T-cells (31). Studies with the J558L plasmacytoma showed eosinophils and macrophages to be the major effector in conferring rejection of tumor cells, as defined by blocking with specific antibodies against granulocytes (32).
**IL-6**

IL-6 is usually classified as a cytokine that is involved in inflammatory responses and it is produced by macrophages, monocytes, as well as activated T-cells (49). It has also been shown to be able to increase cytotoxic activity of T-cells *in vitro* (50). IL-6 has been implicated in both nonspecific as well as specific antitumoral responses. Sun *et al.* (37) working with the B16 melanoma, showed that the antitumor responses were due to mainly nonspecific proinflammatory effectors like macrophages and neutrophils. Porgador *et al.* (36), working with the Lewis Lung Carcinoma D122, however, showed that IL-6 conferred a reduction in both tumorigenicity as well as metastatic competence that was a result of T-cell mediated function. In addition, IL-6 irradiated cells were a potent vaccine toward protecting mice against a subsequent wild type tumor challenge, and moreover, a reduction in established tumor growth could also be observed with concomitant injection of inactivated IL-6 transfectants.

**IL-7**

IL-7 is a cytokine known to have a critical role in the development and maturation of lymphocytes (7). However, this cytokine was also used in the context of antitumoral immunity by Hock *et al.* (38) who showed that the plasmacytoma J558L was completely rejected upon injection of IL-7 expressing tumors into mice. Immunohistochemical analysis showed infiltration of macrophages and CD4+ as well as CD8+ T-cells within the tumor, while subset depletion experiments showed an absolute dependence on CD4+ T-cells as well as macrophages, but not CD8+ T-cells. In contrast, McBride *et al.* (39)
showed an increase in the number of infiltrating CD8+ T-cells relative to CD4+ T-cells within FSA fibrosarcoma tumors expressing IL-7, and splenocytes from animals that had been vaccinated with FSA expressing IL-7 were able to specifically lyse parental FSA tumor cells in vitro, giving evidence for CD8+ T-cell mediated effects.

**IFN-γ**

Various murine tumor models have used IFN-γ expressing tumor cells to induce rejection of tumor variants and induce and subsequent protection toward wild type challenge. Predominantly, CD8+ T-cells have been implicated as the effector subset in the antitumoral response, in models including a neuroblastoma C1300 (51), a fibrosarcoma CMS-5 (33), an adenocarcinoma SP1 (35), a colon carcinoma CT26 (35), a lung carcinoma 3LL (34) and a bladder carcinoma MBT-2 (47). In a number of these studies, MHC class I upregulation was observed on the tumor cells themselves, upon expression of the recombinant cytokine.

Working with a weakly immunogenic fibrosarcoma CMS-5, Gansbacher et al. (33) showed that upon injection of mice with IFN-γ expressing tumor variants, splenocytes exhibited specific cytotoxicity against parental cells over a set of discrete time points. Furthermore, an in vivo protective effect was seen upon injection of survivors with parental unmodified CMS-5 cells. Porgador et al. (34) observed that IFN-γ expression via retroviral infection of two poorly immunogenic clones of the lung carcinoma 3LL turned the clones into high expressors of H-2Kb and caused a specific decrease in tumorigenicity and metastatic growth. In addition, irradiation of IFN-γ expressing clones
and their subsequent injection into mice induced significant protection against parental cells, while injection of IFN-γ expressing clones almost cured mice carrying already established micrometastases. Esumi et al. (35) showed also that while IFN-γ induced MHC I upregulation is necessary for tumor rejection, MHC upregulation by itself is not sufficient and other downstream effects of IFN-γ are integral in making tumor cells more immunogenic.

**TNF-α**

The primary source of TNF-α is usually considered to be the macrophage / monocyte, but various other cell types also secrete it, including neutrophils, T and B lymphocytes, NK cells as well as endothelial cells (49). TNF-α exerts a wide variety of effects on diverse cell types, and plays an important role in defense against infection and tumor growth (7). Asher et al. (42), working with the MCA 205 sarcoma, showed that tumors expressing TNF-α regressed in animals after an initial phase of tumor growth and that this regression was mediated by CD4+ and CD8+ T-cells. Blankenstein et al. (43) demonstrated that J558L plasmacytoma tumor establishment was significantly delayed with TNF-α expression; however, in this case, this effect was blocked by an antibody to type 3 complement receptor (CR3) which blocks migration of inflammatory cells like Macrophages.

**GM-CSF**

A number of early studies involving vaccination models with cytokine gene modified cells showed that expression of certain cytokines were capable of augmenting T-cell
mediated immunity, defined by systemic protection of vaccinated animals after challenge with wild type cells. Because it is known that some tumors are inherently immunogenic, it has been postulated that instead of just having a specific effect of T-cell activity, cytokines might also act nonspecifically by killing the tumor cells and rendering them immunogenic.

Dranoff et al. (8) addressed this issue specifically for the B16 melanoma tumor model where they made stable expressors of IL-2, 4, 5, 6, GM-CSF, IFN-γ and TNF-α, and vaccinated mice with live tumors expressing one of these cytokines. Delays in tumor formation were observed with expression of IL-4, 6, IFN-γ and TNF-α, while only cells expressing IL-2 were completely rejected. Expression of IL-6 eventually resulted in death of animals, as did expression of IL-5 and GM-CSF.

Interestingly, while challenge of mice protected via vaccination with IL-2 expressing tumor variants resulted in 100% mortality, challenge of mice vaccinated with variants expressing IL-2 and GM-CSF induced systemic protection. This led to a postulation that while IL-2 expression mediated a local antitumor response, expression of GM-CSF mediated a more systemic effect. Using irradiated B16 cells, Dranoff et al. showed that vaccination via GM-CSF expression did in fact lead to systemic immunity against wild type cells, while irradiated cells by themselves did not. Thus, irradiated cells conferred a level of protection equivalent to other cytokines, while only GM-CSF induced complete protection. Depletion via antibody administration showed CD4+ and CD8+ cells to be primarily responsible for tumor rejection.
**IL-12**

IL-12, originally called cytotoxic T-lymphocyte Maturation Factor (CLMF) is a heterodimeric cytokine composed of two subunits of 45 KDa and 35 KDa. It was originally isolated by Stern et al. (52) on the basis of its ability to synergize with IL-2 to facilitate cytotoxic lymphocyte reactions, and act as a growth factor for PHA activated human T-lymphoblasts. At about the same time, Kobayashi et al. (53) had also identified the IL-12 activity which they designated Natural Killer Cell Stimulatory Factor (NKSF) on the basis of its ability to induce production of IFN-γ by resting PBMC and act as a proliferative stimulus in combination with PHA and phorbol esters.

After the murine cDNA for IL-12 was cloned (54), Gately et al. (55) showed that in vivo administration of recombinant IL-12 caused a dose dependent enhancement of NK cell lytic activity when assayed in vitro. In addition, IL-12 treated mice had elevated levels of serum IFN-γ. Lastly, mice immunized with allogeneic splenocytes displayed enhanced CTL activity against these cells when IL-12 was administered concomitantly with the immunogen. This showed that IL-12 can enhance NK and CTL activity, and induce IFN-γ expression in vivo. Germann et al. (56) also demonstrated that IL-12 could induce proliferation of Th1 cells activated via IL-2 or anti CD-3, but not Th2 cells.

One of the initial cancer therapy studies with IL-12 was performed by Zitvogel et al. (40) who showed that injection of IL-12 expressing fibroblasts at the site of an established sarcoma MCA 207 could suppress tumor growth in a dose dependent manner, or eliminate it, and induce long term immunity towards challenge. Furthermore, IL-12
delivery by irradiated fibroblasts at a distant site led to efficient rejections of established tumors. Immunohistochemical staining revealed that for mice treated with IL-12 expressing fibroblasts an enhanced number of CD4+ T-cells, CD8+ T-cells and Macrophages were present at the tumor site.

Tahara et al. (41) subsequently used the MCA 207 sarcoma and showed that genetic modification of the tumor cell via retroviral expression of IL-12 also induced tumor rejection when injected intradermally in mice. Furthermore, a protective immunity was observed in most animals challenged on the opposite flank with wild type cells. Three day old established tumors could also be cured by IL-12 expressing tumor cells inoculated at a distal site. Depletion experiments showed both CD4+ and CD8+ T-cells to be important for long term immunity.

**Combination Therapies Utilizing IL-12 and B7-1**

The synergistic effects of the costimulatory molecule B7-1 and the cytokine IL-12 were initially observed by Kubin et al. in a series of *in vitro* experiments involving both mitogen activated, as well as peripheral blood T-cells. In this report (57) Kubin et al. show firstly, that 5 days following activation via PHA, culture of T-cell blasts with IL-12 and anti-CD28 induced proliferation that was far superior to that induced by IL-12 alone. Secondly, cytokine profiles were significantly altered as PHA blasts which were stimulated with IL-12 and CHO cells expressing B7-1 showed increased IFN-γ production relative to cells that were stimulated with only one of these agents, or with
anti-CD3 in conjunction with B7-1. Thirdly, human peripheral blood T-lymphocytes which were incubated with anti CD28 and IL-12 showed high IFN-γ production, while those incubated with either one showed baseline levels. Finally, addition of CTLA-4 Ig to both PBMC as well as PHA blasts reduced the level of IFN-γ produced after incubation with IL-12 and CHO cells expressing B7-1.

Initial antitumor models utilizing IL-12 and B7-1 were established by Coughlin et al. (58) who showed that while SCK mammary carcinoma cells expressing B7-1 were rejected in only 28% of mice, and intraperitoneal administration of IL-12 only induced a delay in wild type tumor onset, the combination of rIL-12 administration with B7-1 gene modification in tumors induced rejection in 92% of mice. Further, while wild type cells could be effectively eradicated by administration of SCK-B7-1 cells on the opposite flank along with rIL-12 administration, rIL-12 by itself had no effect. Antibody depletion experiments showed CD4+ and CD8+ T-cells to be responsible for wild type tumor rejection.

Established tumor models were more successfully treated by Rao et al. (59) who showed firstly, that even though 3 or 6 day old lung metastases of CT26.CL25 (adenocarcinoma) could be effectively treated by administration of recombinant vaccinia virus (rVV) encoding the model tumor antigen β-gal, addition of rIL-12 greatly increased the therapeutic effect. A further significant delay in tumor onset was observed if B7-1 was also expressed by the rVV expressing β-gal, and rIL-12 was administered in mice with
established tumor burdens. This showed that B7-1 synergized with IL-12 in effectively activating T-cells against pre-existing tumors expressing the β-gal model antigen.

Augmentation of B7-1 mediated immunogenicity via intratumoral IL-12 gene expression was demonstrated by Pizzoferrato et al. (60) who showed that while A20 cells expressing B7-1 were delayed in tumor formation (relative to wild type cells), A20 cells expressing both IL-12 and B7-1 were completely rejected in wild type mice. Furthermore, 70% of the surviving animals displayed systemic immunity in that they rejected a challenge of wild type A20 cells on the opposite flank. Interestingly, in this case, vaccination with A20 cells expressing IL-12 only also led to 100% tumor rejection, and an equivalent level of protection against wild type challenge was observed. A synergistic effect between IL-12 and B7-1 was observed in the established tumor model, whereby wild type tumors could be cured by A20/IL-12 or A20/B7-1/IL-12 when the vaccines were injected into the tumor site, but only by A20/IL-12/B7-1 when they were injected at a distal site. Tumorigenicity experiments in nude mice demonstrated that T-cells were responsible for tumor rejection, as A20 cells expressing IL-12 or IL-12 and B7-1 lost their immunogenicity in these immunocompromised mice.

**Gene Transfer Methodologies**

A major issue in designing a gene-modified tumor vaccine, or any other DNA-based vaccine is the type of vector to be used in the expression of recombinant constructs. The choice of vector often depends on the approach of therapy and the type of response that is
desired. The methods of recombinant gene expression can be broadly broken up into viral and non-viral (or physical), as discussed below.

Physical Methods of Gene Transfer

Plasmid DNA Immunization

Many of the original studies examining the effects of in vitro genetic modification of tumor cells on tumorigenesis and subsequent immunity, utilized plasmid DNA as the vector expressing the gene of interest. These genes included IL-2 (44, 45), IL-4 (31), and IFN-γ (51) as well as costimulatory molecules such as B7-1 (6), B7-2 (15), and 4-1BB (23). In addition, a study comparing the efficacy of B7-1 and IL-12, both of which were intratumorally encoded by plasmid vectors, was carried out by Fallarino et al. (61). They showed that while immunization with irradiated P815 variants expressing either IL-12 or B7-1 protected against wild type challenge, established tumors could only be cured by injection of variants expressing IL-12 and not B7-1.

Because plasmids are not as constrained as most viruses in the exact amount of DNA that they need to incorporate, they are good candidates for gene transfer strategies utilizing varying lengths of cDNA’s. As well, these vectors eliminate the need for working with live viruses, minimizing health risks, as well as non specific immune responses in the immunized host. The disadvantage associated with plasmids as vectors is the relatively low efficiency of cell transfection. This is particularly significant in the context of cancer
vaccination where stable cell lines cannot always be established, and primary cells need to be transfected at efficiencies as high as possible.

