Murine Acute Myeloid Leukemia Cells Expressing the Cytosine Deaminase Gene Induce Protective Immunity to Parental Leukemic Cells

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Despite advances in the treatment of AML, long term survival remains poor. Immunotherapy, aimed at eradicating the minimal residual disease leukemic cells that lead to relapse may improve survival. We have investigated the potential utility of an AML vaccine expressing cytosine deaminase (CD). The expression of CD renders cells selectively sensitive to a drug, 5-fluorocytosine (5-FC), that has no effect on normal mammalian cells. We have generated a CD expressing variant of the murine C1498 AML cell line. We determined that in our model, in vivo 5-FC treatment does not eliminate 100% of the CD-expressing cells. Nevertheless, enhanced survival was seen with CD-expression alone and immunogenicity of the CD protein can elicit an immune reaction against parental cells by epitope spreading. C1498 clearance was determined to be mediated mainly by CD8+ T cells. Most importantly, resistance to parental rechallenge and concurrent challenge was seen with mice immunized with CD-expressing cells.
Dedication

This research is dedicated to my father, Reginald Claude Thompson (1942-1998).

Without the lessons he taught me in life I could not have continued after his death.
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List of Abbreviations

5-FC 5-fluorocytosine
5-FdUMP 5-fluoro-2'-deoxyuridine 5'-monophosphate
5-FU 5-fluorouracil
5-FUDP 5-fluorouridine 5'-diphosphate
5-FUMP 5-fluorouridine 5'-monophosphate
5-FUR 5-fluorouridine
5-FUTP 5-fluorouridine 5'-triphosphate
ACV acyclovir
AML acute myeloid/myeloblastic leukemia
APC antigen-presenting cell
ATCC American Type Tissue Collection
β-gal β-galactosidase
BCG bacillus Calmette-Guerin
BMT bone marrow transplantation
C1498-CD CD-expressing C1498 cell line
C1498-neo control C1498 cell line, expressing neo but not CD
C1498-P parental C1498 cell line
CD cytosine deaminase
CD cluster of differentiation
cDNA complementary DNA
CO₂ carbon dioxide
CR complete remission
CTL cytotoxic lymphocyte
DMEM Dulbecco's modified Eagle's medium
DMSO dimethylsulfoxide
DNA deoxynucleic acid
Escherichia coli
ER endoplasmic reticulum
FACS Fluorescent activated cell sorter
FCS fetal calf serum
FITC fluorescein isothiocyanate
GCV ganciclovir
GM-CSF granulocyte-macrophage colony-stimulating factor
GVHD graft versus host disease
GVL graft versus leukemia effect
HLA human leukocyte antigen
HSV-1-TK herpes simplex virus 1 thymidine kinase
ICAM intracellular adhesion molecule
IFN interferon
IL interleukin
IMDM Iscove's modified Dulbecco's medium
IP intraperitoneal
IV intravenous
List of Abbreviations (continued)

LFA      lymphocyte function-associated antigen
LT       lymphotoxin
MHC      major histocompatibility complex
MMB-CD   CD-expressing MMB cell line
MMB-neo  control MMB cell line, expressing neo but not CD
MMB-P    parental MMB cell line
MTT      3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl-tetra-zoliumbromide
NEB      New England Biolabs
Neo      neomycin phosphotransferase
NK       natural killer
NP       influenza nucleoprotein
PBS      phosphate buffered saline solution
SC       subcutaneous
SGVHD    syngeneic graft versus host disease
TCR      T-cell receptor
Th1      T-helper 1
Th2      T-helper 2
TNF      tumour necrosis factor
TK       thymidine kinase
Introduction

Background

While chemotherapy and bone marrow transplantation (BMT) are the mainstay of treatment for acute myeloblastic leukemia (AML), remission is induced in only 60 – 70% of patients with AML and long-term survival is achieved in only 25-50% of patients who achieve complete remission [1]. The majority of patients die of their disease, achieving remission only to relapse at a later date. It is therefore the long-term management of minimal residual disease that in particular needs to be improved, if survival is to be increased.

Several lines of evidence suggest that manipulation of the immune system may be effective in controlling leukemic minimal residual disease. One approach to such immune manipulation - active, specific immunotherapy - would involve the immunization of patients in remission with a vaccine consisting of genetically modified tumour cells. Active specific immunotherapy of AML to control minimal residual disease is an attractive option for two reasons. First, inducing a tumour specific cytotoxic immune response is desirable because of its exquisite specificity, and secondly, there exists the ability to engender immunological memory, protecting the patient from recurrence of the disease [2].

The ability to eliminate the cells used for vaccination by drug treatment after immunization has occurred, would allow live cells to be used for vaccination, while ensuring that leukemic relapse does not arise from the vaccine itself. Several non-mammalian enzymes convert nontoxic prodrugs to toxic metabolites. The transfer of genes encoding these enzymes to mammalian cells produces cells selectively sensitive to
the prodrugs. One of these enzymes, cytosine deaminase (CD) from *Escherichia coli*, converts the prodrug 5-fluorocytosine (5-FC) to the toxic metabolite 5-fluorouracil (5-FU). We have examined the ability of a murine AML cell line engineered to express the CD gene to be eliminated *in vitro* and *in vivo* with 5-FC treatment, and to stimulate a tumour specific immune response against unmodified leukemic cells.

**Acute Myeloid Leukemia**

Acute Myeloid Leukemia (AML) is a neoplastic disease arising from the transformation of a single hematopoietic progenitor [3]. However, AML is a heterogeneous disease, as it encompasses the transformation of any of a large number of myeloid hematopoietic stem and progenitor cells [3]. In addition, transformation can occur at different stages of differentiation, resulting in varying tumour characteristics [3]. Nevertheless, the end result of the disease is the same, an overgrowth of the AML cells in the bone marrow of the patient [3].

While the incidence of disease is approximately 3.6 per 100,000, the mortality rate is 2.5 per 100,000. This high rate of mortality is not due to the inability of patients to achieve remission, as the majority of adult patients with AML achieve complete remission (CR) with standard chemotherapy. Instead, mortality is mainly due to leukemic relapse, with long-term survival being achieved in only 25-50% of patients who achieve CR [1].

Age is an important factor in outcome, with patients older than 55 faring poorly compared to younger patients [1]. Unfortunately, the median age of patients with AML
is approximately 65 years, with the incidence of adult to childhood disease occurring at a ratio of about 4 to 1.

Treatment for AML may include chemotherapy, and bone marrow transplantation (BMT). While BMT may lead to long term survival, these treatments may result in graft versus host disease (GVHD) [4].

The majority of patients with AML die of their disease, achieving initial remission only to relapse at a later date. It is therefore the long-term management of minimal residual disease that in particular needs to be improved if survival is to be increased. New therapies to achieve greater survival for AML patients are desperately needed. Active immunization aimed at enhancing cell-mediated immunity against the leukemia cells is one such option. The cells involved in this type of immunity are T-cells, including CD8\(^+\) T-cells, which specifically recognize and kill the target tumour cell, and CD4\(^+\) T-cells, which provide help in the form of cytokines once activated by a specific antigen [5].

T-Cell Activation

Altered-self cells can be eliminated by a cytotoxic reaction that results in the lysis of target cells. The effector mechanisms of the cytotoxic reactions have been identified and can be divided into two general classifications: cytotoxicity involving antigen-specific T-cells and cytotoxicity involving non-specific cells, such as natural killer cells and macrophages [6]. Since it has been demonstrated in animal studies that, with a few exceptions, T-cell mediated immunity is the critical factor in the rejection of tumours [7], the following discussion will focus on T-cell activation.
Antigen specific T-cell activation requires the interaction of the T-cell with specialized antigen-presenting cells (APCs), which include dendritic cells, activated B cells, monocytes in the peripheral blood, and Langerhans cells and keratinocytes in the skin [6]. However, in the case of a tumour antigen, activation may also occur through interaction with the tumour cell itself, although no doubt less successfully than with interaction with an APC. Three steps are required for T-cell activation – adhesion, recognition and costimulation.

Due to the small size of the T-cell receptor (TCR), its low affinity toward antigen and the limited amount of antigen on the APCs, an adhesion complex is formed to allow the TCR to contact, recognize the antigen and become activated [8]. Primary adhesion molecules on T-cells that contribute to the binding between the T-cell and the APC include CD2, ICAM-1 and LFA-1. Their cell surface bound ligands LFA-3, LFA-1 and ICAM-1 are expressed on potential APCs [9].

The next step is recognition, in which an antigen-specific signal is delivered to the T-cell through the interaction of the T-cell receptor (TCR) with a specific peptide antigen in the context of the major histocompatability complex (MHC) on an antigen-presenting cell (APC). Intracellular tumour proteins can be presented to T-cells as multiple peptide fragments in MHC class I molecules, and shed tumour protein can be taken up, processed and presented as peptide fragments in MHC class II molecules [10]. Cytotoxic T-cells are generally CD8+ and are class I MHC restricted, although in rare instances CD4+ class II restricted T-cells have been shown to function as CTLs [6, 9]. The CD4+ T-cells, referred to as T-helper cells, interact with MHC class II presented peptide fragments.
This interaction then allows the T-cell to respond to secondary APC costimulatory signals such as those mediated by the B7/CD28 system. These costimulatory molecules are believed to function to amplify the signals transduced by the TCR [8].

Finally, activation of cytotoxic T-cells results in increased expression of interleukin-2 (IL-2) receptors. In contrast, activation of T-helper cells results in clonal expansion and the induction of effector functions, such as the production of IL-2. The large increase in secretion of IL-2 induces the proliferation and differentiation of cytotoxic T-cells into functional cytotoxic lymphocytes, leading to the destruction of the antigen bearing cell.

T-helper 1 and T-helper 2 Effector Cells

The induction of an antitumour cytotoxic lymphocyte (CTL) response has been shown to require the participation of CD4+ T-cells, which are referred to as T-helper cells. CD4+ T-cells must recognize antigenic peptides on a professional APC, such as a dendritic cell or macrophage, that presents antigen peptides on MHC class II molecules [11]. The CD4+ cells then secrete cytokines that activate CTL precursors.

CD4+ T-cells can be divided into two different classes based on distinct patterns of cytokine production. T-helper 1 (Th1) cells produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin (LT), while T helper 2 (Th2) cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Both Th1 and Th2 cells secrete IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF-α) [12].