To circumvent the problem of low transfection efficiency and longer periods of time required to establish variants, an alternative strategy using a “gene gun” has been developed (50). This strategy involves the coating of the foreign DNA of interest through precipitation, and bombarding the tissue of interest with these particles through use of a helium powered accelerator. Following delivery, the DNA dissolves within the aqueous environment of the cytoplasm and is available for expression.

*In vitro* modification of tumor cells via plasmid acceleration through a gene gun was demonstrated by Mahvi *et al.* (62). In this report, Mahvi *et al.* initially bombarded an adherent layer of B16 melanoma with DNA encoding GM-CSF, and obtained high expression levels of this cytokine (1000 – 4000 ng/million cells/24 hrs). Further, a vaccination of mice by these cells protected 58% of mice against challenge with wild type B16 cells. Finally, heterogeneous samples of primary human tumor cells could be successfully transfected with hGM-CSF, upon homogenization of the tumor samples and subjecting to DNA bombardment within 24 hours of surgical removal. Expression levels of GM-CSF varied, depending on the patient and type of cancer, and ranged from negligible to 140 ng/million cells/24 hours. Lower levels of expression were attributed to the clonal nature of B16 (and an inherent ability to express cytokines at high levels), greater cell death in primary cells, differential transfection efficiencies depending on
tumor type and stromal elements, as well as cytoplasmic size which restricts the size of the gold particles that can be incorporated.

Thus, genetic manipulation via plasmid DNA is an attractive approach for \textit{in vitro} modification of tumor cells, though traditional methods involving transfection may not be as efficient. Further, approaches using DNA vaccines that have been implemented include direct \textit{in vivo} injection via gene gun in murine models (63) and encapsulation within lipids and direct intratumoral injection into patients (64, 65).

\textit{Viral Methods of Gene Transfer}

\textit{Retrovirus}

Retroviruses are double stranded RNA viruses which make specific contact with target cells through surface envelope proteins on the viral capsid. Following initial binding, the viral envelope fuses with the cell membrane and the RNA molecules enter the cell. The encapsulated viral RNA polymerase then copies the RNA genome into a cDNA molecule, which integrates into the cellular genome to form a provirus (7). The viral genome can be read in overlapping frames, and the main products are \textit{gag} (structural protein), \textit{pol} (reverse transcriptase), and \textit{env} (transmembrane glycoprotein). The viral DNA is flanked at either end by long terminal repeats (LTR) and has a RNA polymerase II promoter site at the 5' LTR (50).
Because retroviruses in their natural form are infectious and pathogenic, a two component approach is used for gene therapy purposes. The recombinant gene is cloned into the sites for the gag, pol, and env proteins, and the cDNA of interest is driven by elements in the 5' LTR (50). Polycistronic messages can also be cloned, utilizing an internal ribosome entry site (IRES) within the message (66). The recombinant retrovirus can then be introduced into a packaging cell line which then provides the proteins that cannot be encoded by the recombinant virus (66). This yields functional retroviruses which are able to infect target cells, integrate into the host genome and express the foreign product, but are unable to complete the lytic life cycle.

Many of the initial murine studies discussed above, involving cytokine gene-modified tumor cells, used retroviruses as the vector expressing the gene of interest. These genes included IFN-γ (33), TNF-α (43), and GM-CSF (8). Subsequently, tumor cells engineered to express retrovirally encoded IL-12 were also employed as a murine vaccine by Tahara et al. (41). This vector encoded recombinant p40 and p35 subunits of IL-12 as well as neomycin as a polycistronic message within the MCA 207 and MCA 102 sarcomas. Animals receiving this tumor vaccine rejected their tumor and were significantly protected against challenge, while animals bearing tumor cells with retrovirus encoding only neomycin succumbed to tumor formation.

Thus, recombinant retroviruses are attractive as vectors for active immunotherapy as they have higher frequency of cell transformation than plasmids, and can generate high levels
of protein expression. In addition, the viral vector itself is non immunogenic, and therapeutic effects are specifically a function of the recombinant genes expressed.

The retrovirus is integrative within the host genome, and while this has the advantage of conferring stable expression for the duration of the treatment period, it carries the disadvantage of conferring constitutive, and even toxic levels of expression if the cells carrying the virus are not eventually destroyed. Furthermore, because of this integrative property, the retrovirus is mutagenic to the cell line it is introduced into. Cells also need to be mitotically active in order to be infected by retroviral vectors. Finally because of the compact nature of the retroviral genome, recombinant genes only up to about 9 Kb can be inserted (50).

Adenovirus

The limitations of the retroviral vector, namely, constitutive gene expression, requirement for mitotically active cells, and mutagenicity toward host cells, have led to the development of another viral vector that circumvents these disadvantages. Adenoviruses have a 36 Kb DNA genome that encodes four early proteins (E1 to E4) and five late proteins (L1 to L5) (50), and like the retrovirus can accommodate approximately 8 Kb of recombinant genetic material (67). Adenoviruses can be rendered replication defective by deletion of the E1 region of the genome (68), thus requiring a complementary cell line (293) to provide the necessary protein products in trans. Further deletion of the E3 region allows the incorporation of additional recombinant sequences.
Unlike the retrovirus, adenoviruses can be grown to high titers (69) and they can infect many cell types regardless of mitotic status (70). Furthermore, the virus exists episomally, eliminating the risk of mutagenesis in the host cell. Also, because the virus does not integrate into the host genome, and is replication defective, the recombinant genes are expressed transiently (71). This eliminates the chance of toxicity that may be incurred by prolonged expression of molecules like cytokines. The only disadvantage associated with Adenovirus is that E1 deleted vectors have been shown to be immunogenic by themselves (72). Repeated administration thus becomes difficult as the immune system reacts specifically to the virus and the advantageous effects of the recombinant gene are not obtained.

Despite the one disadvantage, the Adenovirus is still an efficient vector for antitumor therapy. Warnier et al. (73) showed that a specific CTL response could be elicited against the murine model tumor antigen P815A, after mice were immunized intradermally with adenovirus encoding this molecule, and their splenocytes were restimulated with cells expressing P815A and B7-1. Adenoviruses expressing the irrelevant protein β-galactosidase did not cause mice to develop a CTL response, even following in vitro stimulation.

Adenoviruses encoding the recombinant cytokine IL-12 were used in antitumor therapy by Bramson et al. (74) who showed that direct intratumoral injection of a mammary adenocarcinoma with Adenovirus-mIL-12 produced significant tumor regression, and animals who completely rejected their tumors were immune to secondary challenge. A
transient expression of IL-12 was observed as expected, with IL-12 levels being highest between two and three days post injection.

**Poxviruses**

Poxviruses comprise a large family of DNA viruses that have a wide range of hosts, both vertebrate and invertebrate (75). The most well known poxviruses are variola, responsible for smallpox, and vaccinia, the virus used for vaccination purposes against the former, more pathogenic agent (76).

In contrast to retroviruses and adenoviruses, poxviruses have a large complement of genetic material, approximating about 200 – 300 Kilobase pairs, depending upon the particular virus (76). The genome of the most well characterized poxvirus, vaccinia, has been sequenced (77), and it has been postulated to encode about 260 distinct proteins, most of which are of unknown function. Other poxviruses including canarypox, cowpox and fowlpox, are thought to encode comparable numbers of genes (76).

Morphologically, poxviruses are roughly brick shaped or oval shaped virions about 400 nm in diameter (76). Structurally, viruses are encapsulated at the outermost surface by a network of surface tubules, followed by an outer membrane (75). At the core of the virion is a nucleoprotein complex, which is flanked by two lateral bodies of unknown function. The entire genome is comprised of one linear DNA molecule with the single stranded ends ligated together to form a hairpin loop at either end of the molecule. Also present within the vaccinia nucleoprotein core are intact enzymes, including a
multisubunit RNA polymerase, a transcription factor, a poly A polymerase, a capping enzyme, a methyltransferase and a DNA topoisomerase (76, 78).

Canarypox Virus (ALVAC)

The canarypox virus is a member of a related genus of poxviruses known as avipoxviruses. Like vaccinia, the canarypox virus also has a large genome, measuring about 325 Kb (79). Developed at Virogenetics Corp. (Troy, New York), canarypox virus vectors are appealing as vectors for gene therapy due to a number of reasons. Firstly, they are able to infect a wide variety of cell types (80) with very high efficiencies of infection (81). Also, like other poxviruses, the canarypox vectors can accommodate large genes of up to 25 Kb (82). In addition, poxviruses exist cytoplasmically and so are not mutagenic toward the host cell that they infect (76, 78). Finally, the added advantage of the canarypox virus over other poxviral vectors is that it is replication defective within mammalian cells (80). While the virus can be maintained and propagated efficiently in chicken embryonic fibroblasts (CEF), the canarypox virus life cycle is aborted in mammalian cells before viral DNA replication takes place (83). The advantage of being naturally defective is two fold: firstly, the virus cannot be pathogenic to the entire host system once it has been targeted to a specific cell type, and secondly, genes are only expressed transiently as the virus is diluted out and eventually lost in continually dividing cells such as tumors. This helps to avoid chronic toxicities associated with continued presence of protein products like cytokines. In summary, the advantages of wide tropism, larger genome, episomal expression and replication defect make ALVAC an attractive vector in gene therapy as an alternative to other vectors. The disadvantages associated
with retroviruses, such as the requirement for cycling cells, host mutagenicity and permanent expression are overcome by this vector. Furthermore, an added advantage of using ALVAC over other established Adenovirus vectors is that the size of the recombinant inserts may be increased to 25Kbp compared to about 8Kbp in Adenoviruses.

Generation of Recombinant ALVAC viruses

The ALVAC viral genome consists of a double stranded linear DNA molecule measuring approximately 325Kb (79). Although the genome has been characterized via XhoI restriction mapping, a complete sequence is yet to be published. Recombinant cDNA sequences expressed via ALVAC vectors are driven by promoters that are derived from well characterized vaccinia virus genes (80). Specifically, for ALVAC B7-1 the human B7-1 cDNA is driven by the vaccinia virus promoter H6, and the expression cassette is cloned into the C6 multiple cloning site. For ALVAC IL-12, expression of the p35 subunit is driven by the vaccinia E3L promoter and the expression of p40 is driven by the 42K entomopox promoter. The expression cassettes for p35 and p40 are cloned into the C5 and C6 multiple cloning sites, respectively (see figure 1).

Titration of the ALVAC viruses

The procedure used to titer ALVAC viruses has been described in detail by Puisieux et al. (79). In this procedure, dilutions of viral lysates are added to monolayers of chicken
embryo fibroblast cells (CEF) and incubated at 37°C, 5% CO₂. Subsequently, viral media is removed and media containing 1.2 % agarose is added. 3 days later, media containing 1.2 % agarose is added again, 24 hours after which plaques are visualized and counted.

The ALVAC Virus in Cancer Therapy

The use of recombinant canarypox virus in cancer immunotherapy is gradually becoming widespread. Toso et al. (84) demonstrated that the ALVAC virus was a suitable vector in providing stimulation of tumor infiltrating lymphocytes ex vivo. In this study, irradiated peripheral blood mononuclear cells infected with ALVAC encoding the tumor antigen MAGE-1 specifically stimulated tumor infiltrating lymphocytes from a breast cancer patient to proliferate. In addition, these lymphocytes recognized an allogeneic B-cell lymphoma expressing vaccinia encoded MAGE-1, but not the same cell line bearing wild type vaccinia virus.

The fact that ALVAC encoded proteins could also serve as effective antitumoral vaccines was first shown by Roth et al. (85). In this report, mice immunized subcutaneously with ALVAC expressing mutant human p53, or wild type human p53, were significantly protected against challenge with a fibroblast cell line 10(3)273.1NT24, which expressed a mutant human p53 protein. Interestingly, immunization with ALVAC encoding mutant murine p53 or wild type murine p53 also induced significant protection upon challenge.
with the same cell line. In either case, subcutaneous injection with the non recombinant ALVAC vector did not confer any protection.

Subsequently, Hodge et al. (86), working with ALVAC recombinants expressing the tumor antigen CEA showed that immunization of mice intramuscularly elicited lymphoproliferative as well as cytolytic T-cell responses. Furthermore, significant titers of anti CEA IgG was observed in serum. Finally, 70% of mice which were immunized three times with ALVAC CEA rejected a tumor challenge of the MC38 colon carcinoma, which was expressing CEA. Injection of this cell line in animals receiving an irrelevant vaccine (ALVAC RG (rabies glycoprotein)) resulted in tumor formation.

Very recently, in a clinical trial conducted by Marshall et al. (87), it was shown that after vaccination of patients with ALVAC-CEA, an increase in frequency of cytotoxic T-cell precursors specific for CEA could be observed in 7 out of 9 patients. These patients all carried the HLA-A2 allele, and after three intramuscular vaccinations with ALVAC-CEA their peripheral blood T-cells were able to recognize a HLA matched cell line which expressed CEA.