The two distinct subsets of T-helper cells are associated with different cellular immune functions related to the cytokines that each subset secretes. In fact, some of the
cytokines that the subsets secrete have opposing functions. Th1 clones are well suited to deal with viral infections due to the activation of cytotoxic T-cells and induction of IFN-γ by IL-2 production [6]. Th2 cell clones enhance antibody production from B-cells and induce aspects of an allergic reaction and are more suited to dealing with parasitic infections [6]. In summary, Th1 cells mediate cellular immunity and Th2 cells mediate humoral immunity.

Since it is believed that cellular immunity is the critical factor in the rejection of tumours, it may be important for tumour rejection that naïve CD4+ cells differentiate into the Th1 subtype. Costimulatory molecules have been shown to play an important role in directing the differentiation of naïve CD4+ cells to the Th1 or Th2 subtype. It appears that B7.1 may direct the differentiation to the Th1 subtype while B7.2 costimulation results in the Th2 subtype [5]. Consistent with the idea that the cellular arm of the immune system is critical for tumour rejection, B7.1 has been shown to be superior to B7.2 in generating antileukemia immunity [13]. However, there exists some evidence to suggest that both cellular and humoral immunity can contribute to tumour rejection, as Th2 cytokines (IL-4 and IL-10) have been shown to enhance tumour rejection in some cases [5].

**Non-Specific Tumour Immunity**

As mentioned above, cytotoxic reactions involving non-specific cells, such as natural killer cells (NK cells) and macrophages can also eliminate altered-self cells [6]. NK cells are a subpopulation of lymphocytes that can kill some cancer cells without prior sensitization or the requirement of MHC restriction [7]. NK cells appear to be important
at least for the surveillance of tumours, since mice or humans that have a genetic impairment in NK cells show an increase in incidence in some forms of cancer [6].

Macrophages are often observed to cluster around tumours and their presence can be correlated with tumour regression [6]. Macrophages secrete several products that are cytotoxic to tumour cells including INF-γ, and TNF-α [6]. In addition, it has been shown that macrophages can be activated by bacterial products in vitro to cause selective cytolysis of tumour cells [7]. It is hypothesized that some cancer cells lack a necessary 'danger signal' to activate these cells that is provided by the bacterial products [7]. So, unlike NK cells, the activity of macrophages can be heightened by increasing the antigenicity of the tumour cell.

Tumour Antigens and T-cell Unresponsiveness

It is unknown why the tumour cells involved in minimal residual disease are able to escape detection by the immune system. Tumour antigens, including leukemia specific antigens, have been shown to exist [14], and would be expected to be targets of a rejection response. T-cells should recognize the tumourigenic cells as foreign and induce their cytolysis. Indeed, B and T-cells that recognize antigens expressed on cancer cells have been discovered in patients with cancer [15]. Thus, if there exists tolerance to the tumour, this does not involve the deletion of the immune repertoire against the cancer cell [15].

It is difficult to understand why the human body fails to reject tumour cells. How could an immune system such as ours, the product of millions of years of evolution, fail to detect and eradicate such harmful cells? In reality, the failure of our immune system in
this area is probably a reflection of its development in the face of two opposing forces, the need to destroy foreign cells and harmful pathogens, and the need to avoid destroying its own cells. A number of mechanisms exist to prevent autoimmune responses by the immune system, and it seems that it is these very fail-safes that unfortunately allow some tumour cells to go unchecked. In order to prevent autoimmunity, the response of the immune system against self components, there exist mechanisms of self tolerance. Although mature self-reactive T-cells and B-cells may exist, their activity is regulated, either through clonal anergy or clonal suppression [6]. A breakdown in this regulation can lead to autoimmune diseases that can cause serious, sometimes fatal, damage to cells and organs [6]. Nevertheless, in the case of a person dying of cancer, it may be desirable to interrupt the regulation of self-reactive T-cells and attempt to direct them against the tumour cells, which are altered-self cells.

In general, tumour cells are not very effective at inducing immunity [16]. Although all tumours may have antigens that can be recognized by the immune system, these antigens can vary from potent antigens to antigens with low immunogenicity [15]. Even when the tumour antigen is potent, it appears that T-cell activation requires the antigen to be presented in the correct context, in the presence of costimulatory molecules. It is hypothesized that when T-cells interact with cognate antigen in the absence of a costimulatory signal, induced unresponsiveness (anergy) or death by apoptosis (clonal deletion) results [17]. More recently, this idea has been modified from an all or nothing event to the possibility that there can exist degrees of activation, based on the nature of the stimulus, from fully activated, partially activated, to anergized T-cells [18].
Nature of Tumour Antigens

Many tumour antigens appear to be self-antigens that are altered or expressed out of their normal cellular and developmental context, with the exception of viral gene products in virus-associated malignancies. Examples of tumour antigens against which T-cell responses have been identified are: oncogene products activated by mutation or rearrangement, mutated tumour suppressor genes, reactivated embryonic gene products not expressed in adult tissues, tissue specific self antigens expressed by tumour cells and viral gene products [11].

These tumour antigens may be self-determinants that are cryptic due to expression at a sub-threshold level, thereby allowing the antigen specific T-cell clone to escape deletion in the thymus [19]. Once these epitopes are presented at higher concentrations, or in the correct context, the antigen specific T-cells may become activated.

Vaccines

The idea of a vaccine brings to mind a vaccine being used to immunize an individual against a pathogenic virus. This treatment involves treating a patient prior to potential exposure to the virus. The treatment of a cancer patient with a tumour vaccine is somewhat different, in that the vaccine is used after the antigenic insult, and that the target of a tumour vaccine is a usually a T-cell and not a B-cell response [11]. In one version, the vaccine is produced using the patient’s own tumour cells, which will hopefully stimulate the patient’s immune system to eradicate any remaining tumour cells. Using whole cells in tumour vaccines precludes the need to have identified the tumour antigen(s) at the genetic level. The individual’s tumour is simply used as the source for the tumour antigen(s).
The use of cancer vaccines dates back to 1893 when surgeon William Coley reported tumour regression in patients injected with pyogenic bacterial extracts [20]. Coley hypothesized that the toxins had non-specifically stimulated the immune system, resulting in enhanced immune responses specific for the tumour [20].

The tumour vaccination strategy of the past often included the injection of whole irradiated tumour cells with an adjuvant such as bacillus Calmette-Guerin (BCG). The use of adjuvants mixed with human tumour cells has been reported to be successful in treating melanoma and renal cell carcinoma, resulting in regression of the tumour [20]. In fact, it has been shown that introduction of BCG alone into the bladder of patients suffering from superficial bladder carcinoma improves survival [16]. It is unclear exactly how this treatment works, but it does involve inflammatory responses [16]. The inflammatory response may provide an important milieu for antigen presentation and induction of an immune response [16]. It is also believed that in cases in which an antitumour response is induced in this manner, the immune system and the tumour are in some sort of a balanced state [11]. For such balanced cases, even inefficient strategies to activate the immune system may be able to tip the balance towards immune-mediated tumour eradication [11].

Present day vaccine strategies often involve the transfection of tumour cells with cDNAs encoding costimulatory molecules. Increasing the expression of costimulatory molecules and cytokines on tumours may be a method of overcoming the unresponsiveness of tumour-antigen specific T-cells. A number of laboratories have had success in augmenting solid tumour-specific T-cell activation in a specific and selective fashion by gene transfer to tumour cells. By this approach, the presentation of tumour
antigens has been enhanced by the overexpression of the immunomodulatory cytokines [21-23] or costimulatory molecules [13, 24, 25] or both [26]. In some cases, in addition to enhanced clearance of modified tumour cells, a tumour specific immune response capable of protecting the animal from a second challenge with unmodified tumour cells has also been induced by this strategy [21, 23-26]. Among the cytokines used for this purpose, IL-2 (which induces proliferation of cytotoxic CD8 T-cells), IL-12 (which activates T helper type 1 and cytotoxic CD8 T-cells) and GM-CSF (which stimulates granulocyte and macrophage growth) have been consistently effective in showing reduced tumour growth or elimination by the immune system [27, 28].

Because costimulatory molecules are only required for T-cell activation and not T-cell effector function [10], a vaccine comprised of tumour cells engineered to express costimulatory signals could be used to stimulate the T-cell response resulting in destruction of unmodified tumour cells. Such a vaccine may also engender immunological memory that could protect the patient from recurrence of the disease [2].

**AML Cells Are Well Suited to a Vaccine Strategy**

One therapeutic approach in the treatment of leukemia, bone marrow transplantation (BMT), provides insights which suggests that the immune system may be receptive to modulation by gene transfer into AML. The transfer of bone marrow allows a patient to survive increased doses of chemotherapy and radiation that are lethal to bone marrow. An additional benefit of allogeneic BMT results from the cell-mediated interactions of the donor derived immune cells with residual tumour cells in the host [29].

Two effects are observed following BMT – the graft-versus-leukemia effect (GVL) and graft-versus-host disease (GVHD). GVHD results from donor T-cells
attacking the host tissue. Since GVHD can be lethal, many groups have attempted to reduce the incidence of GVHD, using a variety of techniques such as T-cell depletion. However, patients undergoing T-cells depleted BMT have higher relapse rates than those with the non-depleted BMTs, regardless of the severity of GVHD [29]. Similarly, the risk of leukemia relapse following BMT is inversely proportional to the degree of GVHD experienced [29]. As well, transfusions of lymphocytes from donor marrow have been shown to exert a strong GVL effect in patients with recurrent chronic myeloid leukemia (CML) and relapsed AML [30].

The GVL effect is believed to result from a T-cell reaction against leukemia-specific antigens, or from a GVHD reaction against minor histocompatibility antigens presented by HLA class I and II antigens [30]. A number of minor histocompatibility antigens are known to be expressed predominately on progenitor cells [30], and therefore may be expressed on leukemia cells, which are transformed progenitor cells. The fact that leukemia relapse rates in patients receiving BMTs from identical twins (syngeneic BMT) are significantly higher than in those receiving BMTs from an HLA-identical supports this notion [29].

Taken together, these observations show that an immune response to tumour cells is possible, at least in the allogeneic setting. There is also evidence that a similar immune response may be possible in the autologous setting. Rodents treated with the immunosuppressive drug cyclosporin A (CsA) following syngeneic or autologous BMT develop a disease similar to GVHD [31]. The development of this syngeneic GVHD (SGVHD) is linked with the production of an antitumour response. Mice injected with lymphoma cells following irradiation and syngeneic BMT had 73% long term survival if
treated with CsA compared to 25% without CsA treatment [31]. However, these survivors showed no long-term memory immunity, as all of the mice succumbed to rechallenge [31]. These results show that it is possible to stimulate a syngeneic immune system to respond to tumour cells, although the effector mechanisms responsible for this murine GVHD mediated GVL remain unknown.