**Active Immunotherapy with Tumors Genetically Modified via ALVAC Vectors**

In an experimental approach similar to the one undertaken in this project, Kawakita et al. (81) looked at the *in vivo* growth of the RM1 murine prostate cancer model, after infection with recombinant ALVAC vectors. Cells were infected with ALVAC IL-2, ALVAC B7-1, ALVAC IFN-γ, or ALVAC TNF-α, or no virus at all, and injected
subcutaneously. Tumor growth was unaltered relative to cells alone in all cases except with ALVAC TNF-α, where a delay in tumor growth was observed. Complete tumor rejection was only observed with a combination of viruses encoding TNF-α and IL-2, while other combinations with ALVAC TNF-α had no effect.

To determine whether this primary rejection with cells bearing ALVAC TNF-α and ALVAC IL-2 conferred immunity systemically, animals were vaccinated with irradiated cells bearing the two recombinant viruses, and then challenged with wild type cells after ten days. No protection, or delay in tumor formation, was observed after challenge.

SCID mice, which have no mature T or B cells also rejected a tumorigenic dose of RM1 cells infected with ALVAC IL-2 and ALVAC TNF-α, indicating that primary tumor rejection is not mediated by specific immune effectors. In parallel, no CTL activity against RM1, or recombinant ALVAC infected RM1-variants, was observed with splenocytes from wild type mice that were vaccinated with RM-1 bearing ALVAC IL-2 and ALVAC TNF-α.

More recently, Puisieux et al. (79) showed that infection of TS/A mammary adenocarcinoma cells with ALVAC recombinants generated some nonspecific immunity when tumor variants were injected subcutaneously. Cells were infected with ALVAC β-galactosidase, ALVAC IL-12, ALVAC IL-2, ALVAC GM-CSF, or ALVAC IFN-γ, or no virus at all. Significant survival was observed only in mice receiving cells bearing ALVAC IL-12 at 30 days post injection. Interestingly, there was also a delay in tumor
formation in animals receiving cells bearing ALVAC β-gal, relative to animals receiving cells alone.

A subsequent approach involved multiple intratumoral injections of pre-established 7-day tumors with ALVAC IL-12 virus, or ALVAC β-gal, or PBS alone. At 46 days, animals being treated with PBS had all died, while animals given ALVAC IL-12 survived at a frequency of 100%. Surprisingly there was also a 30% survival in mice receiving ALVAC β-gal.

In conjunction with treatment of established tumors with recombinant ALVAC vectors, mice that displayed a non-necrotic tumor were further challenged with a lethal dose of wild type cells on the opposite flank. 70% of mice that were receiving ALVAC IL-12 within their original tumor cleared the contralateral challenge completely. Again, ALVAC β-gal also conferred some protection as 30% of mice receiving ALVAC β-gal into the original tumor cleared the contralateral challenge.

Immunohistochemical staining of tumors treated with ALVAC β-gal and ALVAC IL-12 revealed a dense infiltrate of MAC-1 positive cells, indicating a presence of monocytes or macrophages. In addition, in tumors treated with ALVAC IL-12, CD4+ and CD8+ T-cells were also observed.

Injection of TS/A tumor cells into Nude mice proved to be lethal in all three scenarios of treatment consisting of injections with ALVAC IL-12, ALVAC β-gal or PBS. Thus, in
contrast to the study by Kawakita et al. cited above, this experiment showed a complete dependence on T-cells for tumor rejection.

**Current Experimental Approach**

The focus of this project was to characterize the mode of action of the ALVAC virus vector within a lymphoid tumor model. Generally, we aimed to observe whether a live tumor vaccine bearing ALVAC alone, ALVAC B7-1, ALVAC IL-12 or both ALVAC B7-1 and ALVAC IL-12 would induce an anti-tumoral reaction, through specific T-cell activation and if so, whether primary rejection of tumor variants would confer systemic immunity toward wild type challenge. The approach specifically incorporated the molecules B7-1 and IL-12 for two reasons: firstly, both IL-12 as well as B7-1 are known to have potent effects in specifically augmenting T-cell activation and/or killing through unique mechanisms. Activation of tumor specific T-cells via these molecules may in turn lead to the formation of T-cell memory against tumor cells, facilitating systemic immunity. Secondly, previous models established in our laboratory have shown the efficacy of tumor vaccines expressing both IL-12 and B7-1, through plasmid and retroviral expression vectors. Using the same molecules in the context of a novel viral vector allows us to assess the differential effects of the viral vector alone in augmenting cancer therapy.

For the purpose of this experiment, we used the early T-cell lymphoma model STF10. We hypothesized that the ALVAC encoded molecules of B7-1 or IL-12, or both, would indeed render the tumor cells more immunogenic, while the vector alone would not affect
the intrinsic properties of the tumor cells. Our in vivo experiments show that first of all, the ALVAC virus by itself is 100% immunogenic in that STF10 tumor cells bearing the non-recombinant virus, are completely rejected in immunocompetent mice. Secondly, the parental as well as the recombinant ALVAC vectors expressing B7-1 or IL-12 can induce a protective effect against wild type tumor cells, when the initial cellular vaccine is boosted before challenge. Thirdly, cytotoxic effectors specific for wild type tumor cells can be detected in mice that have been immunized and boosted with STF10/ALVAC. Finally in athymic nude mice, STF10 tumor cells bearing ALVAC vectors are rejected to a significant extent, even though not completely as in wild type mice. This indicates that primary rejection of tumor variants is mediated by both T-cells as well as T-cell independent mechanisms.
Material and Methods

Cell Lines
The mature B-cell lymphomas A20, K46J, M12, the melanoma B16-F1, the pro B-cell line NFS-70 and the mastocytoma P815 were purchased from American Type Culture Collection (ATCC). The SCID thymoma line ST was derived from a spontaneous tumor in a C.B-17 SCID mouse and was kindly provided by Dr. Gillian Wu (Ontario Cancer Institute, University of Toronto, Toronto, Canada). The acute myeloid leukemia cell line C-1498 was kindly provided by Dr. Andre Schuh (Department of Medical Biophysics, University of Toronto, Toronto, Canada).

All cell lines were maintained in culture media (CM) of RPMI 1640 + 10% Fetal Calf Serum (FCS) + 50 μM 2-mercaptoethanol, at 37°C, in 5% CO₂, with the exception of C-1498 and B6-F1 which were maintained in DMEM + 10% FCS.

To establish subclones, SCID Thymoma (ST) cells were plated into 96 well plates at concentrations of 30, 3 and 0.3 cells / well in complete media. Plates corresponding to each dilution were set up in duplicate. After 14 days, 8 ST subclones were observed on a plate corresponding to 0.3 cells/well, and these were individually expanded. Two representative clones were designated STF10 and STG4, and maintained in conditions identical to those for the parental culture. B16-F1 was similarly cloned via limiting dilution, and one representative subclone was designated B16-F1.2. B16-F1.2 was maintained under conditions identical to those for the parental culture.
Infection of cell lines via ALVAC vectors

All parental and recombinant ALVAC viruses were acquired from Virogenetics Corp., Troy, New York. For infection, cell lines except B16-F1 were plated at a concentration of $5 \times 10^5$ cells / mL in RPMI 1640 media with 2% Fetal Calf Serum (FCS). C-1498 was plated at the same concentration, using DMEM + 2% FCS, while B16-F1 was plated at 50% confluency within DMEM + 2% FCS. ALVAC B7-1 (vCP 1334, Virogenetics, New York) was added at a multiplicity of infection (MOI) of 20 Pfu / cell. Cells were incubated for 10 hours at 37°C and 5% CO₂, after which they were washed in serum free media, and recovered overnight in their respective culture medium (CM) at 37°C and 5% CO₂.

Flow Cytometric Assessment of Cell Surface Marker Expression

For indirect immunofluorescent analysis, $1 \times 10^6$ cells were centrifuged for 8 minutes at 800 rpm, washed twice in phosphate buffered saline (PBS), and then treated with 1 ug of the indicated primary antibody. An equal number of control cells were treated with 1 ug of isotypic control antibody. Cells were incubated on ice for 30 minutes, and then washed twice in PBS. Subsequently, cells were treated with polyclonal fluorescein isothiocyanate (FITC) – conjugated goat anti mouse IgM / IgG secondary antibody. Cells were then washed twice with PBS and then resuspended into 400 μL of 2% paraformaldehyde / PBS fixative.

For direct immunofluorescent analysis, cells were treated with the indicated Phycoerythrin (PE)- or Fluorescein isothiocyanate (FITC)– conjugated antibody
(Pharmingen), and a corresponding PE- or FITC- conjugated anti TNP isotypic control antibody. Again, cells were incubated on ice for 30 minutes, and then washed twice with PBS, after which they were fixed in 400 µL of 2% paraformaldehyde fixative.

Antibodies used for staining included purified hamster IgG anti CD3, rat (r) IgG2b anti CD4, rIgG2a anti CD8, mouse (m) IgG2a anti H-2K\(^d\)D\(^d\), mIgG2a anti I-A\(^d\), rIgG2a anti B7-1, mIgM anti hB7-1, rIgG2a anti B7-2, polyclonal FITC-conjugated goat anti mouse IgM/IgG, or FITC-conjugated Goat anti hamster IgG. CD44, CD25 and HSA were assayed for by treating cells with PE-conjugated rIgG2b anti CD44, rIgG2b anti CD25, or FITC conjugated rIgG2b anti HSA. All antibodies as well as non-specific isotypic controls were purchased from Pharmingen, except for anti CD3, FITC-Goat anti hamster IgG and the corresponding isotypic control, which were purchased from Cedarlane.

Fluorescence emitted by specifically bound antibody was measured by flow cytometry on a FACScalibur cytometer (Becton Dickinson) and data were analyzed using CellQuest Software (Becton Dickinson).

**Immunooassay for IL-12 Production**

Prior to *in vivo* inoculation of mice with STF10 vaccines bearing parental and recombinant ALVAC vectors (see below) supernatant from virally infected cells were assayed for the presence of IL-12 via Enzyme Linked ImmunoSorbent Assay (ELISA), using the OptEIA kit (Pharmingen) according to manufacturer's protocol. All values presented in Table 1 are expressed as ng/mL/million cells/24 hours.
Reverse Transcription (cDNA synthesis)

10^7 cells from indicated samples were spun down and cellular mRNA was extracted using a RN-easy Kit (Qiagen) according to manufacturer’s protocol. For reverse transcription, 2 ug of RNA was used from each sample.

5 µL (2 ug) of RNA was added to 1.5 µL of random hexamer (300 ng / µL), along with 5.5 µL of sterile water, and incubated at 65°C for 5 minutes. Subsequently, 4 µL of 1st strand 5 x buffer, 2 µL of 0.1M DTT, 1 µL of 20 mM dNTP, and 0.5 µL of RNase Inhibitor (RNguard) was added on ice, and then incubated at room temperature for 15 minutes. Each sample was done in duplicate. Following incubation at room temperature, one of the duplicates from each sample was treated with 1 µL of Superscript II reverse transcriptase (for +RT reactions). Samples were incubated for 1 hour at 37°C followed by 1 hour at 48°C. Superscript II was then deactivated by incubation at 95°C for 5 minutes. RNase A was then added to each tube, and samples were incubated at 37°C for 30 minutes, followed by incubation at 95°C for 5 minutes. The final volume of each sample was then brought up to 40 µL with sterile water.

PCR for gene expression

2.5 µL of the sense primer, and 2.5 µL of the antisense primer (both initially at 2.5 pmol / µL) were added to 3 µL of indicated cDNA template, along with 5µL of 10 x PCR buffer, 5 µL of 10 mM dNTP and 0.25 µL of Taq polymerase (Qiagen). For TdT cDNA amplification, samples were denatured for 5 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, reannealing at 61°C for 30 seconds and extension at
72°C for 30 seconds. Subsequently, a final extension took place at 72°C. For RAG-1, RAG-2 and HPRT amplification, samples were denatured at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 48 seconds, extension at 72°C for 45 seconds. Subsequently, a final extension at 72°C took place for 10 minutes.

Murine TdT cDNA was amplified to a 124 bp product using sense primer 171 with sequence 5' ATATGCTTGCCAGCGAAGAACC 3' and antisense primer 172, with sequence 5' GAG ATT TCA GTA CAG AGG ACG C 3'. TdT Primers were kindly provided by Dr. Gillian Wu. Murine RAG-1 cDNA was amplified to a 545 bp product using a sense primer of sequence 5' CCAAGCTCGAGACATTCTCTACCTGC 3' and an antisense primer of sequence 5' CTGGATCCGGAATCTCTGGCTGCTC 3'. Murine RAG-2 cDNA was amplified to a 471 bp product using sense primer 5' CACATCCACAAGCAGGAAGTACAC 3' and antisense primer 5' GGTTCCAGGACATCTCCTACTAAG 3'. Murine HPRT was amplified using sense primer 5' GCTGGTGAAAAGGACCTCT 3' and antisense primer 5' CACAGGACTAGAACAACCTGC 3'.