The nature of AML cells may also render them well suited to a vaccine. First, for as yet undetermined reasons, myeloid leukemias respond better than do lymphoid leukemias to donor lymphocyte transfusion therapy [30]. Second, AML cells express high levels of MHC I and II: one study demonstrated 100% MHC I expression and 81% MHC II expression on human AML cells [32]. This high expression of MHCs allows the cells to display antigen to T-cells, a necessary component for the activation of T-cells. And third, AML cells may be closely related to, if not the transformed counterparts of APCs, suggesting that AML cells are more likely than other tumour cells to express the components required for stimulation of T-cells. The same study mentioned above showed that the costimulatory molecule B7.2 was detected in 28% of AML cases [32]. AML cells are also likely to express primary adhesion molecules required for initial adhesion: 22% of the human AML cases studied expressed ICAM-1 [32].

**Suicide Gene Systems**

The use of live cells in tumour vaccines appears to be more efficacious than irradiated cells in certain circumstances [5]. Although there have been reports of enhanced tumour immunity using irradiated tumour cells, the use of live cells has been required in some cases [5]. In a murine myeloid leukemia model using cells engineered
to express either B7.1 or B7.2 protective immunity was only induced with injection of live cells and not irradiated cells [13]. Although the reason that live cells are required is not clear, it could be that radiation-induced damage to the antigen-processing machinery of the cells, compromises the cells' ability to present the necessary antigens to the specific T-cells [5].

In situations in which the use of a live vaccine is required to induce protective immunity - possibly the case for the myeloid leukemias - a method of controlling the growth of the vaccine is desirable. The ability to eliminate selectively such cells can be obtained at least partially by introducing a suicide gene into the vaccine.

Two metabolic suicide gene systems have been studied extensively: the Herpes simplex virus 1 thymidine kinase (HSV-1-TK) / Ganciclovir and cytosine deaminase / 5-FC suicide enzyme and drug combinations. The suicide gene systems are based on the principle that an enzyme normally absent in mammalian cells can convert a nontoxic prodrug to a toxic drug. When these transduced mammalian cells are placed into a host, treatment with the prodrug will result in the selective death of the transduced cells due to local toxicity produced by the cells themselves, without the generation of systemic toxicity.

HSV-1-TK has a substrate specificity that is distinct from normal eukaryotic cellular thymidine kinase. Unlike mammalian TK, which specifically phosphorylates thymidine to generate thymidine monophosphate, the HSV-1-TK can produce the monophosphate forms of the antiviral nucleoside analogs acyclovir (ACV) and ganciclovir (GCV). Other cellular kinases further metabolize the monophosphates, ultimately resulting in the generation of a highly toxic form of the prodrug. Cytosine
Deaminase is a bacterial enzyme that deaminates cytosine to form uracil but can also convert the prodrug 5-FC into a toxic metabolite 5-FU. These endproducts lead to the disruption of cellular DNA synthesis and ultimately cell death [33]. One advantage of using the CD system over the HSV-1-TK system is the ability to reserve the prodrugs for the HSV-1-TK for the treatment of possible herpes infections. Ganciclovir and acyclovir are the only effective agents to treat herpes infections while fungal infections can be treated by a number of alternative agents to 5-FC.

**Mechanism of 5-FC Action**

The transfer of the cDNA encoding the suicide enzyme CD to mammalian cells confers a novel chemosensitivity to 5-FC. The function of the CD enzyme is to deaminate cytosine to form uracil, but it is also able to convert the relatively nontoxic prodrug 5-FC into a toxic metabolite 5-FU. 5-FU has been widely used as a chemotherapeutic agent, but is limited by adverse effects associated with systemic delivery. Modified CD-expressing cells results in high local concentrations of 5-FU produced by intratumoural conversion of 5-FC to 5-FU, thus abrogating the multiple adverse effects of systemic administration of 5-FU. Unlike 5-FU, systemic administration of 5-FC is free of complications.
Figure 1. Mechanism of 5-FC and cytosine deaminase action. 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; 5-FUR, 5-florouridine; 5-FdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate; 5-FUMP, 5-fluorouridine 5'-monophosphate; 5-FUDP, 5-fluorouridine 5'-diphosphate; 5-FUTP, 5-fluorouridine 5'-triphosphate [34]

Processing of rRNA and mRNAs is disrupted by the incorporation of 5-FUTP into RNA in place of uridine triphosphate. Thymidilate synthetase is irreversibly inhibited by 5-fluoro-2'-deoxyuridine 5'-monophosphate (5-FdUMP), resulting in impaired DNA biosynthesis. The inhibition of DNA synthesis in CD-expressing cells increases with increasing concentrations of 5-FC [35]. Cell death due to CD/5-FC killing is believed to occur via apoptosis [35]. Consistent with this idea, CD/5-FC killing results in the release of mono- and oligonucleosomes [35, 36] and production of a nucleosomal stepladder pattern of DNA fragmentation [37]
Use of 5-FU as a Chemotherapeutic Agent

5-FU is the standard chemotherapeutic agent for the treatment of colorectal cancer. Colorectal cancers have increased susceptibility to 5-FU, based on the more efficient usage of uracil by these cells compared to normal cells [38]. While a number of other agents and various drug combinations have been examined for use in colorectal carcinoma, none have demonstrated superior response rates to 5-FU [39]. This is in spite of the fact that 5-FU treatments result in only 10% to 20% response rates [39]. Unfortunately, 5-FU causes a large number of side effects including anorexia, vomiting, stomatitis, diarrhea, esophagitis, proctitis, gastrointestinal ulceration and bleeding, leukopenia, thrombocytopenia and alopecia [38]. In fact, the toxic side effects can be the limiting factor in determining the dose of 5-FU [40]. Due to the low therapeutic index of 5-FU, the use of the CD/5-FC is attractive because a high local concentration of 5-FU can be delivered to the tumour site, thereby avoiding the systemic side effects [38]. In contrast to 5-FU, 5-FC is not toxic to humans at therapeutic doses [38]. A phase I study for use of the CD/5FC system in metastatic colon carcinoma of the liver is currently underway [38].

Bystander Effect

The bystander effect generally refers to the prodrug-induced death of tumour cells that are not expressing a suicide gene, but are in close proximity to cells that do express the suicide gene product. This bystander effect has been seen with both CD and TK expressing cells, but it appears to have different mechanisms of action in the two cell types. The bystander effect in the HSV/TK system has been investigated in great detail.
When tumour cells expressing HSV-TK are exposed to GCV \textit{in vitro} and are in contact with unmodified tumour cells, an antitumour effect is seen against the unmodified cells. However, it has been shown that this antitumour effect is not seen if the cells are separated by a 0.4 \textmu m membrane. The bystander effect is not due to diffusion of ganciclovir phosphate generated by thymidine kinase, since ganciclovir phosphate is unable to pass through cell membranes in substantial quantities. Thus, it is believed that this \textit{in vitro} bystander effect is due to the transmission of the toxic nucleotide from dying HSV-TK cells to unmodified tumour cells via either gap junctions or apoptotic vesicles [41].

An \textit{in vitro} bystander effect has also been shown in the CD/5-FC system. However, this bystander effect differs from that of TK/GCV in that it does not require direct cell-to-cell contact [42, 43]. As 5-FU is membrane permeable, it is able to diffuse out of the producing cell [44], and thereby affect neighbouring cells. \textit{In vitro} inhibition of cell growth can be seen when only 10\% of cells express CD [45].

In addition to the bystander effect seen \textit{in vitro}, there appears to exist a different form of bystander effect \textit{in vivo} that is mediated by another mechanism, likely the immune system. It has been shown that an intact immune system is required for an \textit{in vivo} bystander effect with the HSV-TK/GCV system [46, 47]. It has also been noted that the antitumour response to dying HSV-TK modified tumour cells resembles the inflammatory response to microbial pathogens [48]. This involves an initial inflammatory response that leads to an immune response, possibly mediated by the apoptotic death of the engineered tumour cells and the release of soluble factors that would affect the tumour microenvironment. Indeed, tumour necrosis and an increase in
mononuclear and T-cells have been seen after HSV-TK/GCV treatment which appear to coincide with localized production of TNF-α within the tumours [46]. Not only is this in vivo 'bystander effect' responsible for the elimination of unmodified cells during GCV treatment, it may also be responsible for partial or full protection against rechallenge with unmodified cells [47]. This protection is dependent upon the cells being killed in vivo by GCV, as irradiated tumour cells do not offer such protection [47, 49]. The manner of cell death following GCV treatment may lead to different immune effects than when cells are lethally irradiated, possibly as a result of more efficient uptake and presentation of tumour antigens by antigen presenting cells.

**Model of AML**

The C1498 cell line was originally derived from a female C57BL/6 mouse. The cell line is myelomonocytic, expressing a granulocyte marker (gran-1) and two macrophage markers, Mac 1, Mac 3 [22]. In addition, C1498 is MHC class I positive, but class II negative, and expresses ICAM-1, ICAM-2, and LFA-1, but neither B7.1 or B7.2 [22](see Figure 3 for MHC and B7 expression). Boyer et al. [22] showed that C1498 is sensitive to both NK and specific CTL lysis in vitro. In addition, they demonstrated that both of these methods of lysis are dependent upon LFA-1 binding to ICAM-1 and ICAM-2 present on the C1498 cells.

The route by which the leukemia cells are injected (intravenous, intraperitoneal, or subcutaneous) appears to have a great effect upon the immune response, and thus is an important consideration for the AML model. Subcutaneous (SC) or intraperitoneal (IP) cell injection result in antigens being concentrated at a site of professional APC
residence. SC injections would result in the antigens being exposed to Langerhans cells in the skin. Langerhans cells are known to process antigens, migrate to draining lymph nodes, differentiate into dendritic cells which can then present antigen to T-cells. Dendritic cells, present in the skin and peritoneum, are known to be the most potent stimulator of T-cells. Using C1498 cells Boyer et al. showed that SC immunization was superior to IP immunization in inducing immune resistance [22]. In addition, intravenous (IV) cell injection is an immunization route that fails to induce an effective T-cell immune response with C1498 cells [22].