Positive control for TdT amplification comprised of a murine genomic sample from mouse tail DNA. Genomic Positive controls contained 1 or 8 µL of 25mM MgCl₂, while negative controls contained water in place of template. Positive controls for RAG-1, RAG-2 and HPRT consisted of two cDNA preps of the pro-B cell line NFS-70, while negative controls contained water in place of template.
20 μL of amplification products of TdT, RAG-1, RAG-2 and HPRT were mixed with 4 μL of 6x loading buffer and electrophoresed on 1.5% agarose gel at 90 Volts for one hour. A 1Kb reference marker (Gibco BRL) was electrophoresed in parallel.

Growth Kinetics of ALVAC-infected STF10 cells
To determine whether infection via ALVAC vectors affected the growth rate of STF10 cells, 1 million STF10 cells were infected with ALVAC B7-1 as outlined above, and following recovery, were replated at 2 X 10⁵ cells/mL in CM (t=0). As a control, 1 million STF10 cells were treated similarly, without ALVAC B7-1 infection. Population density (concentration in vitro) was then assayed at timepoints of t = 24, 48, 72 and 96 hours via trypan blue exclusion assay. Further analysis was not undertaken due to cell death in both cultures due to overwhelming population density.

Stability of ALVAC Encoded products over time in ST
Stability of ALVAC encoded products over time was assayed in both a continually dividing culture as well as a non-dividing culture. 6 million ST cells were infected with ALVAC B7-1, as above. Subsequently, half the culture (3 million cells) was separated and irradiated at 10,000 cGy and replated at 5 x 10⁵ cells / mL in fresh media. Post irradiation, aliquots were taken at 24, 48, 72 and 96 hours, and assayed for B7-1 as described above. The cells did not divide further after irradiation and after 96 hours, were completely non-viable. In parallel, after infection and recovery, the non irradiated cells were replated in fresh media at 5 x 10⁵ cells / mL and aliquots were taken at 24, 48,
72, and 96 hours to assay for B7-1 expression. Since this culture was continually dividing, cells were split and maintained at $5 \times 10^5$ cells / mL at each of these timepoints. Two further timepoints of 120 hours and 144 hours were assayed for B7-1 expression, with this culture.

**Tumorigenicity in Wild Type BALB/c mice: Vaccination and Challenge**

8-10 week old BALB/c female mice were purchased from Charles River Laboratories (Canada) and housed at the animal facility of the Division of Comparative Medicine, University of Toronto. All experiments were conducted in accordance with the University of Toronto Animal Care guidelines.

STF10 cells were plated at $5 \times 10^5$ cells / mL in RPMI 1640 + 2% FCS and infected *in vitro* (as above) with the indicated ALVAC parental or recombinant virus, and incubated for 10 hours at 37°C, 5% CO$_2$. All infections were carried out at an MOI of 20, except when combining ALVAC IL-12 and ALVAC B7-1 where an MOI of 10 was used for each respective virus. Following infection, cells were washed twice and recovered overnight in complete medium (CM).

Following recovery, cells were washed 4 times in serum free media, resuspended into serum free media at a concentration of $5 \times 10^6$ cells / mL, and injected subcutaneously into the right flank of five corresponding groups of mice. A dose of $1 \times 10^6$ cells was administered per mouse, with five mice per group. Tumor growth *in vivo* was monitored, and animals were sacrificed when maximum tumor diameter reached 2 cm.
Surviving animals were challenged at day 55 with $1 \times 10^6$ wild type STF10 cells on the opposite (left) flank. As a positive control for tumor growth, naive age-matched female BALB/c mice were also injected with wild type STF10 cells.

To determine whether a vaccination regimen incorporating a cellular boost would induce greater survival after wild type challenge, a protocol utilizing two vaccines before wild type challenge was undertaken. Mice were immunized with the indicated cellular vaccines consisting of STF10 cells infected with parental or recombinant ALVAC vectors. In this experiment, instead of using cells doubly infected with ALVAC B7-1 and ALVAC IL-12 as one of the vaccine groups, a mixture of equal numbers of ALVAC B7-1-infected and ALVAC IL-12-infected cells was used. 8 female BALB/c mice were used per group, except in the group receiving STF10/ALVAC IL-12, where 16 mice were used.

34 days post vaccination, a new batch of STF10 cells were infected with parental or recombinant ALVAC vectors. On day 35, animals surviving the initial vaccination were boosted with the exact same tumor vaccine (as they had received initially) via subcutaneous injection on the same (right) lateral flank. A boost of the same vaccine was administered to all animals except those that had been immunized with STF10/ALVAC IL-12, where only 8 out of 16 were boosted, and 8 were left unboosted. A positive control for tumor formation was included by the injection of wild type STF10 cells into 8 age-matched, previously unvaccinated BALB/c female mice.
On day 56 post initial vaccination (or day 21 post boost), all mice surviving the initial vaccine as well as the boost (where applicable) were injected on the left lateral flank with wild type STF10 cells at a dose of one million cells per mouse. Once again, positive controls for tumor formation were included via the injection of wild type cells into previously unvaccinated BALB/c mice.

In experiments using athymic nude mice 8-10 week old female BALB/c nu/nu mice were purchased from Charles River Laboratories and maintained as above, except under sterile conditions. A similar tumorigenicity assay was performed, whereby STF10 cells were infected with the indicated ALVAC virus, and injected subcutaneously on the right lateral flank, at a dose of $1 \times 10^6$ cells per mouse. Again, five mice were used per group, and animals were euthanized when maximum tumor diameter reached 2 cm.

A follow-up tumorigenicity experiment with nude mice incorporated 8 mice per group, with one modification, in that instead of doubly infecting one group of cells with ALVAC B7-1 and ALVAC IL-12, a mixture of an equal number of ALVAC B7-1-infected cells and ALVAC IL-12 infected cells, was used.
Statistical Analysis

Kaplan Meier survival curves were compared using the log-rank test in the SPSS 6.1 Macintosh version statistical package. Differences are considered statistically significant if p < 0.05.

Cytotoxic T-lymphocyte Assays

Preparation of Stimulator Cells

STF10 cells were plated at a concentration of $5 \times 10^5$ cells / mL in RPMI 1640 + 10% FCS + 50 uM 2-Me (CM) and infected with ALVAC at an MOI of 20. and then grown overnight at 37°C, 5% CO₂. Following incubation, cells were washed twice in serum free RPMI and resuspended in culture media (CM). Subsequently cells were irradiated at 10,000 cGy, washed, and resuspended at $1 \times 10^5$ cells / mL in CM. 1 mL was subsequently plated out in 24-well tissue culture plates.

Preparation of Effector Cells

Because STF10/ALVAC IL-12 consistently induced greatest antitumor protection after one vaccination, mice were immunized with STF10 / ALVAC IL-12 via subcutaneous injections and one mouse was euthanized at each timepoint of 13, 17, 27 and 35 days post injection to examine in vitro reactivity. In parallel, a naive mouse was also euthanized at each timepoint. Animals were dissected under sterile conditions and spleens were removed and resuspended in serum free RPMI. Subsequently, a single cell splenocyte suspension was created by homogenizing spleens and passing the homogenate through a
0.22 um cell strainer. The strainer was then washed twice with 7 mL of serum free RPMI, and the flow through was pooled with the splenocyte suspension.

The splenocyte suspension was then centrifuged and washed twice with serum free RPMI. After the last wash, splenocytes were resuspended in 1 mL of Tris-NH4-Cl, pH 7.2 and incubated at 37°C for 2 minutes to lyse red blood cells. Following RBC lysis, the cells were washed once again in serum free RPMI and resuspended in culture media (CM) of RPMI + 10% FCS + 50 uM 2-Me at a concentration of 5 x 10^6 cells / mL. 1 mL of this solution was then added to the 1 mL of the stimulator culture that had been previously set up as indicated above. Each stimulator – effector well was set up in duplicate. Cells were stimulated in vitro for 5 days, at 37°C, 5% CO₂.

Preparation of Target Cells

4 days after setting up the stimulator – effector co-culture, 4 x 10^6 STF10 cells were plated at 5 x 10^5 cells / mL in culture media (CM). 2 x 10^6 of these cells were infected overnight with ALVAC IL-12 at MOI of 20 and 2 x 10^6 were grown uninfected, at 37°C, 5% CO₂. Following overnight incubation, 10 uCi of ³H-Thymidine was added per million cells for each group and cells were further incubated for 4 hours at 37°C, 5% CO₂. Subsequent to labeling, cells were washed in serum free RPMI and resuspended in RPMI + 5% FCS at a concentration of 1 x 10^5 cells / mL.
**CTL Setup**

Effector cells from immunized mice and naive mice that were being stimulated with STF10 / ALVAC IL-12 were removed from the stimulation plate and resuspended via vigorous pipetting into RPMI 1640 + 5% FCS at a concentration of $1 \times 10^7$ cells / mL. 150 µL samples containing $1.5 \times 10^6$ cells were pipetted into 96 well plates, in duplicate for lysis reactions against STF10 and STF10 / ALVAC IL-12 target cells. Effector cells were then serially diluted 3 times by taking out 50 µL and resuspending into 100 µL of CTL medium RPMI 1640 + 5% FCS. 100 µL of targets (at $1 \times 10^5$ cells / mL) were then aliquoted into corresponding wells to yield final effector : target ratios of 100:1, 33:1, 11:1 and 3:1.

Plates were centrifuged for 8 minutes at 800 rpm (without brakes) and incubated at 37°C, 5% CO₂, for 4 hours. Following incubation, labelled unlysed cells were harvested onto Unicell plates using a cell harvester. The Unicell plates containing the unlysed cells were washed, dried and baked at 42°C for 40 minutes. Subsequently, the porous membranes of the plates were sealed at the back, and 25 µL of scintillant was added to each well. The tops of the plates were then heat sealed and counts were read on a Packard-Top Count scintillation counter. % Specific Lysis was calculated as outlined by Matzinger *et al.* (88), using the formula:

$$\text{% Specific Lysis} = 100\% \times \frac{\text{spontaneous cpm} - \text{experimental cpm}}{\text{spontaneous cpm}}$$
Differential Effects of in vitro stimulation on STF10 Lysis

To determine whether unmodified STF10 cells differentially activated STF10 (parental tumor) specific splenocytes from immunized mice, stimulation co-cultures were set up as above, against both STF10/ALVAC as well as unmodified STF10 cells. Responder cells were obtained from mice that had been immunized with STF10/ALVAC and then boosted with the same tumor vaccine. Mice were at 21 days post boost, at the time of splenocyte harvest. Targets of STF10 or STF10/ALVAC were set up in the manner described.
Results

**Heterogeneity in Tropism / Viral Gene Expression**

To determine whether the tropism or the gene expression of ALVAC encoded genes was restricted to various subsets of lymphoid cells, a range of tumor cell lines were tested for their ability to take up the ALVAC virus and express the recombinant human B7-1 gene. The cell lines tested included an acute myeloid leukemic line C1498, a melanoma B16, mature B-cell lymphomas K46J and A20, a mastocytoma P815, a pro B-cell line NFS-70, a thymic lymphoma ST, as well as two ST subclones STF10 and STG4. As figure 2 shows, A20, K46J, P815 and C1498 cells were completely negative for recombinant gene expression, while the B16 cell line displayed a very high frequency of cells positive for human B7-1. In addition, the level of B7-1 molecule expression in this cell line was also very high. NFS-70 and ST were the only murine lymphoid cell lines that showed positivity for B7-1 expression, at frequencies of 83% and 94%, respectively. STF10 and STG4, which were subcloned from the parental ST population, could also be successfully infected with ALVAC B7-1. Even though the subclones exhibited a high frequency of cells positive for B7-1 expression, there was some heterogeneity in the level of B7-1 expression by individual cells in the population. Despite the heterogeneity, the clonal STF10 was chosen as a tumor model rather than the bulk ST to minimize the level of cellular heterogeneity that may result from successive mutations that take place in individual cancer cells within the original tumor bulk.
Surface Marker Phenotype of STF10

We were interested in evaluating the role of ALVAC based vectors in mediating immunogenicity in lymphoid malignancies. For this reason, the thymic lymphoma subclone STF10 was chosen as a tumor model. Because the parental thymic lymphoma (ST) arose from a SCID mouse, the cell line was thought to be derived from early T-cells. To confirm the developmental stage from which this thymic lymphoma arose, the cell line was screened for the presence of early T-cell markers.

As figure 3 shows, STF10 was CD44+, CD25-, HSA+, CD4 low, CD8 low, and CD3-. In addition, the cell line was B7-1 low, B7-2 low, MHC class I (H-2KdDd) + and MHC class II (I-A^d) low. Further analysis of the intracellular enzyme constitution of STF10 revealed positivity for recombination activating gene 1 (RAG-1) and recombination activating gene 2 (RAG-2), as well as terminal deoxynucleotidyl transferase (TdT) (figure 4). These results confirmed that STF10 was derived from an early double negative thymic precursor T-lymphocyte.