We have chosen to use IP injection in order to increase the interaction of the leukemia cells with APCs. Although SC injection has been shown to be more effective in inducing immune resistance with C1498 cells, with the cell doses we will be studying SC injection may lead to tumour ulceration, necessitating euthanization of the mice. Therefore with our mouse model, using the C1498 cell line injected IP, we plan to examine: the ability to eliminate cells engineered to express CD in vivo by 5-FC, whether protective immunity to challenge with parental cells can be induced by immunization with CD expressing cells lines and 5-FC treatment, and to determine the immunological basis for any protective immunity seen.
Methods

Cell Lines

The C1498 murine myeloid leukemia cell line was derived from a C57BL/6 mouse. C1498 cells express MHC class I but not class II, and express LFA-1, ICAM-1 and ICAM-2 but neither B7.1 or B7.2 [22]. The cell line has been used to characterize murine anti-AML immunity [22]. Cells were grown in tissue culture dishes (Sarstedt) at 37°C in a humidified 5% CO₂ atmosphere using Iscove’s modified Dulbecco’s medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Sigma), and 50 units/ml penicillin-streptomycin (Gibco BRL). C1498 is a spontaneous leukemia.

The MMB3.19 (MMB) murine myeloid leukemia cell line was used as a control during cytotoxic lymphocyte analysis. MMB cells are also derived from a C57BL/6 mouse and express class I and class II MHC molecules as well as LFA-1, B7.1 and B7.2 [4]. The MMB3.19 cell line is a c-myc retrovirus transformed leukemia.

Cell Culture and Gene Transfer

Leukemia cells were infected with the pCD2 retroviral vector obtained from Dr. Craig Mullen (MD Anderson). This virus contains the E. coli CD gene modified for expression in mammalian cells under the transcriptional control of the Moloney murine leukemia virus long terminal repeat, as well as the neomycin phosphotransferase (NeoR) gene driven by the simian virus 40 early region promoter. GP+E-86 cells (helper cells for pCD2) were infected using the superfect transfection method (Gibco BRL) as follows: Cells were seeded 3 x 10⁵ cells per 35 mm tissue culture dish in 4 ml IMDM + 10% FCS, and were incubated overnight at 37°C in a CO₂ incubator. DNA for transfections was
prepared using the Qiagen maxiprep kit (Qiagen). 2μg of DNA in 10μl H₂O were mixed with 85 μl OPTI-MEM I medium (Gibco BRL) and 15μl of Superfect (Gibco BRL). This mixture was incubated at room temperature for 10 minutes then diluted in 0.8 ml DMEM + 10% FCS. The GP+E-86 cells were rinsed in serum free medium, and the DNA solution was added onto the cells. After 3.5 hours of incubation in the 37°C CO₂ incubator, the medium was replaced with 4 ml of DMEM and 10% FCS. Two days later, the cells were subcultured, 400 μg/ml G418 (Gibco BRL) were added, and selection was continued thereafter for two weeks. To collect virus particles for transfection, cells were seeded onto 100 mm dishes and grown to subconfluence. The medium was removed from the cells and fresh DMEM + 10% FCS was added. After O/N incubation in the 37°C CO₂ incubator the supernatant was removed and filtered.

Cells to be infected (C1498 and MMB) were seeded at 2 x 10⁵ cells/ml in 100 mm dish and incubated overnight in a 37°C CO₂ incubator. They were then resuspended in Iscove’s + 10% FCS + 8 μg/ml polybrene at 2 x 10⁶ cells/ml. 1ml of cells were mixed with 2ml of virus supernatant and seeded into 100mm dishes. After incubation for 3.5 hours in the 37°C CO₂ incubator, 20 ml Iscove’s +10% FCS were added to the cells. Two days later, the cells were subcultured, G418 (Gibco BRL) was added (1mg/ml for C1498 cells and 0.5mg/ml for MMB cells), and selection was continued thereafter for two weeks. Individual clones were selected by limiting dilution in methylcellulose. The selected clones were subjected to FACS analysis to characterize cell surface markers.

To construct a control vector that did not express CD, the pCD2 vector was digested with EcoR1 and Bam H1 (NEB) to excise the CD gene. The DNA ends were blunted using the Klenow fragment of E. coli DNA polymerase I (NEB) and the blunt
ends were ligated using T4 DNA ligase (NEB). Cells were infected with this control virus using the lipofectin transfection method and selected as explained above.

**RNA Extraction**

Total RNA was isolated from log-phase cells using TRIzol reagent (GIBCO, Life Technologies), a modification of the single-step isolation developed by Chomczynski and Sacchi [50], according to the manufacturers protocol. The final RNA pellet was dissolved with DEPC-treated water (diethylpyrocarbonate, SIGMA). All RNA preparations were subjected to a DNAse digestion step prior to cDNA synthesis to remove any remaining genomic DNA. For each µg of RNA, 3 units of RQ1 RNase-free DNAse (Promega) and 40 units of RNase inhibitor (RNAguard, Amersham Pharmacia Biotech) were incubated in a buffer containing 10mM Tris-HCl, pH8.3, 50mM KCl and 1.5mM MgCl₂ in a 50 µl reaction volume at 37°C for 2-5 minutes. The RNA was then purified using an equal volume chloroform extraction. RNA, contained in the aqueous layer, was precipitated at -80°C by adding 0.1 volume of 3M sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol along with 40 units of RNase inhibitor. Finally, the RNA pellet was resuspended in RNase-free water.

**cDNA synthesis**

cDNA synthesis on 1 µg total RNA was carried out using 40 ng of random hexamers (Amerham Pharmacia Biotech) and mouse-Moloney leukaemia virus reverse transcriptase (Superscript-RT, GIBCO, Life Technologies) in a reaction volume of 20µl under conditions recommend by the manufacturer with the exception of the following modifications: 40 units of RNase inhibitor and DTT to 0.1M were added to the RNA
priming step. Reactions were terminated by heat inactivation of the RT at 95° for 5 minutes.

**PCR reactions**

PCR reactions were carried out in 50 μl reactions under standard Perkin Elmer-Cetus (Norwalk, Conn.) conditions; 50mM KCl, 10mM Tris-HCl pH9.0, 0.1% TritonX-100, 0.2mM dNTPs (GIBCO, Life Technologies), 50 pmol of each primer {CD-1: TTGCTGGAAGAGGCG'TACG, CD-2: CGACGTTTGGATACGTATCG, CD expected product size: 376bp) (mHPRT-1: GCTGGTGAAAAAGAACCTCT, mHPRT-2: CACAGGACTAGAAACACCTGC, HPRT expected product size: 249bp)}, 1.25 U Taq Polymerase (GIBCO, Life Technologies) and 1μl cDNA. The PCR regimen of 94°C denaturation for 45 seconds, 56°C primer annealing for 45 seconds and 72°C extension for 45 seconds was run for 35 cycles proceed by a 5 minute 94°C hot start and followed by an 8 minute extension using a Perkin Elmer-Cetus model 480 thermocycler. 10μl of each product were size-separated electrophoretically on a 1.7% agarose gel containing 0.5μg/ml ethidium bromide in 1x TAE buffer (40mM Tris-acetate, 1 mM EDTA pH8.3). Controls included the cDNA reaction mix with no reverse transcriptase added to check for genomic contamination and a no template PCR control to check for PCR artifacts and contamination.

**The MTT Assay**

The MTT assay is a simple colorimetric assay for the measurement of cell proliferation. The test is based on the enzymatic reduction of the tetrazolium salt MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetra-zoliumbromide] by living, but not by
dead cells [51]. The reactions can be carried out in 96 well plates and the reaction product, a purple coloured formazan crystal, is soluble in dimethylsulfoxide (DMSO). The dissolved product is measured colorimetrically in a multiwell plate reader [52]. This assay has previously been used successfully to determine chemosensitivity in leukemia cells [53, 54].

**Measurement of Growth In Vitro**

Cells were seeded in triplicate in a 100µl volume per well in 96 well plates at 5 x 10⁴ cells/ml for C1498 cells and 10⁴ cells/ml for MMB cells. One plate each was incubated for 1 day, 2 days, 3 days, 4 days and 5 days at 37°C in a humidified atmosphere containing 5% CO₂. At the end of the incubation period, 20µl of a 5mg/ml solution of MTT (Sigma) in PBS were added to each well. The plate was then incubated for four hours at 37°C in a humidified atmosphere containing 5% CO₂. The supernatant was removed from each well and the purple crystals were dissolved in 100µl DMSO (Caledon). The absorbances at 570nm were read in a Thermomax microplate reader (Molecular Devices). The absorbances were corrected by subtracting the mean of the background absorbance of wells that had only contained media from the mean of the absorbance for each condition.

**In Vitro Sensitivity to 5-FC**

Leukemia cells were plated into 96 well plates (5 x 10³ cells per 100 µl for MMB cells, 1 x 10³ cells per 100 µl for C1498 cells). Various concentrations of 5-FC (ICN Canada) dissolved in PBS were added in 100 µl to each well to produce a range of final 5-
FC concentrations from 100-1000 μg/ml of 5-FC. The plates were incubated for 4 days at 37°C in a humidified atmosphere containing 5% CO₂, and survival to 5-FC was determined using the MTT assay. The mean of the corrected absorbances of the replicate wells exposed to 5-FC was divided by the mean of the wells that had received no 5-FC to calculate the percentage cell survival.

**In Vivo Sensitivity to 5-FC**

Healthy 6-8 week old female C57BL/6 mice were injected IP with varying doses of either C1498-parental (C1498-P) or C1498-CD cells, and survival was followed. Additional mice were then injected IP with 10⁷ cells and thereafter received 5-FC beginning one week after cell injection. A one week delay was chosen to allow the cells time to stimulate the immune system before their elimination by 5-FC. Using a mouse model of adenocarcinoma, Consalvo et al. [55] determined that beginning 5-FC treatment seven days post challenge resulted in the greatest tumour regression of CD-expressing cells. Mice received IP injections of 3ml of 5-FC (dissolved 12.5mg/ml in phosphate-buffered saline) twice daily for 10 days. Survival was followed.

**Antibodies for FACS**

Four antibodies were used to characterize the murine tumour cells by FACS: antimouse CD45R (B7.1, Pharmingen #01121D); antimouse GLI (B7.2) antigen, purified from the HB-253 cell line (ATCC, Rockville, Maryland), grown in RPMI + 10% FCS; antibody reactive to H2 (MHC I), purified from the TIB 126 cell line (ATCC, Rockville, Maryland), grown in DMEM + 10% FCS; and antibody reactive with Ia antigen, I-Ab, d,
q and I-Ed, k (MHC II), purified from the TIB-120 cell line (ATCC, Rockville, Maryland), grown in DMEM + 10% FCS.

Plates of monoclonal antibody producing cells were grown to over-confluence and the supernatant was separated from the cells. Protein G Sepharose 4 Fast Flow (Pharmacia Biotech) was packed into columns and used to purify the antibodies from the supernatant as described in instructions provided by Pharmacia Biotech[56].

**FACS Analysis**

Cells to be analyzed were harvested, spun down at 1000 rpm, and then resuspended at 10⁶ cells/100µl in 1% FCS in PBS. Primary antibody (1µg of anti-B7.1, anti-B7.2, anti-MHC-I or anti-MHC-II) was added to the cells, and the cells were kept on ice for 30 minutes. The cells were then washed twice by adding 500µl of 1% FCS in PBS and spinning down at 1000 rpm at 4°C. The cells were resuspended in 100µl 1% FCS in PBS and the secondary antibody (1µg of goat-anti-mouse FITC) was added. The cells were incubated in the dark at 4°C for 30 minutes. The cells were then washed twice as before. Finally, the cells were resuspended in 300µl 1% FCS in PBS and were analyzed in a FACScan machine (Becton Dickenson).

**In Vivo Antibody Blocking**

For selective immunosuppression, female 6-8 week old C57/Bl6 mice (Charles River) received six IP injections of 0.2 ml PBS containing 100µg of anti-CD4 (TIB207 hybridoma, clone GK1.5, anti-L3T4, American Type Tissue Collection [ATCC]), anti-CD8 (TIB210 hybridoma, anti-Ly-2.2 of CD8α, ATCC), or anti-NK1 (HB191
hybridoma, clone PK136, ATCC) on the day preceding and 4hrs, 3 days, 7 days, 10 days and 14 days after challenge with leukemia cells.

HB191, TIB 207 and TIB 210 cells were grown in RPMI (Gibco) + 15% FCS, Iscove’s Modified Dulbecco’s Medium (IMDM)+ 20% FCS and IMDM + 10% FCS respectively. Antibodies were purified as described in Antibodies for FACS below and were then quantified using the Bio-Rad protein bioassay (Bio-Rad Laboratories).

As a control for the in vivo antibody blocking of CD4+ and CD8+ cells, antibodies that bound to these molecules via a different epitope were used to demonstrate depletion by FACS analysis of splenocytes from the blocked animals and control unblocked animals. Mice were sacrificed the day after the last injection of blocking antibodies and the splenocytes were obtained for FACS analysis. The anti-CD4 blocking with TIB-207 (LY 2-2) was demonstrated with a rat monoclonal antibody to mouse clone RM4-4 (Pharmingen), and the anti-CD8 blocking with TIB-210 (anti-Ly-2.2 of CD8a) was demonstrated with a rat monoclonal antibody to mouse CD8b (LY3.2) (Caltag). Depletion of mouse NK cells by PK136 had already been shown to be effective at the doses used by other investigators, with greater than 95% abrogation of NK cell lysis of the NK sensitive target YAC [22].

**JAM CTL Assay**

The JAM assay is a method of determining the cytolyis of cells based on the measurement of the DNA retained by living cells as opposed to the measurement of the components lost by the dying cells [57]. The DNA of the target cells is labeled by tritiated thymidine uptake. Dying cells degrade their DNA into small segments and these
DNA fragments can pass through the filters onto which the cells are collected for analysis. The DNA of the non-degraded cells remains intact and is captured onto the filters. The level of radioactivity on the filters is measured and the percent lysis of the cells is determined by the equation: (labeled cells – target cells)/ labeled cells.

Specifically, mice were sacrificed and their spleens were obtained. The spleens were crushed into a single cell suspension, using a mortal and pestle, which was then rinsed twice in 10 ml IMDM + 10% FCS. After centrifugation, the pellet was resuspended in red blood cell lysis buffer (3M Tris-NH₄Cl pH 7.2) and incubated at 37°C for three minutes. 10ml of IMDM + 10% FCS were added and the non-lysed cells were pelleted, and then resuspended in IMDM + 10% FCS. Cells were counted and resuspended to a concentration of 5 x 10⁶ cells/ml. In the meantime, stimulator cells (either MMB or C1498 cells) at a concentration of 10⁶ cells/ml rads in 15ml tubes (Falcon) were irradiated at 10,000 rads in a Gammacell 1000 Elite cell irradiator (MDS Nordion). The irradiated stimulator cells were resuspended in IMDM + 10% FCS to a concentration of 2.5 x 10⁵ cells/ml.

Cocultures of stimulator cells and effector cells were set up by adding 1ml of spleen cells and 1ml of a chosen stimulator cells (both in IMDM + 10% FCS) into a well of a 24 well plate. The ratio of spleen cells to stimulator cells ratio was 20:1 for C1498 cells and. These effector to simulator cell ratios had previously been determined by others to be optimal [22, 58]. The cocultures were incubated for 5 days at 37°C in a humidified atmosphere containing 5% CO₂.

Target cells were seeded onto 24 well plates at a concentration of 2.5-5 x 10⁵ cells/ml on the fourth day of the coculture and incubated overnight at 37°C in a
humidified atmosphere containing 5% CO₂. The target cells were then labeled by adding 5μl of 1mCi/ml [methyl-³H] thymidine (Amersham Pharmacia Biotech) to each ml of target cells. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 4 hours, and then pelleted and resuspended in an amount of IMDM + 10% FCS that would dilute the cells 5 times, resulting in a concentration of approximately 10⁵ cells/ml.

The cocultured spleen cells were collected, pelleted and concentrated by resuspension into a volume of IMDM + 10% FCS that was 1/5 of the volume in which they had been cocultured. 150μl of the spleen cells were added to each well in rows A or E of a 96 well plate. 100μl of IMDM + 10% FCS were added to all the other wells in the plate. A 3 fold serial dilution of 4 rows of wells was then achieved using a multichannel pipetor. 50μl of each of the wells containing the spleen cells were resuspended several times with the wells in the row below, then 50μl of the wells in this row were diluted in the row below, and again with the last row. Finally, the extra 50μl in the last row were discarded. 100μl of the labeled target cells were then added to three columns of wells, resulting in triplicates of each different effector to target ratio (100:1, 33:1, 11:1 and 3:1). These 96 well plates were incubated for 4 hours at 37°C in a humidified atmosphere containing 5% CO₂. After the 4 hour incubation, cells were harvested onto Packard Ultrafilter GF/C filter plates using a Filtermate 196 cell harvester (Packard). Filter plates were dried at 40°C for 40 minutes and the bottoms of the plates were sealed with adhesive stickers (Packard). 25ml Microscint scintillation fluid (Packard) were added to each filter well and the top of the filter plates were sealed using Topseal-S Microplate
Heat Sealing Film (Packard) in a Micromate 496 Plate Sealer (Packard). Finally, filter bound <sup>3</sup>H was determined in a Topcount Microplate Scintillation Counter (Packard).

**Rechallenges**

Mice that had survived IP challenge with C1498-CD cells for 90 days were rechallenged IP with $10^6$ C1498-parental cells in 200μl PBS. Survival was followed.

**Mixing Experiments**

Female 6-8 week old C57/B16 mice (Charles River) were injected IP with a mix of parental and CD-expressing cells at three mixing doses, 10:1, 1:1, and 1:10 CD:P cells in 400μl PBS. Mice were injected IP with $10^7$ CD-expressing cells and $10^6$ parental cells, or with $10^6$ CD-expressing cells and $10^6$ parental cells, or with $10^5$ CD-expressing cells and $10^6$ parental cells. Mice injected with some of the mixtures (10:1 and 1:1 for C1498 cells and 10:1 for MMB cells) were also treated with 5-FC as described above in *In Vivo* Sensitivity to 5-FC. Survival was followed.

**Nude Mice**

Female 6-8 week old nu/nu nude mice (Charles River) were injected IP with $10^6$ C1498-CD or C1498-parental cells in 200μl PBS. Survival was followed.
Results

Construction of CD-expressing murine AML cells

A murine AML cell line, C1498, was infected with a retrovirus expressing CD, and single cell clones were selected. The parental cell line will be referred to as C1498-P and the CD-expressing cell line will be referred to as C1498-CD. The AML cell line was also infected with a retrovirus identical to the one above, except that the DNA encoding the CD gene had been excised, to produce a control cell line. This control cell line will be referred to as C1498-neo in that it expresses this selection marker, as does the CD-expressing cell line.

The parental, control and CD-expressing cells demonstrated similar growth kinetics in vitro (Figure 2) and similar expression of cell surface determinants (Figure 3). Expression of CD mRNA by C1498-CD cells was demonstrated (Figure 4).

Sensitivity to 5-FC In Vitro

Survival of the cells was plotted following exposure to 5-FC for four days (Figure 5). Both cell lines displayed inhibition of cell proliferation dependent on 5-FC concentration. However, the cells expressing CD were much more strongly inhibited. Approximately 82% of C1498-CD cells were killed at a concentration of 0.1 mg/ml 5-FC while only 5% of C1498-parental cells were killed at this same concentration. At the highest 5-FC concentration studied, 1mg/ml, 95% of C1498-CD cells were killed while 19% of C1498-P cells survived.
Figure 2. Growth *in vitro* of the three C1498 leukemia cell lines. Growth over 5 days of C1498-P (circles), C1498-CD (squares) and the control leukemia cell that expressed neomycin but not cytosine deaminase (C1498-neo) (triangles) as determined by MTT assay is shown. Error bars represent one standard deviation and may be hidden behind the data point symbols if the standard deviation is small. None of the curves are significantly different from each other ($P > 0.05$).
Figure 3. Results of FACS analysis for cell surface determinants of C1498 cells. Shown are plots of relative cell count versus log FITC fluorescence. FACSs of unstained cells and cells exposed to the secondary FITC antibody only are shown as controls (the same secondary FITC antibody was used with all four detection antibodies). FACSs of cells incubated with detection antibodies for MHC I, MHC II, B7.1 and B7.2 and then the detection antibody are shown. All three cell types express MHC I but not MHC II, B7.1 or B7.2. Values of the mean flourescences for each FACS are shown.
Figure 4. Expression of CD mRNA by C1498-P, C1498-CD and C1498-neo cells. C1498-P cells are shown in lanes 2-5, C1498-CD cells are shown in lanes 7-10 and C1498-neo cells are shown in lanes 12-15. Lanes 6 and 11 are unloaded and lanes 1 and 16 show a 1Kb ladder. The extracted mRNA underwent PCR with no RT with mHPRT primers (lanes 2, 7 and 12) with RT and with mHPRT primers (lanes 3, 8 and 13), with no RT and with CD primers (lanes 4, 9 and 14) and with RT and with CD primers (lanes 5, 10 and 15). While all cells contain similar amounts of HPRT-specific RNA, only C1498-CD cells, as expected contain demonstrable CD-specific transcripts.
Figure 5. Survival of C1498 cells after 4 days exposure to 5-FC in vitro. Survival of C1498-P cells (circles) and C1498-CD cells (squares) following exposure to concentrations of 5-FC ranging from 0.1mg/ml to 1mg/ml and as determined by MTT assay is shown. Both cell lines were derived from single cell clones. Survival at each dose of 5-FC was assayed in quadruplicate. The % survival was calculated by dividing the mean of the absorbances of the replicate wells exposed to 5-FC by the mean of wells that had received no 5-FC.
**In Vivo Dose Survival Studies**