Growth Kinetics of ALVAC Infected STF10 cells

To determine whether ALVAC infection affected the growth rate of STF10 cells, 1 million STF10 cells were infected with ALVAC B7-1 and subsequently, population growth was monitored over time, starting from a density of 2 \( \times 10^5 \) cells/mL. Figure 4 shows that compared to an uninfected control population, STF10 cells that have been infected with ALVAC vectors do not display growth kinetics that are notably different.
Both survival curves have similar trends indicating that infection via ALVAC vectors has no effect on the survival and proliferation of STF10 cells.

**MHC-class I expression on ALVAC infected STF10 cells**

To observe the effect of the intracellular ALVAC virus on MHC expression, the levels of H-2K$^d$ and H-2D$^d$ were assayed at timepoints of 6 hours post initial exposure, and 24 hours post infection. As shown in figure 5, there is no downregulation of MHC class I molecule expression due to the presence of ALVAC. Specifically a slight increase is observed after poxviral infection, in that the geometric mean fluorescence from antibody tagged MHC molecules is 17 and 19 for infected cells at 6 hours and 24 hours respectively, and 14 and 15 for uninfected cells at 6 and 24 hours respectively.

**Stability of ALVAC encoded Products over Time**

Because the ALVAC virus is non-integrative into the host genome, and is replication incompetent in mammalian tissue, recombinant products encoded by this virus will be transiently expressed. To determine the duration of expression of ALVAC encoded products, in both a rapidly dividing population, as well as in a non dividing population, the STF10 tumor model was used to monitor ALVAC B7-1 gene expression over time. The number of cells positive for human B7-1 was assayed via cell sorting of both a log phase as well as an irradiated, non dividing culture.

As shown in figure 7, in a continually dividing culture, ALVAC encoded B7-1 expression was maximal at two days post infection (48 hours), as all the cells originally
infected with ALVAC B7-1 were still positive for the recombinant molecule. However, by 72 hours post infection, the expression of human B7-1 is lost, and expression levels of the recombinant molecule return to baseline. In contrast, in a culture where replication of the cells is halted via irradiation, the presence of ALVAC encoded products can be detected right up to the point at which the cells undergo apoptosis (> 96 hours). In addition, the number of cells encoding ALVAC B7-1 remains fairly constant, at between 70 - 80% for the duration of this assay, though the level of protein expression (assayed by fluorescence intensity) gradually decreases over time. These cells undergo apoptosis at this time presumably due to an excess of DNA damage as a result of prior irradiation. Viral infection does not induce apoptosis by itself, as the population which was infected, but not irradiated, expanded continuously, leading eventually to the dominance of virus-free daughter cells over virally infected cells.

This shows that firstly, the replicative index of the chosen cell line is an important factor in how long cells can express ALVAC encoded products, as a population that is not replicating maintains expression from the ALVAC virus longest. Secondly, the temporal regulation and the half-life of the actual product being encoded are important factors as even though cells may be positive for ALVAC B7-1, and hence the ALVAC virus, the level of the recombinant product declines over time.

**Decreased Tumorigenicity of STF10 cells Modified by ALVAC Vectors**

To determine if genetic modification of STF10 tumor cells by introduction of genes encoding IL-12 and / or B7-1 could enhance antitumor immunity, STF10 tumor cells
containing ALVAC B7-1 or ALVAC IL-12, or both ALVAC B7-1 and ALVAC IL-12 were injected into syngeneic mice. As controls, STF10 tumor cells bearing non-recombinant ALVAC virus, and STF10 tumor cells with no virus were also injected in parallel into syngeneic mice. All immunizations were via subcutaneous injection in the right flank.

While various previous models have used irradiated cells as the initial vaccine inoculum in order to achieve protective immunity against wild type tumor cells, we wanted to examine whether a primary response could be generated against the vaccine before we examined the issue of protective immunity. As a result, in all our experiments, vaccination is carried out using live tumor cells, as opposed to irradiated cells so that in the absence of an effective response against the vaccine, a clear indication is obtained via tumor formation.

Control mice receiving STF10 cells alone all succumbed to tumor within 20 days post injection, while mice receiving STF10/ALVAC B7-1, STF10/ALVAC IL-12 or STF10/ALVAC B7-1/ALVAC IL-12, all remained completely tumor free, up to 55 days post injection. Surprisingly, mice that received STF10 cells infected with the non recombinant ALVAC virus (STF10/ALVAC) also rejected their tumors and remained 100% tumor free up until day 55 (Figure 8).

Since all animals that received STF10 cells containing either parental or recombinant ALVAC virus rejected their tumors at 100% frequency, all animals were challenged with
an injections of wild type STF10 cells in the opposite (left) lateral flank, to determine if systemic immunity against the wild type cells was conferred. As controls, previously unvaccinated (naive) mice were also injected with wild type STF10 tumor cells.

Following challenge, 4/5 control (previously unimmunized) mice developed tumors. Tumor formation was also observed in 5/5 mice immunized with ALVAC B7-1, 4/5 mice immunized with STF10/ALVAC, 4/5 mice immunized with STF10/ALVAC IL-12, and 3/5 mice immunized with STF10/ALVAC B7-1/ALVAC IL-12 (Figure 9). Mice that were vaccinated with STF10/ALVAC IL-12 initially seemed to display a delay in tumor formation when challenged with wild type cells. However, log-rank statistical analysis revealed that compared to survival of naive mice, none of the STF10 vaccines (including STF10/ALVAC IL-12) were efficacious in conferring a significantly greater level of protection after wild type challenge (p > 0.05 in all cases).

Tumorigenicity in Nude Mice

To determine whether the rejection demonstrated in the above experiment was mediated by T-cells, a tumorigenicity experiment was carried out in BALB/c nude mice, which lack mature T-cells.

STF10 cells were infected as above with either a) ALVAC B7-1, b) ALVAC IL-12, c) ALVAC B7-1 and ALVAC IL-12, d) ALVAC, or e) No virus, and injected subcutaneously into nude mice. In contrast to results with wild type mice, STF10 variants bearing ALVAC B7-1, or ALVAC IL-12, or both were not 100% immunogenic,
and formed tumors in 2/5, 4/5 and 2/5 mice, respectively. STF10 cells infected with non recombinant ALVAC parental vector were 100% immunogenic in nude mice, as all mice receiving this cellular variant rejected their tumor vaccine (Figure 10A). However, relative to tumor formation via unmodified STF10 tumor cells, tumor formation via STF10 cells infected with either parental or recombinant ALVAC vectors was still delayed to a significant extent (p < 0.05). There was no significant difference in the rejection of STF10/ALVAC, relative to STF10/ALVAC B7-1, STF10/ALVAC IL-12, or STF10/ALVAC B7-1/ALVAC IL-12.

Similar results were obtained upon repeating the experiment whereby mice received either a) STF10 alone, b) STF10/ALVAC, c) STF10/ALVAC B7-1, d) STF10/ALVAC IL-12, or e) a mixture of STF10/ALVAC B7-1 and STF10/ALVAC IL-12. In agreement with the initial experiment, tumors resulting from STF10 cells bearing either parental or recombinant ALVAC vectors were significantly delayed in formation, relative to those resulting from uninfected STF10 cells (p < 0.05 in all cases) (Figure 10B). As well, though there was a significant delay in tumor formation when STF10 cells were modified with ALVAC viruses, rejection was not 100% as seen with wild type mice, suggesting the possible role of T-cells in tumor variant rejection.

Taken together, these preliminary findings point toward three important conclusions. Firstly, in contrast to previous assumptions, the ALVAC vector is by itself immunogenic, in the context of a subcutaneously administered whole cell tumor vaccine that has been in vitro modified. Secondly, as nude mice reject the STF10 variants to some extent relative
to unmodified controls, but not as efficiently as wild type mice, the effector mechanism responsible for rejection of STF10 tumor variants is multifactorial. Finally, T-cells are involved to some extent as there is greater mortality in nude mice receiving STF10 vaccines; however, the vaccination regimen employed is ineffective in conferring systemic antitumor immunity. Even though there appears to be a slight delay in tumor formation in mice vaccinated with STF10/ALVAC IL-12, this delay is not statistically significant, with a sample size of n=5.

**Boosting with whole cell vaccines bearing ALVAC vectors confers antitumoral immunity**

Because the initial vaccination regimen employing one tumor cell vaccine injection followed by a lethal challenge at day 55 did not yield a statistically significant protective response with any of the tumor vaccines, the protocol was modified in two main respects. Firstly, the STF10/ALVAC B7-1/ALVAC IL-12 vaccine was replaced with a vaccine made up of a mixture of STF10/ALVAC B7-1 cells and STF10/ALVAC IL-12 cells at equal amounts. This was done to keep the infection level via each respective virus consistent, as an infection procedure that subjects both viruses to the same culture at the same time could not control for the infection of every cell by the two viruses at equimolar ratios. Secondly, in order to examine whether the vaccination regimen could be improved to afford better protection upon challenge with wild type tumor cells, a prime and boost approach was utilized.

Animals were injected at day 0 with either a) STF10, b) STF10/ALVAC, c) STF10/ALVAC B7-1, d) STF10/ALVAC IL-12, or e) STF10/ALVAC B7-1 &
STF10/ALVAC IL-12. All animals survived except those that received STF10 cells alone (not shown), and on day 35, all animals were boosted with the same respective vaccine. As controls, naive mice were injected with STF10 cells alone, and half the mice which previously had received STF10/ALVAC IL-12 were left unboosted. Again, naive mice receiving STF10 alone died by day 21 (not shown). All other mice survived, as expected upon vaccination with STF10 cells modified with parental and recombinant ALVAC viruses.

21 days after boosting, all animals were challenged on the opposite flank with wild type STF10 cells to determine whether systemic immunity had been conferred. However 6 days after challenge, one mouse out of eight, which had been initially vaccinated with STF10/ALVAC IL-12 but left unboosted succumbed to tumor formation on the right flank. Because the challenge was administered on the left flank, and the kinetics of tumor formation argued against this tumor being formed as a result of challenge, this mouse was taken out of the sample of survivors that were challenged.

As shown in figure 11, all naive animals that received STF10 cells succumbed to tumor with expected kinetics. Animals that had been vaccinated and boosted with STF10/ALVAC, STF10/ALVAC B7-1, STF10/ALVAC IL-12 or both STF10/ALVAC IL-12 and STF10/ALVAC B7-1 showed significantly delayed tumor formation (relative to naive mice) in all cases (p < 0.01). This showed that after boosting with whole cell vaccines bearing parental or recombinant ALVAC vectors, some measure of systemic immunity against STF10 cells had been induced. Furthermore, survival frequencies were

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also increased relative to naive mice, as mice vaccinated with STF10/ALVAC, STF10/ALVAC B7-1, STF10/ALVAC IL-12, or STF10/ALVAC IL-12 and STF10/ALVAC B7-1 showed overall survival rates of 50%, 12.5%, 25% and 25% respectively. Notably, STF10/ALVAC by itself was a potent immunogen, in that priming and boosting with STF10/ALVAC gave a level of protection that was comparable to that with other vaccines incorporating recombinant ALVAC vectors in STF10 cells.

Also of interest is the fact that even though a previous vaccination with STF10/ALVAC IL-12 (figure 9) failed to elicit a protective response after challenge, this time one vaccination with STF10/ALVAC IL-12 was significantly protective against tumor formation, relative to naive mice (p < 0.01) when the sample size was increased to n=8. In addition, a double vaccination with STF10/ALVAC IL-12 does not confer a protective effect that is superior to that mediated by a single vaccination with STF10/ALVAC IL-12, as mice vaccinated twice displayed kinetics of tumor formation that were comparable to that seen in mice vaccinated only once.

In Vitro Antitumoral Reactivity

Mice were immunized with STF10/ALVAC IL-12, and their splenocytes were assayed at various timepoints for reactivity against both wild type STF10 and STF10/ALVAC IL-12. Spleens were taken at days 13, 17, 27 and 35 post immunization, and lymphocytes were stimulated against STF10/ALVAC IL-12 before they were incubated with STF10 and STF10/ALVAC IL-12 targets for lysis. Spleens from naive mice were also processed similarly, as negative controls.
Significant *in vitro* cytolytic activity could be observed from mice only on day 27, and only against STF10/ALVAC IL-12 targets, at effector : target ratios of 100:1 and 33:1 (figure 12B). Specific lysis of uninfected STF10 tumor cells was not observed at this timepoint (figure 12D), indicating that STF10 specific CTL-precursors were either absent or in very low frequency in the population of splenocytes. Lymphocytes from naive mice, stimulated similarly with STF10/ALVAC IL-12 were not able to lyse STF10 or STF10/ALVAC IL-12.