Mice were injected IP with varying doses of the murine AML cells, and survival was followed (Figures 6). (Mice were not treated with 5-FC in these experiments). In general, mice receiving larger doses of the murine AML cells died sooner than those receiving smaller doses, and survival was enhanced in mice receiving injections of CD-expressing cells rather than parental cells. A significant enhancement of survival of mice injected with murine AML cells expressing CD was seen at all cell doses with the C1498 cells (Figure 6). Median survivals increased from ~22 days to ~49 days at a dose of 10^7 cells, from ~27 days to 43 days at a dose of 5x10^6, from ~32 days to 49 days at a dose of 10^6 and from 31 days to 57 days at a dose of 5x10^5. Additionally, although no mice injected with any of the above doses of C1498-P cells survived past 41 days, a proportion of mice receiving any of these doses of C1498-CD cells survived to 90 days. The percentage of mice surviving to 90 days ranged from approximately 18% at a dose of 5x10^5 C1498-CD cells to 40% at a dose of 10^7 cells.

The survival of mice injected with C1498-neo cells (Figure 7) was not significantly different than that of animals receiving C1498-P cells (a median of survival of 28 days compared to ~22 days) at the 10^7 cell dose. Similarly, mice injected with 10^6 C1498-neo cells demonstrated survival similar to that of mice injected with this same dose of C1498-P cells with a median of survival of ~31 days compared to ~32 days.

**Sensitivity to 5-FC In Vivo**

Based on these initial studies, a dose of 10^7 cells was chosen for subsequent experiments so that potential enhanced survival with 5-FC treatment could be easily
Figure 6. Kaplan-Meier analysis of the survival of mice injected IP with varying doses of C1498-P and C1498-CD cells. Mice injected with either C1498-P or C1498-CD cells do not show any significant differences ($P > 0.05$) among cell doses. However, there is a significant difference ($P < 0.05$) between the survival of mice injected with the same dose of C1498-P cells compared to that of C1498-CD cells.
Figure 7. Kaplan-Meier analysis of the survival of mice injected IP with either C1498-P or the control leukemia cell that expressed neomycin but not cytosine deaminase (C1498-neo). The C1498-neo $10^6$ and $10^7$ curves are not significantly different from their C1498-P $10^6$ and $10^7$ counterparts ($P > 0.05$).
observed. Median survivals at a dose of $10^7$ cells were: \(\sim 22\) days for C1498-P and \(\sim 49\) days for C1498-CD.

Mice were then injected IP with $10^7$ C1498-P or C1498-CD cells. After seven days, these mice received IP injections of the prodrug 5-FC (3 ml of a 12.5mg/ml solution) twice daily for ten days. Mice injected with the CD-expressing cells demonstrated lengthened survival when treated with 5-FC (Figure 8). Mouse survival was enhanced by 5-FC treatment from a median survival of \(\sim 49\) days (with 40% of mice surviving to 90 days) to approximately 64% of mice surviving to 90 days. In contrast, treatment with 5-FC did not increase survival of mice injected with parental cells significantly. Mice injected with $10^7$ C1498-P demonstrated median of survival of \(\sim 22\) days that increased to \(\sim 25\) days with PBS treatment (Figure 8).

Mice injected with CD-expressing cells but treated with PBS showed a decreased rate of survival compared to mice injected with the same dose but not treated. Survival decreased from 40% surviving to day 90 to all mice dying by day 39 (Figure 8).

**Mice Surviving CD Challenge are Rechallenged with Parental Cells**

Mice that had been injected with C1498-CD cells, and which had survived to day 90, were rechallenged with $10^6$ C1498-P cells (Figure 9). The majority of mice (about 86%) which had survived injection of $10^7$ C1498-CD with 5-FC treatment survived to 90 days post parental cell rechallenge. In comparison, only 25% of mice that had survived this same initial challenge without having received 5-FC survived to 90 days post parental cell rechallenge. However, all of the mice that had survived an initial challenge
Figure 8. Kaplan-Meier analysis of the survival of mice injected with $10^7$ C1498 cells with and without 5-FC or PBS treatment. Mice were injected IP with $10^7$ C1498 cells (either parental or CD expressing). One week later a selection of each mouse group received 5-FC (IP injection of 37.5 mg of 5-FC twice a day for 10 days) or PBS treatment. There is no significant difference between the two C1498-P curves (5-FC treatment or not). However, a significant difference ($P < 0.05$) is seen among all other curves.
Figure 9. Kaplan-Meier analysis of the survival of mice that had survived 90 days post challenge with C1498-CD cells to rechallenge with $10^6$ C1498-P cells. Survivors of challenge with $10^7$ C1498-CD cells with (white circle) or without (grey circle) 5-FC treatment, with $5 \times 10^6$ C1498-CD cells (square) or with $10^6$ C1498-CD (triangle) cells were injected IP with $10^6$ C1498-P cells and survival was followed. The survival of naïve mice injected with the rechallenge dose is shown for comparison (diamond). The naïve mouse curve is statistically different from the C1498-CD $10^7$ with 5-FC curve, as well as the C1498-CD $5 \times 10^6$ curve ($P < 0.05$).
of $5 \times 10^6$ C1498-CD or $10^6$ C1498-CD cells (without 5-FC treatment) (Figure 9) survived the rechallenge with $10^6$ C1498-P cells.

**Cell Mixtures**

Mice were injected with mixtures of CD-expressing cells and parental cells (the stable dose being $10^6$ parental cells) in ratios of 10:1, 1:1 and 1:10 (Figures 10 and 11).

While mice receiving an injection of $10^6$ C1498-P cells alone die by day 42, 45% of the mice receiving a mixture of $10^7$ C1498-CD cells and $10^6$ C1498-P cells were alive at day 90 (Figure 10). This level of survival is similar to mice receiving $10^7$ C1498-CD cells alone (survival 40% at day 90). However, when mice injected with this same mixture were also treated with 5-FC survival mirrored that of mice injected with $10^6$ C1498-P, with a median survival of 30 days and none surviving past 90 days.

Mice receiving a 1:1 dose of $10^6$ C1498-CD and $10^6$ C1498-P or a 1:10 dose of $10^5$ C1498-CD and $10^6$ C1498-P showed the same survival rate as mice injected with $10^6$ C1498-P (Figure 11). However, 12.5% of mice injected with the 1:1 dose survived to 90 days similar to the percent survival of mice injected with $10^6$ C1498-CD of 18%. Adding 5-FC treatment to the 1:1 dose did not improve survival, but rather resulted in the death of all mice by day 34.

**Antibody Blocking In Vivo**

Mice injected with the murine AML cells expressing CD were also injected with monoclonal antibodies directed against CD8+ T-cells, CD4+ T-cells, or NK cells, in order to elucidate which arm of the immune system may be responsible for the increased survival of mice injected with CD-expressing cells compared to parental cells.
Figure 10. Kaplan-Meier analysis of the survival of mice injected with a 10:1 mixture of C1498-CD and C1498-P cells. Survival of mice injected with $10^7$ C1498-CD and $10^6$ C1498-P cells and treated (down triangles) or not treated with 5-FC (up triangles) is shown. Survival of mice injected with $10^6$ C1498-P cells (circles) or $10^7$ C1498-CD cells (squares) is shown for comparison. The C1498-P and C1498-CD control curves are significantly different ($P < 0.05$). The mixture without 5-FC is significantly different from the C1498-P control but not the C1498-CD control ($P > 0.05$). The mixture with 5-FC treatment is significantly different from the C1498-CD control but not from the C1498-P control. The mixture without 5-FC is significantly different from the mixture with 5-FC ($P < 0.05$).
Figure 11. Kaplan-Meier analysis of the survival of mice injected with a 1:1 or 1:10 mixture of C1498-CD and C1498-P cells. Survival of mice injected with 10^6 C1498-CD and 10^6 C1498-P cells and treated (diamonds) or not treated with 5-FC (up triangles) or injected with 10^5 C1498-CD and 10^6 C1498-P cells (down triangles) is shown. Survival of mice injected with 10^6 C1498-P cells (circles) or 10^6 C1498-CD cells (squares) is shown for comparison. The C1498-P and C1498-CD control curves are significantly different from each other (P<0.05). None of the other curves are significantly different from the C1498-P curve.
Mice injected with $10^6$ C1498-CD cells demonstrated decreased survival when injected with anti-CD4 antibodies (median survival shifted to 35 days from 43 days). An even greater decreased survival was seen when mice were injected with antibodies directed against CD8+ T-cells (median survival shifted to 23 days from 43 days, Figure 12). Conversely, mice injected with antibodies blocking NK cells showed increased 90 day survival from 9% to 43%.

FACS analysis was used to demonstrate that the blocking antibodies used did indeed deplete the CD4+ or CD8+ cells (Figures 13 and 14). The dosage of the specific NK blocking antibody used had been previously shown to deplete NK cells[22].

Nude Mice

Nude (athymic) mice were injected with $10^6$ murine AML cells. Although the median survival of nude mice injected with C1498-CD or C1498-P was similar (42 days and 44 days respectively), while all mice injected with C1498-P died by day 52, 25% of the mice injected with C1498-CD survived to day 90 (Figure 15).

Cytotoxic Lymphocyte Assay

Initial cytotoxic lymphocyte assays were performed using CTLs generated from the spleens of mice that had survived challenge with C1498-CD cells ($10^7$, $5 \times 10^6$ or $10^6$ cells), as well as rechallenge with C1498-P cells. The splenocytes were harvested at approximately 100 to 120 days post C1498-P rechallenge. The splenocytes were cocultured for five days with C1498-P cells and the ability of these mice to lyse C1498-P cells was assessed. Splenocytes from all of the survivors could lyse C1498-P cells, while those of naïve mice could only show a low level of lysis (16%) at the 100:1 E:T ratio, and
Figure 12. Kaplan-Meier analysis of the effect of antibody blocking on the survival of mice injected with C1498-CD cells. Survival of mice injected IP with $10^6$ C1498-CD cells alone (circles), or in conjunction with blocking antibodies; anti-NK antibodies (squares), anti-CD4 antibodies (triangles) or anti-CD8 antibodies (diamonds). The anti-CD8 curve is significantly different from the control C1498-CD curve ($P< 0.05$) while the anti-CD4 and anti-NK antibody blocking curves are not significantly different from the control C1498-CD curve ($P> 0.05$).
Figure 13. FACS analysis to demonstrate efficiency of antibody blocking of CD4⁺ cells. Shown are plots of relative cell counts versus log FITC fluorescence. Figure A, B and C are FACSs of the spleen of a PBS injected mouse. A shows the FACS of the control spleen cells without incubation with the detection antibody (negative control for PBS injected mice), B the same spleen cells after incubation with the detection antibody (rat monoclonal antibody to mouse clone W). Figure C shows the same naïve spleen cells after incubation with the antibody that will be used to block CD4⁺ cells (TIB207) and then incubation with the detection antibody, demonstrating that the blocking antibody does not impede binding of the detection antibody. Figure D shows the FACS of spleen cells of mice that have been injected with the blocking antibody (negative control, detection antibody not used), and figure E shows these spleen cells after incubation with the detection antibodies. By comparing figure E to figure B we see that the CD4⁺ cells are indeed blocked. Values of the means fluorescences for the MI gate of each FACS is shown.
Figure 14. FACS analysis to demonstrate efficiency of antibody blocking of CD8^+ cells. Shown are plots of relative cell counts versus log FITC fluorescence. Figure A, B and C are FACSs of the spleen of a control PBS injected mouse. A shows the FACS of the control spleen cells without incubation with the detection antibody (negative control for PBS injected mice), B the same spleen cells after incubation with the detection antibody (rat monoclonal antibody to mouse CD8b). Figure C shows the same naive spleen cells after incubation with the antibody that will be used to block CD8^+ cells (TIB210) and then incubation with the detection antibody, demonstrating that the blocking antibody does not impede binding of the detection antibody. Figure D shows the FACS of spleen cells of mice that have been injected with the blocking antibody (negative control, detection antibody not used), and figure E shows these spleen cells after incubation with the detection antibodies. By comparing figure E to figure B we see that the CD8^+ cells are indeed blocked. Values of the means fluorescences for the MI gate of each FACS is shown.
Figure 15. Kaplan-Meier analysis of the survival of nude mice injected with C1498-P or C1498-CD cells. Mice were injected IP with $10^6$ C1498-P (black circles) or $10^6$ C1498-CD cells (white circles). The two curves are not significantly different ($P > 0.05$).
no lysis at the other E:T ratios (Figure 16). Overall, the levels of lysis were highest in the 5x10^6 C1498-CD challenge survivors.

Cytotoxic lymphocyte assays were then performed using CTLs generated from the spleens of mice that had been injected with 10^7 C1498-CD cells with and without 5-FC treatment. Mice were injected with cells on day 0, 5-FC treatment occurred from day 7 to day 17 and splenocytes were harvested for CTL assays on day 20. After coculture (with either C1498-CD or C1498-P cells), cytolysis of C1498-CD, C1498-P, and the control MMB-CD and MMB-P cells was determined. The numbers shown for the mice that had been injected with C1498 cells are the averages of CTLs from two to four mice. This pooling of results from separate mice has contributed to the high standard deviation seen. All cytolysis percentages listed in the following results will refer to the 100:1 effector to target ratio.

All mice demonstrated high levels of lysis of C1498-P (ranging from 33% to 48% lysis), regardless of whether the mice had been treated with 5-FC or not, or which cells (-CD or -P) had been used in the CTL coculture step (Figures 17-20). A high level of C1498-CD lysis was also seen in all groups (ranging from 29% to 45%). Lysis of MMB-CD cells by splenocytes from non treated mice was absent when C1498-P coculture was used, and increased to an average of 24% for mice when the coculture step used C1498-CD cells. With mice that had undergone 5-FC treatment, lysis of MMB-CD was low using splenocytes (9%) that had been cocultured with C1498-P but quite high for those cocultured with C1498-CD (32%). The level of lysis of MMB-P was low for all conditions (ranging from 0 to 5%).
Figure 16. Cytolysis of C1498-P cells by splenocytes of mice that had survived challenge with C1498-CD cells and rechallenge with C1498-P cells. Spleens from mice that had survived challenge with C1498-CD (10^7, 5x10^6 or 10^6 cells), as well as rechallenge with 10^6 C1498 P cells were cocultured with C1498-P cells. Lysis of C1498-P cells by a 10^7 C1498-CD survivor (circles), 5x10^6 C1498-CD survivors (squares), 10^6 C1498-CD survivor (triangles) and naïve mice (diamonds) are shown. Average and one standard error of the mean are plotted. If possible, data was pooled from the splenocytes from identically treated mice (n = number of mice) and each E:T ratio was assayed in triplicate per mouse spleen.
Figure 17. Cytolysis of C1498-P cells by splenocytes from mice immunized with C1498-CD cells. Lysis of C1498-P cells by splenocytes of mice that received no 5-FC treatment is shown, after coculture with either C1498-P cells (circles) or C1498-CD (diamonds). Lysis of C1498-P cells by splenocytes of mice that received 5-FC treatment is also shown, after coculture with either C1498-P cells (squares) or C1498-CD cells (triangles). Average and one standard error of the mean are plotted. Data was pooled from the splenocytes from identically treated mice (n = number of mice) and each E:T ratio was assayed in triplicate per mouse spleen.
Figure 18. Cytolysis of C1498-CD cells by splenocytes from mice immunized with C1498-CD cells. Lysis of C1498-CD cells by splenocytes of mice that received no 5-FC treatment is shown, after coculture with either C1498-P cells (circles) or C1498-CD (diamonds). Lysis of C1498-CD cells by splenocytes of mice that received 5-FC treatment is also shown, after coculture with either C1498-P cells (squares) or C1498-CD cells (triangles). Average and one standard error of the mean are plotted. Data was pooled from the splenocytes from identically treated mice (n = number of mice) and each E:T ratio was assayed in triplicate per mouse spleen.
Figure 19. Cytolysis of MMB-P cells by splenocytes from mice immunized with C1498-CD cells. Lysis of MMB-P cells by splenocytes of mice that received no 5-FC treatment is shown, after coculture with either C1498-P cells (circles) or C1498-CD (diamonds). Lysis of MMB-P cells by splenocytes of mice that received 5-FC treatment is also shown, after coculture with either C1498-P cells (squares) C1498-CD cells (triangles). Average and one standard error of the mean are plotted. Data was pooled from the splenocytes from identically treated mice (n = number of mice) and each E:T ratio was assayed in triplicate per mouse spleen.
Figure 20. Cytolysis of MMB-CD cells by splenocytes from mice immunized with C1498-CD cells. Lysis of MMB-CD cells by splenocytes of mice that received no 5-FC treatment is shown, after coculture with either C1498-P cells (circles) or C1498-CD (diamonds). Lysis of MMB-CD cells by splenocytes of mice that received 5-FC treatment is also shown, after coculture with either C1498-P cells (squares) C1498-CD cells (triangles). Average and one standard error of the mean are plotted. Data was pooled from the splenocytes from identically treated mice (n = number of mice) and each E:T ratio was assayed in triplicate per mouse spleen.
Discussion

Human AML is a markedly heterogeneous disease due to the fact that it arises from the transformation of hematopoietic progenitor cells that retain the potential for varying degrees of differentiation. This heterogenicity is evident from the variable expression of molecules on the surface of AML cells. According to one study examining the expression of MHC and costimulatory molecules in human leukemias, while all AML are positive for MHC class I antigen, only 80% are positive for MHC II [32]. The expression of the B7 class of costimulatory molecules is even lower among the samples studied, with 2% expressing B7.1 and 28% expressing B7.2 [32].

The AML cell line we have used for these experiments, the C1498 cell line, expresses MHC I but not MHC II nor B7.1 or B7.2, and is a good murine AML model to use in initial in vivo vaccine experiments in that it is fairly representative of human AML. We believe that these preliminary experiments will give us insights into the applications of CD in the AML setting. Hopefully these studies will facilitate the use of CD-expressing vaccines in humans by illuminating the potential benefits, and possible pitfalls of this treatment option.

The goals of this research were to develop and optimize in a murine system a suicide gene based tumour cell vaccination strategy for the treatment of AML. First, we wanted to determine if the expression of CD would allow us to use live cells as a vaccine by ensuring the elimination of these cells by 5-FC treatment. We examined the ability of a murine AML cell line engineered to express the CD gene to be eliminated in vitro and in vivo with 5-FC treatment. Secondly, we were interested in determining whether any immunogenic effect might be caused by CD-expression and elimination by 5-FC. We
were particularly interested in whether cells modified to express CD could stimulate a tumour specific immune response against unmodified leukemic cells. We assessed the ability of AML cells expressing CD to confer long-term protection to rechallenge with parental cells, as well as resistance to coinjected parental cells in a murine model.

5-FC mediated killing of CD-expressing cells in vivo is incomplete

One of the initial goals of this work was to facilitate the use of a live vaccine by determining if the expression of CD would allow for the elimination of vaccine cells by 5-FC treatment. It would be optimal if all of the vaccine cells could be killed so that relapse could not arise from the vaccine. However, despite the enhanced survival seen with 5-FC treatment in mice injected with CD expressing cells, a number of the mice still die indicating that in our model not all of the cells are killed by 5-FC (Figure 8).

Curiously, the survival of C1498-CD injected animals was decreased in mice that received control PBS injection rather than 5-FC. While IP injections did not shorten the survival of animals that received parental cells, this observation nevertheless suggests that repeated IP injection (20 injections over 10 days) in itself may be associated with increased mortality. It is possible, therefore, that this injection effect masks, in part, the degree of enhanced survival of animals injected with CD-expressing cells observed following 5-FC treatment. It is conceivable, therefore, that survival would be enhanced even further, if the 5-FC could be delivered in an alternative fashion.