Because *in vivo* experiments had previously shown that immunizing and boosting mice with the STF10/ALVAC variant also increased antitumor responses against STF10 tumor cells upon challenge at day 21, animals were immunized with STF10/ALVAC, boosted with the same respective tumor vaccine, and then assayed for splenic reactivity against STF10 and STF10/ALVAC at day 21. In addition, the differential *in vitro* effects of stimulation were also examined in that lysis of the uninfected STF10 cell line was assayed after *in vitro* stimulation of splenocytes with either wild type STF10 or STF10/ALVAC.

As shown in figure 13, splenocytes from naive mice were unable to lyse uninfected STF10 cells when incubated with STF10 *in vitro*. However, specific cytotoxic activity against uninfected STF10 cells was observed from splenocytes of mice immunized with STF10/ALVAC, after stimulation *ex vivo* with STF10 cells (figure 13A). In addition, specific activity against STF10/ALVAC could also be elicited from splenocytes
stimulated with wild type STF10, with comparably higher killing at the lower effector : target ratio of 30:1 (figure 13B).

In contrast, stimulation of splenocytes with STF10/ALVAC yielded results that were similar to previous experiments, in that specific cytolytic activity of splenocytes from immunized mice was not appreciably greater than that elicited from splenocytes of naive mice, when assayed against STF10 cells (figure 13C). There was also a higher level of background killing by splenocytes from naive mice.

Taken together, these data imply that STF10 specific CTL precursors are indeed present at day 21, in the splenocyte population of mice that are immunized and boosted with STF10/ALVAC. Their clonal expansion, and their subsequent detection is dependent on the stimulus provided, i.e., they are selected more efficiently by uninfected STF10 cells and cannot be detected if splenocytes are stimulated against STF10/ALVAC.
Discussion

The most significant observation described here is that the ALVAC vector itself is immunogenic and can elicit a systemic cellular immune response. That is, in the context of whole cell tumor vaccines with STF10 cells, the ALVAC vector was 100% immunogenic and the additional benefits from the encoded B7-1 or IL-12 could not be detected. Initial experiments indicated that protection against challenge with STF10 could be elicited at appreciable, though statistically insignificant levels, with STF10 cells modified with ALVAC IL-12. Repeating the in vivo experiment with a greater sample size indicated that vaccination with STF10/ALVAC IL-12 is indeed significantly protective; however, boosting with this vaccine does not confer a greater protective effect.

Although protection from ALVAC IL-12 modified STF10 cells was not enhanced by boosting the initial immunization, tumor protection was enhanced by boosting using all the other STF10/ALVAC immunogens. A vaccination and boost with STF10/ALVAC is significantly protective against wild type challenge, at levels that are comparable to that induced by vaccinating and boosting with STF10/ALVAC IL-12. Thus, STF10/ALVAC is a potent immunogen in eliciting an anti-STF10 response by itself, and in this model there is no added benefit of IL-12 or B7-1 expression.

Experiments in nude mice indicate that rejection of ALVAC – infected STF10 variants is mediated in part by T-cells, due to reduction in rejection efficiency, but other effector
mechanisms are also responsible as ALVAC—infected cells are not completely tumorigenic in nude mice. Lastly, in mice immunized with STF10/ALVAC, splenic cytotoxic T-cells specific for wild type STF10 tumor cells can be detected in vitro but this detection is dependent on stimulation (ex vivo) with STF10, and is abrogated on stimulation with STF10/ALVAC.

**Immunogenicity of the ALVAC Vector**

We have shown that both the ALVAC recombinants and the non-engineered ALVAC vector can induce immunogenic responses in immune competent as well as athymic nude mice. In wild type mice, tumor cells bearing either parental or recombinant ALVAC vectors were always rejected at a frequency of 100% except in one case where one 1 out of 16 mice injected with STF10/ALVAC IL-12 developed a tumor. As well, in nude mice, STF10 cells bearing parental or recombinant ALVAC vectors were rejected at varying, though statistically significant, frequencies relative to unmodified STF10 cells which were 100% tumorigenic.

These results indicate that the ALVAC virus by itself is immunogenic and can elicit an immune response without the added expression of recombinant genes like IL-12 and / or B7-1 when applied in a whole cell vaccine. Immunogenicity of the ALVAC virus in antitumor models was also demonstrated by Puisieux *et al.* (79) who showed firstly, that whole cell immunization with TS/A adenocarcinoma cells bearing ALVAC β-gal caused a delay in tumor onset, but did not confer total rejection as did TS/A cells bearing ALVAC IL12. In our case we saw complete tumor rejection with the ALVAC virus,
comparable to that with ALVAC IL-12. This discrepancy may be resolved by considering the fact that the multiplicity of infection employed by Puisieux et al. (79) was 5 Pfu/cell, whereas the MOI used here was 20 Pfu/cell. A lower MOI may lead to a lower efficiency of infection by ALVAC in vitro, providing fewer stimulating signals and targets in vivo during tumor rejection. Cells bearing ALVAC IL12 would in this case be better rejected because IL-12 is secreted and can modulate effectors in the local environment to a greater extent than can the parental virus by itself.

Immunogenicity of the ALVAC virus was also demonstrated in an additional experiment by Puisieux et al. (79), whereby intratumoral injection with the non-specific virus ALVAC β-gal showed increased survival of mice relative to those injected with PBS. However, mice injected with ALVAC IL-12 showed increased survival relative to any other group. Again, this discrepancy may be resolved by the mode of administration: intratumoral injection only allows the virus to access those cells that are in close proximity to the site of injection. As a result, only a few cells receive the vector, and any immune response directed towards the vector alone is directed only to those cells, and not the majority of the tumor mass. Injection with ALVAC IL-12, however, may have a better effect because in this case even though a few cells receive the vector, IL-12 can affect the entire local environment once secreted. Effectors all around the tumor can be activated even though only a subset of cells produces IL-12. In our case, the tumor cells are modified in vitro, where parental viruses can interact with tumor cells more homogeneously, allowing most cells to be infected before injection into mice.
Having established that the ALVAC virus is immunogenic, it still remains to be elucidated which aspect of the virus is providing the immune stimulus. The primary immune response may be directed against the virions and the protein components themselves, or may be a function of the viral DNA, which becomes exposed once the virus uncoats intracellularly. The related poxvirus, vaccinia, is naturally immunogenic, and various antigenic determinants have been characterized (89). In this report, it was shown that antibodies specific for polypeptides from the viral core as well as the viral envelope could be detected in sera from immunized humans. Because the poxviruses are related in structure, it is possible that antigenic determinants are present in ALVAC both at the core, which consists of structural proteins and enzymes, as well as the viral coat. While T-cell determinants have yet to be characterized for either vaccinia or ALVAC, it is possible that initial antitumoral reactivity by T-cells can be a function of activation against viral epitopes. These viral epitopes may be presented either indirectly by APC which pick up determinants from apoptotic / necrotic tumor cells, or directly by the tumor cells that harbour the virus.

Alternatively, the nucleotide sequence of the viral genome itself may contain elements that are immunostimulatory. For example, unmethylated CpG sequences have been shown to elicit responses from various arms of the immune system. Krieg et al. (90) showed that unmethylated bacterial DNA, as well as synthetically manufactured oligonucleotides bearing a motif of a CpG island flanked by two 5' purines and two 3' pyrimidines induce murine B-cells to activate and secrete immunoglobulin. The response is potent in that close to all splenic B cells react in this manner. Stacey et al. (91) showed
that macrophages (both primary and cell line) also react to CpG motifs by secreting iNOS, and Ballas et al. (92) demonstrated enhanced NK killing \textit{in vivo} and \textit{in vitro}, in response to CpG motifs. Finally, dendritic cells have also been implicated in the response to CpG motifs in that murine fetal skin derived dendritic cells undergo maturation, upregulate MHC class II and B7-2, and secrete IL-12 at amounts far greater than when activated by LPS (93). This activation of dendritic cells as a result of CpG treatment has been shown to be able to provide a positive stimulus for allogeneic T-cells to proliferate \textit{in vitro} (93).

Thus, if the genome of the ALVAC virus contains CpG motifs, then it may be a mode through which it is exerting its immunogenicity. Antigen presenting cells consisting of macrophages and dendritic cells may take up cellular debris from apoptotic tumor cells, and instead of presenting viral epitopes, they are presenting of tumor antigen epitopes with the concurrent activation of costimulatory molecules. The upregulation of costimulatory molecules in conjunction with tumor antigen presentation may serve to activate previously anergized T-cells against tumor epitopes. Once activated, CD8+ T-cells can reject the tumor efficiently.

\textbf{Antitumoral Protection following Vaccine Boost}

We showed that systemic antitumor protection against wild type STF10 tumor cells could be enhanced by boosting the initial STF10 cellular vaccine. This was true for immunogens containing both parental or recombinant ALVAC vectors, except those modified with ALVAC IL-12, which proved to be protective after a single vaccination.
Thus, in a double vaccination protocol, STF10/ALVAC is as protective as all other tumor vaccines, and the protective effect can be attributed to the virus alone.

These results indicate that potent T-cell memory against STF10 can be induced by vaccination with STF10/ALVAC by itself; however, this is dependent on a boost with the relevant tumor vaccine. This agrees with the view that T-cells need to be exposed to tumor or viral antigens more frequently in order to maintain immunity against tumor cells, i.e., T-cell memory is dependent on the increased presence of tumor antigens over time. If the host encounters an antigen at day 0, clears it and does not see it again until day 55, the subsequent response is not as potent as that when the antigen is encountered again partway through this period at day 35. The issue of whether T-cell memory is mediated by long lived lymphocytes that have encountered antigen once, versus those that continually need to mount a low level immune response against persistent antigen to maintain memory, was addressed by Kundig et al. (94). Immunization with a completely immunogenic Vesicular Stomatitis Virus carrying the nucleoprotein N (VSV-N), and subsequent challenge with pathogenic vaccinia viruses containing the same immunogenic CD8+ restricted protein N (Vacc-N), caused mice to lose T-cell mediated reactivity against N protein (and hence Vaccinia) over time. Further, the level of immunity is a function of immunization dose, in that at lower doses of the original immunogen, the immunity is lost faster.

Further, the role of antigen persistence was demonstrated (94), by vaccinating with a low dose of VSV-N and challenging with Vacc-N with or without boosting with EL-4 cells
carrying N protein. Without boosting, mice were completely susceptible to challenge at day 44; however a boost at day 40 abrogated this susceptibility, and that multiple boosts before this time point were not necessary.

Thus, T-cell memory responses against peripheral antigens may indeed be a function of the time following initial antigen clearance as well as frequency of antigen encounter. If the antigen is completely cleared from the periphery, then T-cell responses against the antigen can only be observed if splenic T-cells are reactivated. According to Kundig et al, in order to maintain peripheral immunity, the immunogen must persist in low amounts such that at the time of challenge, T-cells are already present in the periphery and activated. This was demonstrated by showing that LN lymphocyte reactivity against antigen is lost over time, while splenic activity is not, and that LN reactivity can be regained by a boost.

An analogous situation may be applicable in antitumor responses, especially in the model that is presented here. Challenge with wild type STF10 too long after initial vaccination may result in tumor formation due to the fact that T-cells have already cleared the initial vaccine and have left the periphery. If however, a boost is administered, an additional stimulus is provided for trafficking T-cells to remain activated in the periphery. At the time of challenge, these activated T-cells may be sufficient to mediate antitumor activity.
Protection against Tumor Epitopes as a Result of Viral Immunogenicity

Since wild type STF10 cells can in fact be cleared after vaccinating and boosting with STF10 cells bearing parental and recombinant ALVAC vectors, it can be concluded that a memory response can be induced via the activation of STF10 tumor specific T-cells upon systemic challenge. While experiments in nude mice confirm that there are additional T-cell independent mechanisms responsible for STF10 variant clearance, the presence of a protective immunity against unmodified tumor cells suggests T-cell involvement. How a T-cell response against STF10 cells can be gained as a result of a virally infected cellular vaccine is still unclear, but a number of possibilities exist. If, as discussed above, the viral DNA is immunostimulatory, then during rejection of STF10 cells bearing ALVAC vectors, tumor antigen specific T-cells that recognize cellular epitopes are being activated. This would require tumor antigen uptake, processing and presentation by APC that have upregulated their costimulatory molecule and cytokine expression levels, in response to ALVAC sequences. In this way, previously anergized or ignorant T-cells are activated against tumor epitopes, and can efficiently reject even wild type tumor cells upon challenge.

Alternatively however, the ALVAC protein epitopes may themselves be the primary immunogenic stimulus that is being presented to T-cells. Regardless of this, what we are observing is that T-cell activation against non-viral, tumor epitopes can also be induced. This transference of specificity may be explained either by the action of cross reactive T-cells that are activated by viral epitopes and can now recognize tumor epitopes, or by the phenomenon of epitope / determinant spreading.
The phenomenon of epitope spreading has usually been associated with autoimmune disorders in animal models of disorders such as insulin dependent diabetes (95) as well as relapsing remitting experimental autoimmune encephalomyelitis (R-EAE) (96), which is a Th1 mediated demyelinating disease responsible for chronic CNS damage. R-EAE can be induced in mice by the administration of an immunodominant peptide epitope from the proteolipid (PLP) molecule (96), or an epitope from myelin basic protein (MBP) (97). The initial reports of epitope spreading came from experiments whereby immunization of mice with the MBP protein allowed the isolation of proliferative T-cell clones specific for the immunodominant epitope Ac1-11, as well as non cross reactive T-cell clones specific for additional determinants in peptides 35-47, 81-100 and 121-140 (97). Importantly however, reactivity to these epitopes could also be observed upon immunizing mice with only MBP Ac1-11 and not the whole protein. Determinant spreading across different molecules was demonstrated by Cross et al. (98) who injected mice with epitope p87-99 of MBP, and found non cross reactive LN lymphocyte reactivity against epitopes of the PLP protein during acute or chronic stages of disease.

Both intermolecular as well as intramolecular epitope spreading were demonstrated by McRae et al. (96) where firstly, adoptive transfer of T-cells specific for MBP epitope 84-104 resulted in generation of T-cells in vivo that were specific for the PLP determinant 139-151. Secondly, adoptive transfer of T-cells of mice primed with PLP 139-151, resulted in reactivity against a non cross reactive intramolecular epitope PLP 178-191, during acute phase of EAE.
While the above studies are examples of epitope spreading as a result of initial priming via "self" epitopes, priming via viral epitopes has also been shown to give rise to determinant spreading. Like R-EAE, multiple sclerosis (MS) is also a T-cell mediated autoimmune demyelinating disease, and it has been found that infection of mice with Theiler's murine encephalomyelitis virus (TMEV) induces a chronic CD4+ T-cell mediated disease that models progressive MS (99). Miller et al. (99) have shown that injection of TMEV intracerebrally results in disease at about 40-50 days. Starting about 7 days post injection, and for the duration of the disease, infected mice continue to display T-cell proliferative behaviour as well as DTH responses against viral epitopes only. In contrast, there are no DTH or proliferative responses against a panel of myelin epitopes. However, at 30 days post onset (i.e., 80 days post viral infection), T-cell responses to myelin epitopes appear sequentially. First, responses are seen against PLP 139-151, followed by responses against epitopes 56-70 and 178-191 by day 164. According to Miller et al. (99), the kinetics of this differential specificity argue against a cross reactivity of T-cells against both cellular and viral epitopes as a result of molecular mimicry. If indeed there was cross reactivity, then reactivity against myelin epitopes should also have been detected at timepoints when reactivity against virus was detected.

The model put forth by Vanderlugt et al. (100) explains epitope spreading as a cascade resulting from recognition of a single epitope. Antigen presenting cells first present either the administered peptide epitope or viral epitope to CD4+ helper cells. These T-cells activate the APC's to upregulate costimulatory signals and at the same time initiate
an inflammatory response. This results in destruction of cells carrying these epitopes. However, following tissue destruction, cellular debris is taken up by the same group of activated APC’s. Subsequent presentation of cellular epitopes by the activated APC in turn can lead to priming of T-cells against self-determinants.

T-cell mediated immunity against unmodified STF10 tumor cells may also arise from determinant spreading after initial priming with ALVAC epitopes. APC’s picking up tumor debris can present ALVAC epitopes from the initial cellular vaccines to CD4+ Th1 helpers, leading to APC activation. The APC in turn could activate CD8+ T-cells against cells bearing ALVAC epitopes, while Th1 helpers could initiate an inflammatory response. The resulting destruction of ALVAC bearing tumor cells could lead to the uptake of cellular debris by activated dendritic cells and B-cells. These APC’s which still express upregulated costimulatory molecules can now prime T-cells against cellular epitopes. This in turn can induce a protective antitumor response when animals are challenged with wild type tumor cells.

**T-cell Independent Antitumor Mechanisms**

In the experiments presented here, mice were shown to be significantly protected from challenge after two respective vaccines of STF10 cells bearing parental or recombinant ALVAC vectors. While this level of antitumor immunity implicates immunological memory, and hence, T-cell activity, primary tumor vaccines consisting of STF10 cells bearing parental and recombinant ALVAC vectors were also significantly rejected in homozygous nude mice which lack mature T-cells. Again, the added expression of IL-12
or B7-1 or both did not confer any additionally protective effect over that mediated by the vector alone. That this rejection of STF10 variants is not as potent as in wild type mice is evidence that T-cells are indeed involved in primary rejection; however, the significant level of protection over mice receiving unmodified cells suggests the added role of other effectors, such as macrophages, NK cells or neutrophils.

One of the first studies of T-cell independent reactivity against gene modified tumor vaccines was carried out by Golumbek et al. (31) who showed that while growth of Renca cells in nude or SCID mice resulted in 100% mortality within 20 days, Renca cells expressing IL-4 did not grow at all in nude or SCID mice until after two months post injections. Histologic examination of the injection site in wild type mice showed a presence of macrophages, as determined by both gross characteristics, as well as Mac-1 positivity. This showed that in some cases, the initial phases of tumor rejection may be carried out by non specific effectors, though T-cells may be required for ultimate clearance.

A more specific model of T/B – independent NK - mediated antitumoral reactivity was demonstrated by Alosco et al. (101) where murine fibrosarcoma CMS-5 cells either did not grow, or regressed in SCID mice. This tumor rejection was ablated when mice were treated with anti-asialo GM-1, which reduces NK activity. In parallel, in vitro cytotoxicity of splenocytes was reduced against all of CMS-5, CMS-5 + IL-2 and YAC-1 cells when mice were treated with anti-asialo GM-1.
T-cell independent antitumor reactivity as a result of non specific responses against a viral vector was observed by Siders et al. (102) using a renal carcinoma metastasis model. In this report, Siders et al. established a metastatic hepatic malignancy by intrasplenic injection of Adenovirus expressing IL-12, or β-gal. In wild type mice, the number of metastases were cured by 94% with injection of Ad IL-12 (relative to no treatment), and by 25-40% with injection of Ad β-gal. Staining of hepatic sections revealed the existence of CD3+ T-cells, as well as lysozyme containing cells (neutrophils or macrophages) around hepatic blood vessels of animals treated with Ad vectors.

To clarify the mode of antitumoral reactivity, the experiment was repeated in SCID mice, where it was observed that treatment with Ad β-gal reduced the number of metastases by 46% while treatment with Ad IL-12 reduced the number of metastases by 95%. This showed that firstly, an antitumoral response could by elicited by administration of the virus alone, although not as efficiently as with IL-12 expression, and that the response may not be T or B cell mediated. NK-cell depletion in SCID mice abrogated the antitumoral activity in response to Ad β-gal, but Ad-IL-12 still allowed for efficient rejection of tumors. This showed that in this scenario, IL-12 exerts a greater effect in mediating tumor rejection that is greater than that afforded by the vector alone. However, even the effect that is mediated by the vector is significant, and may not be entirely dependent on T-cells.

A similar model may be applicable in rejection of tumor cells bearing ALVAC vectors. While T-cells may be responsible for ultimate clearance of tumors, such as in the case
with Golumbek et al. (31), other effectors such as macrophages, neutrophils or NK cells may be responsible for the partial clearance that is observed in nude mice. Because there is no enhancement of the antitumor response with ALVAC-IL-12 and/or ALVAC B7-1 over ALVAC alone, the immunogenicity of the tumor cells in nude mice can be attributed to the virus alone. In nude mice, non adaptive effectors such as macrophages and NK cells may be recognizing the tumor cells as targets that have been modified by the virus in such a way as to be more likely candidates for killing. The viral effects on the tumor cells are unclear at this point, but in addition to possible T-cell stimulatory effects as discussed above, they are definitely immunostimulatory towards other effectors as well.

In Vitro CTL Activity

In the in vitro CTL experiments presented here, splenocytes from mice immunized with STF10/ALVAC IL-12 react against STF10 tumor cells infected with ALVAC IL-12 but not STF10 alone, when stimulated in vitro with STF10/ALVAC IL-12. Subsequently, a side-by-side comparison of different in vitro stimuli of either STF10/ALVAC or STF10, showed that stimulation by STF10 alone allowed generation of T-cells specific for both STF10/ALVAC as well as unmodified STF10, while stimulation with STF10/ALVAC reduced the amount of observed reactivity against unmodified STF10. Thus, it can be concluded that STF10-specific CTL’s can be amplified in vitro from spleens of immunized mice, however, their amplification is dependent on the mode of stimulation. Stimulation via STF10/ALVAC (or STF10/ALVAC IL-12) allows the generation of T-cells specific only for STF10 infected with ALVAC viruses, while stimulation via STF10
allows amplification of CTL's specific for either STF10 or STF10/ALVAC. Incidentally, cells that were stimulated with STF10 only displayed higher killing of STF10/ALVAC targets, relative to STF10 targets, at lower effector to target ratios. This may be due to the fact that these splenocytes consist of T-cells that are specific for STF10 epitopes, as well as T-cells specific for viral epitopes, and what we are observing is a synergistic effect of these two groups of cells, during lysis of STF10 cells bearing ALVAC. Alternatively, however, there is an increase in MHC class I expression (around 33%) in STF10 cells after infection via recombinant ALVAC vectors. The higher level of MHC I could provide a greater number of targets for T-cells that are specific for tumor epitopes, leading to greater overall killing.

The fact that wild type tumors can be killed by splenocytes after stimulation via STF10 shows that splenic T-cells specific for STF10 are indeed present around day 25; however, their in vitro amplification is somehow being blocked during stimulation with STF10 cells that have been infected with ALVAC. This inhibition of STF10-specific T-cell amplification may be explained either by an inefficient presentation of tumor epitopes during stimulation via STF10/ALVAC (and hence an inefficient expansion of STF10-specific T-cells), or an inherently low level of tumor cell specific T-cells in the original splenic population.

Suboptimal levels of T-cell activation due to differential antigen presentation was demonstrated by Gallimore et al. (103) using LCMV viral antigens as immunogens. In this report, it was shown that immunization of vaccinia immune mice with vaccinia
encoding a subdominant LCMV glycoprotein epitope (GP117) protected mice when challenged with LCMV bearing GP117. However, the same mice were not protected when they were challenged with LCMV bearing GP117 as well as two other immunodominant epitopes GP33 and GP276. Stability assays showed the GP117 – H-2b complex to have a half life of less than 2 hours, while the GP33 - H-2b and GP-276 - H-2b complexes had half-lives greater than 4 hours. Furthermore, peptide elution experiments showed that while MC57 cells infected with the LCMV variant bearing GP117 but not GP33 and GP276 gave an active fraction for GP117 as expected, cells that had been infected with wild type LCMV did not display GP117. This suggested that in the presence of other immunodominant epitopes in the cell, subdominant epitopes may not be presented as efficiently. Gallimore et al. (103) propose a model whereby the presentation of a subdominant epitope may be “competed out” either due to higher dissociation rate from MHC molecules or due to differential processing via the ER.

Similarly, in STF10 cells that have been infected with ALVAC viruses, it may be possible that viral epitopes are being presented to T-cells more efficiently during ex vivo stimulation of splenocytes from immunized mice. This difference in efficiency may be due to a number of reasons, including a higher affinity of the viral epitopes for MHC class I, more efficient processing of viral epitopes via the ER, or the presence of a greater number of viral epitopes that can effectively compete out the cellular epitopes for binding to MHC. Regardless, cellular epitopes are excluded to the end-effect that in vitro, STF10 epitope specific T-cells are not being expanded when stimulated STF10/ALVAC or STF10/ALVAC IL-12.
Alternatively, in animals that have been vaccinated with STF10 infected with parental or recombinant ALVAC vectors, cytotoxic T-cell clones specific for TRA epitopes may be fewer in number than those specific for ALVAC epitopes, and hence are not being efficiently expanded when stimulated with STF10/ALVAC. A significant difference between the total number of peripheral T-cells that are specific for one antigen versus another antigen may arise due to CD8+ T-cell competition, a phenomenon described by Freitas et al. (104).

Freitas et al. have demonstrated that reconstitution of irradiated hosts with a mixture of transgenic (Tg) and non-transgenic (non-Tg) lymphocytes allows establishment of peripheral CD8+ T-cells at frequencies that are dependent on the specificity of the transgenic TCR. Generally, in chimeras reconstituted with mixtures of Tg and non Tg cells at low ratios of Tg/ non Tg, mature transgenic CD8+ cells in the periphery were rare or absent. In chimeras with higher initial frequencies of Tg bone marrow cells, the percentage of CD8+ single positive Tg cells was significant in the peripheral pool, but the frequency was dependent on the TCR specificity. T-cells that were specific for HY antigen constituted less than 10% of the peripheral (splenic and LN) population (relative to non Tg cells), and T-cells specific for LCMV P14 antigen constituted greater than 50% of cells in periphery, when reconstituted separately with non transgenic cells. A subsequent experiment, which looked at the effect of mixing the two transgenic lymphocyte precursors, showed that at low anti HY: anti P14 precursor ratios, anti HY T-cells in the periphery were rare. In contrast at high ratios, anti HY cells constituted the
majority of peripheral CD8+ T-cells after the first weeks of reconstitution, but at 3 weeks, both populations reached identical sizes. Finally, at 16 weeks, P14 transgenic cells became the dominant population. In vivo BrdU staining revealed that while proliferation of peripheral CD8+ transgenic was poor relative to wild type cells overall, in chimeras injected with both anti HY and anti P14, there was an increased accumulation of stained anti P14 cells relative to anti HY cells. Further, the majority of the anti P14 cells are activated (CD44+) while only 10 – 15% of anti HY cells are activated. These results led to the postulation that competition does exist in selection of T-cells and that cellular dominance may occur by preferential cell activation. In other words, a state of activation may confer a “selective advantage” in establishing a dominant population (104).

This model may be applicable in explaining why in vitro stimulation of splenocytes from immunized mice with STF10/ALVAC preferentially amplifies cells specific for STF10/ALVAC and not STF10. In the in vivo situation T-cells specific for tumor epitopes may initially constitute a smaller proportion of the entire population relative to other T-cells which are specific for non self peptides such as ALVAC epitopes. During immunization with STF10/ALVAC, the anti-ALVAC T-cells may be activated more efficiently than anti STF10 cells due to reasons such as immunodominance. This in turn could result in the dominance of ALVAC specific T-cells over STF10 specific T-cells in the spleen. When these splenocytes are harvested and stimulated with STF10/ALVAC, anti-ALVAC T-cells preferentially expand due to the selective advantage gained by their superior numbers and activation status. This leads to masking of STF10 specific T-cells as these cells cannot establish a dominant population. In contrast, when stimulating with
STF10 only, anti-ALVAC T-cells are not re activated *ex vivo* and this time, anti STF10 T-cells may regain a selective advantage due to their preferential activation.
Future Directions

The usefulness of the ALVAC vector in immunotherapy of cancer has been demonstrated by other investigators through use of murine models. In previous reports, therapeutic effects have been gained through the expression of recombinant genes via the ALVAC vector, while the vector itself has been shown to be moderately immunogenic by itself. This report established the inherent immunogenicity of the ALVAC virus vector further, in a model whereby the virus itself is 100% immunogenic, as well as protective towards wild type tumor challenge.

A natural progression of this work would be to extend this particular tumor model in order to make it more representative of actual human malignancies, where disease may often be well established before gene therapy is applied. Thus, the efficiency of the ALVAC vector has to be evaluated in the treatment of established tumors whereby wild type STF10 cells are inoculated within the model animal, and treatment begins at a subsequent timepoint. The nature of the treatment could involve the intratumoral injection of ALVAC viruses bearing IL-12 or B7-1, to directly modify the existing tumor cells such that the local immune effectors are subsequently activated. Alternatively, the treatment could consist of an injection of radiation-inactivated tumor cells expressing IL-12 or B7-1, and in this scenario, effectors are activated initially by the gene modified vaccines. If systemic immunity is conferred, a subsequent response may then be directed against the established wild type tumor.
To ultimately make this vector suitable for cancer therapy in humans, a balance has to be established in minimizing the immunogenicity of the ALVAC virus, while maximizing the therapeutic effects of the recombinant genes. Factors affecting the level of immunogenicity may include attributes unique to the model itself, such as tumor type and MHC background, but also general treatment approaches such as route of administration, dose of virus, as well as model of tumor establishment and treatment. So far the cancer treatment models utilizing ALVAC have not been established with the differential effects of these characteristics in mind. Thus, while the ALVAC virus is still an attractive vector for cancer gene therapy, the relative effects of each of these parameters should be established with regard to ALVAC immunogenicity. While clinical trials utilizing ALVAC are already underway, parameters influencing viral immunogenicity need to be better defined before the ALVAC virus vector is applied more extensively for therapeutic purposes.
References


complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. 

*Proc Natl Acad Sci USA* 90:11307.


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<td>Tumorigenicity in Wild Type</td>
<td>Boost</td>
<td>Uninfected</td>
<td>4.6 ± 0.3</td>
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<td>BALB/c (prime / boost)</td>
<td></td>
<td>ALVAC</td>
<td>5.3 ± 2.1</td>
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<td></td>
<td></td>
<td>ALVAC B7-1</td>
<td>4.2 ± 1.2</td>
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<tr>
<td></td>
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<td>ALVAC IL-12</td>
<td>61.7 ± 5.7</td>
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<tr>
<td>Tumorigenicity in Nude Mice</td>
<td>Vaccination</td>
<td>Uninfected</td>
<td>1.7 ± 0.1</td>
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<td></td>
<td></td>
<td>ALVAC</td>
<td>0.5 ± 0.2</td>
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<td>ALVAC B7-1</td>
<td>1.8 ± 0.7</td>
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<td>ALVAC IL-12</td>
<td>28.8 ± 2.5</td>
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<td>ALVAC B7-1/ALVAC IL-12</td>
<td>31.9 ± 0.9</td>
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<tr>
<td>Tumorigenicity in Nude Mice</td>
<td>Vaccination</td>
<td>Uninfected</td>
<td>2.0 ± 0.5</td>
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<td></td>
<td>(Repeat)</td>
<td>ALVAC</td>
<td>Below Detection</td>
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<tr>
<td></td>
<td></td>
<td>ALVAC B7-1</td>
<td>1.6 ± 0.3</td>
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<tr>
<td></td>
<td></td>
<td>ALVAC IL-12</td>
<td>102.2 ± 8.3</td>
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All Values are expressed in ng/mL/million cells/24 hours
Figure 1: Genomic Organization of recombinant ALVAC viruses

The ALVAC virus vector consists of a linear double stranded DNA molecule measuring about 325 Kbp. The multiple cloning sites C5 and C6 have been inserted as shown above. The ALVAC hB7-1 construct consists of a human B7-1 cDNA driven by the vaccinia H6 promoter inserted into the C6 MCS. The ALVAC mIL-12 construct consists of two p35 subunit cDNA sequences each driven by one E3L vaccinia promoter, and a p40 subunit cDNA driven by the entomopox 42K promoter. The p35 and p40 subunit constructs are inserted into the C5 and C6 loci, respectively.
Figure 2: Heterogeneity in ALVAC Viral Transduction

To determine the tropism exhibited by the Canarypox virus ALVAC, cell lines of different lineages and stages were assayed for the ability to successfully take up the virus and express the virally encoded protein **B7-1**. Clear histograms represent cells that were specifically stained by anti-human **B7-1** antibody, while shaded histograms represent staining via an isotype matched non specific antibody. Result indicate that recombinant **B7-1** is expressed only in select tissues represented by NFS-70, B16, ST, as well as two ST subclones STF10 and STG4. A20, K46J, C1498 and P815 are completely negative for ALVAC encoded products.
Figure 3: Surface Marker Phenotype of STF10

STF10 cells were stained with antibodies specific for the surface-expressed proteins indicated, and then assayed by cell cytometry as outlined in Materials and Methods. Clear histograms represent specific staining for the marker indicated, while shaded histograms represent irrelevant staining of STF10 via a non-specific isotype matched antibody.
Figure 4: RT-PCR analysis of ST-F10, and STF10 infected with ALVAC, for TdT, RAG-1, RAG-2, and HPRT. ST-F10 yields the expected size PCR product for RAG-1, RAG-2, as well as TdT. The positive control for PCR amplification of TdT consisted of a genomic sample from mouse tail DNA. Positive controls for amplification of RAG-1, RAG-2, and HPRT consisted of various cDNA preps of the mouse Pro-B Cell line NFS-70.
Figure 5: Growth Characteristics of STF10 Cells after infection via ALVAC vectors

STF10 cells were infected with ALVAC B7-1 as outlined in Materials and Methods, and then replated in culture media at a concentration of 20,000 cells/mL. Growth was monitored over a period of 4 days, and was compared to the growth rate of a STF10 culture that was plated at the same concentration, but was uninfected with ALVAC B7-1. Results indicate that infection of cells via recombinant ALVAC vectors does not alter the growth characteristics of the STF10 cells as the rate of expansion of the two cultures is similar.
Figure 6: Effect of ALVAC viral infection on MHC class I expression via STF10 cells

STF10 cells were infected with ALVAC B7-1 as outlined in Materials and Methods, and assayed for the expression of MHC I at timepoints corresponding to 6 hours into viral infection, and 24 hours following viral infection. Results indicate that there is no downregulation of classical MHC class I (H-2K<sup>d</sup> and H-2D<sup>d</sup>) as the geometric mean fluorescence of infected cells is 17 at 6 hours, and 19 at 24 hours, compared to uninfected cells which display a geometric mean fluorescence of 14 at 6 hours and 15 at 24 hours.
which expression levels start declining and is lost completely at four days.

Four days (< 96 hours) post-injection, whereas for a rapidly dividing culture, expression is optimal for about two days (48 hours), after expression all discrete lymphocytic. Results indicate that in a non-dividing culture, ALVAC encoded products can be detected by FACS up to 96 hours post-injection. However, when the other half was kept in log phase, both cultures were assayed for ALVAC encoded B7-1.

Induction of ALVAC product stability: Scid thymoma bulk culture was transduced with ALVAC B7-1 and half the culture was irradiated. Because the ALVAC viral vector is non-replicative in mammalian tissue, the expression of ALVAC encoded products will be transient as shown in the following figure.

**Figure 7: Stability of ALVAC Encoded Products Over Time**

- **Horizontal Axis:** Hour post-injection
- **Vertical Axis:** Percentage of Cells Positive for ALVAC B7-1
- **Graph:** Shows the percentage of cells positive for ALVAC B7-1 over time, with data points indicating the stability of the encoded products.

The graph illustrates the percentage of cells positive for ALVAC B7-1 over time, with data points indicating the stability of the encoded products.
Figure 8: Survival of Mice Post STF10 Tumor Variant Vaccination

STF10 cells were infected with one of a) No Virus b) ALVAC c) ALVAC B7-1 d) ALVAC IL-12 or e) ALVAC IL12 & ALVAC B7-1, and injected subcutaneously into mice (using 5 mice per vaccine). Mice receiving cells alone succumbed to tumor, whereas mice that received cells containing either parental or recombinant ALVAC vectors, all rejected their tumors at a frequency of 100%.
Percentage Survival

Survival of Mice Post Wild Type STF10 Challenge After One Vaccination

Initial STF10 Tumor Vaccine

Naive Control

ALVAC B7-1/IL-12

ALVAC IL-12

ALVAC B7-1

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Figure 10: Tumorigenicity of STF10 Variants in Nude Mice

STF10 cells were infected with parental or recombinant ALVAC viruses as described in Materials and Methods, and injected subcutaneously into nude mice. All animals receiving cells alone succumbed to tumor in both experiments (A & B). Animals receiving STF10 cells containing either parental or recombinant ALVAC vectors displayed tumor rejection at statistically significant (p < 0.05) frequencies, relative to mice receiving cells alone, in both independent experiments.
Mice were primed and boosted with cellular vaccines of a) STF10 / ALVAC b) STF10 / ALVAC B7-1 c) STF10 / ALVAC IL-12 d) STF10/ALVAC B7-1 and STF10 / ALVAC IL-12. In addition, one group of mice was only primed with STF10 / ALVAC IL-12, but left unboosted. Vaccinations and boosts were administered subcutaneously, on the right flank, with the boost taking place 35 days after the initial vaccination. 21 days post the boost, mice were challenged subcutaneously on the left flank with wild type STF10 cells and assayed for survival. Naive controls that had not been previously vaccinated all developed tumors, while all other vaccinated groups displayed improved survival relative to naive mice (p < 0.05 for all groups relative to naive mice).
Figure 12: Specific Activity of Splenocytes from STF10/ALVAC IL-12 immunized mice. Mice were immunized with STF10/ALVAC IL-12 and spleens were harvested on days 13, 17, 27 and 35. Splenocytes were stimulated with STF10/ALVAC IL-12 and specific killing against STF10/ALVAC IL-12 (A & B) or wild type STF10 (C & D) cells was evaluated. Cytolytic activity was observed on day 27 against STF10/ALVAC IL-12 cells (B) and not STF10 cells (D). No cytolytic activity against either type of cells was observed on day 13 (not shown), day 17 (A & C) and day 35 (not shown).
**Figure 13: Effects of Differential Stimulation on Splenocyte Reactivity against STF10:** Mice were immunized with STF10/ALVAC and then boosted with the same vaccine. 21 days following the boost, splenocytes from an immunized mouse and a naïve control were harvested and stimulated with STF10 (A & B) or STF10/ALVAC (C). Results indicate that cytolytic precursors specific for STF10 are indeed present in the splenocyte culture (A), in addition to those specific for STF10/ALVAC (B); however, their detection is dependent on stimulation with wild type STF10. Stimulation via STF10/ALVAC does not yield efficient cytotoxicity against wild type STF10, relative to naive controls (C).