It is unclear however, what the implications of these results are for the possibility of using live vaccines to treat human disease. The alteration of a number of the factors used in our model, such as vaccination dose, timing between injection of tumour cells and
the start of 5-FC treatment, the timing of 5-FC or the dose of 5-FC, or method of 5-FC delivery could potentially result in enhanced killing of the CD-expressing cells. In particular, the dose of CD-expressing cells that we used, $10^7$ cells, was quite high. It is possible that the use of a lower dose of $5 \times 10^6$ or $10^6$ CD-expressing cells would result in complete tumour cell eradication with 5-FC, while preserving the immune-stimulatory effect.

Recent research has suggested that the conversion of 5-FC to 5-FU by bacterial CD is less efficient than is the conversion by yeast CD [59, 60]. The use of yeast CD for enzyme/prodrug gene therapy rather than bacterial CD may also improve 5-FC killing of CD expressing cells in our model. Another method being explored in order to increase enhance the antitumour effect of 5-FC is the coexpression of the enzyme phosphoribosyltransferase together with CD [61, 62]. Phosphoribosyltransferase converts 5-FU to 5-fluorouridine 5'-monophosphate directly, rendering cells more sensitive to 5-FU. Other experimenters have used the coexpression of CD and TK (either separately or as a fusion protein) to enhance tumour cell killing [34, 63]. The use of both suicide genes in conjunction with 5-FC and GCV treatment has been shown to result in synergistic toxicity [64-66].

Moreover, it may be that when lower doses of CD-expressing variants of some cells are used, 5-FC killing may not even be required. The use of cells engineered to express additional factors, such as cytokines, may be even more stimulatory to the immune system. Such combinatorial vaccines may prove to be more immunogenic, allowing the immune system to eliminate larger doses of tumour vaccines either in combination with 5-FC or without 5-FC treatment.
Cytosine Deaminase is Immunogenic

The expression of cytosine deaminase by live tumour cells and the consequent elimination by 5-FC was meant to be the first step in developing a multifactorial and highly immunogenic AML vaccine. However, we discovered that the expression of CD alone rendered the C1498 cells immunogenic.

Enhanced survival was seen in mice injected with C1498-CD cells compared to animals receiving parental cells (Figure 6). This enhanced survival was quite dramatic, with a proportion of mice injected with CD expressing cells surviving past 90 days while those injected with parental cells did not live past day 42. The level of enhanced survival was irrespective of the cell dose used.

To determine whether the enhanced survival noted above could be attributed to CD expression, or could have resulted, at least in part, from other components of the retroviral vector used, or from the retroviral infection itself, mice were also injected with the control cell line (C1498–neo) that had been infected with a retrovirus identical to pCD2, except that the DNA encoding the CD gene had been excised. Survival of C1498-neo injected mice was similar to that observed after C1498-P injection (Figure 7). Thus, the enhanced survival observed in C1498-CD injected mice likely results primarily from CD expression itself, and not from some other component of the retroviral vector. In addition, the difference in mouse survival following injection of these lines cannot be attributed to differences in their rates of growth as all cell lines (parental, CD and neo) demonstrated similar in vitro growth (Figure 2). Similarly, differences the immunostimulatory abilities of the various cell lines cannot be accounted for by
differences in the expression of MHC class I and class II molecules or of the costimulatory molecules B7.1 and B7.2 which was similar among the parental, CD-expressing and neo control lines (Figure 3).

It is likely therefore that the increased survival of the mice injected with CD-expressing cells without exposure to 5-FC is due to the immunogenicity of the CD protein itself, but not of neomycin phosphotransferase, or indeed any other antigen derived from the retroviral vector. The peptides derived from cytosine deaminase could be processed, presented on MHC molecules, and recognized by T-lymphocytes. Consistent with this idea, other investigators have shown a decrease in the tumourigenicity of cancerous cells associated with the expression of a foreign gene. For example, tumour cells expressing bacterial β-gal have been shown to elicit an immune response by cytotoxic T-cells recognizing β-gal derived peptides [67]. In addition, glioma cells transduced with the thymidine kinase gene show a decrease in tumourigenicity [68].

In order to characterize the immune effector populations mediating the clearance C1498 cells, we assessed the survival of mice in which CD4+, CD8+ or NK cells had been depleted by antibody injection, and of nude mice following challenge with C1498 cells (both parental and CD-expressing cells). Nude mice are deficient in T-cells, but have normally functioning NK cells and macrophages. If T-cell function were solely responsible for the enhanced survival of mice injected with CD expressing cells compared to parental cells, this effect should be abrogated in nude mice. We found that the median survivals of nude mice receiving the same dose of CD expressing or parental C1498 cells was similar (Figure 15). This is in sharp contrast with what is seen in wild-type animals, in which a great divergence in the median survivals for mice injected with
C1498-CD cells compared to C1498-P cells was observed. Ultimately, however, a percentage of nude mice injected with C1498-CD cells survived to 90 days, while none of the animals receiving parental cells survived past 52 days, reminiscent of the enhanced survival following C1498-CD cells previously observed in wild-type mice (Figure 6). Thus, it is possible that C1498 clearance is multifactorial, with T-cells playing a role early in the response to C1498-CD cells but with non-T cell effectors clearly playing a role in enhanced long-term survival. Consistent with this notion, Boyer et al. have previously documented the existence of both C1498-specific NK and CD8+ T-cells [22].

We used the technique of antibody depletion to further examine the role of CD8+ and CD4+ T-cells as well as of NK cells. Not surprisingly, CD8+ T-cells do seem to play a role in the enhanced survival of mice injected with 10^6 C1498-CD cells. CD8+ depleted mice died more quickly, compared to non-depleted mice, when injected with C1498-CD cells. Additionally, CD4+ T-cells seem to play a lesser role, if any, in the enhanced survival of mice injected with C1498-CD cells since CD4+ depleted mice also died more quickly than non-depleted mice (although this difference was not statistically significant with the number of mice in our study). It should be noted that as antibody blocking was not complete (Figures 13 and 14), the involvement of CD4+ T-cells in the enhanced survival of mice injected with CD expressing cells cannot be absolutely ruled out. Nevertheless, the lack of CD4+ T-cell involvement is consistent with findings by Boyer et al. who determined with in vitro CTL analysis that only the blockage of CD8, but not CD4, had any effect on CTL activity with C1498 cells[22].

While the survival of animals receiving anti-NK antibodies was not significantly different statistically from the control C1498-CD animals, there appeared to be a trend
toward long survival in the NK-depleted arm. It is possible therefore, that the non T-cell effectors may also modulate C1498-CD clearance although the nature of this effect remains obscure. Taken together, the C1498-CD nude mouse and NK-depletion experiments therefore may suggest that in addition to the existence of cytolytic tumour-specific NK cells, there may also exist a population of NK cells capable of negatively-regulating C1498-specific T-cell responses, as has been demonstrated to exist in another murine tumour model [69]. Alternatively, the slight enhancement in survival of the NK cell-depleted animals may be due to the depletion of the very small subset of T-cells that express NK1.1 [70]. These cells express high amounts of IL-4, which strongly promotes the development of a Th2 response [70]. Therefore the depletion of these cells would lead to a shift in immunological response towards a Th1 type response which is generally believed to be more effective in eradicating tumour cells.

**Immunization With CD-Expressing Cells Can Confer Protection to Parental Cell Challenge**

The primary goal in engineering leukemia cells to express CD was to facilitate the use of a live cellular vaccine. It was encouraging, therefore, that CD expression enhanced leukemia clearance (Figure 6) and that this effect was enhanced by 5-FC treatment (Figure 8). However, these effects were peripheral to the main objective of this strategy. As mentioned above, the forced expression of a foreign protein by tumour cells might be expected to be immunogenic and lead to the enhanced clearance of tumour cells. Moreover, the killing of CD-expressing leukemia cells by 5-FC would be expected to enhance survival further, simply as a reflection of the drug-related reduction in cell
dose. In any event, the potential immune stimulatory effects of CD expression would be of clinical utility only if enhanced leukemia killing were not restricted to the clearance of genetically modified vaccine cells, but also extended to the enhanced clearance of unmodified cells. To determine if this were the case, we assessed whether an initial challenge with CD-expressing cells conferred resistance to subsequent rechallenge with parental cells.

We discovered that survival after C1498-CD challenge did result in protection to rechallenge with C1498 parental cells. After surviving 90 days post injection with $10^7$ C1498-CD cells (without 5-FC treatment (Figure 9)) mice were rechallenged with $10^6$ C1498 parental cells. Subsequent long-term survival was achieved in ~25% of animals. Interestingly, the mice that had been additionally treated with 5-FC demonstrated even greater protection to rechallenge with parental cells, with long-term survival being achieved in ~85% of cases. While the nature of this 5-FC effect cannot be determined from this experiment, these data are consistent with the notion that 5-FC mediated cell killing may result in enhanced tumour antigen presentation [71, 72].

However, mice that had survived challenge with $10^6$ and $5 \times 10^6$ C1498-CD cells (but without 5-FC treatment) (Figure 9) also demonstrated relatively enhanced resistance to C1498-P cells that mirrored that of mice receiving $10^7$ C1498-CD cells with 5-FC. Thus, it is possible that lower doses of CD-expressing cells were more immunogenic than were higher doses, and that the major effect of 5-FC at a dose of $10^7$ cells was merely to decrease the cell inoculum.

Taken together, therefore, these data indicate that immunization with CD-expressing cells did confer long term protection to subsequent parental cell challenge,
and that treatment with 5-FC seemed to enhance this effect. While this result is encouraging, to be of utility in the management of minimal residual leukemic disease, a leukemia cell vaccine must be capable of inducing resistance to not only subsequent parental challenge but more importantly to pre-existing parental leukemia cells. While our observation to date did indicate that vaccination with CD-expressing cells could induce resistance to parental cells, the relevance of post-vaccination parental cell challenge to minimal residual disease remained unclear. We therefore proceeded to assess whether CD expressing cells (+/- 5-FC killing) could similarly induce the clearance of pre-existing (or at least of co-injected) parental cells. Specifically, we assessed the survival of animals injected with 10:1, 1:1, and 1:10 ratios of both CD-expressing and parental cells. As shown in Figure 10, animals injected with a mixture of $10^7$ CI498-CD cells and $10^6$ CI498-P cells demonstrated increased survival that mirrored that of animals receiving $10^7$ CI498-CD cells alone (40% survival at day 90), rather than the shorter survival of animals receiving $10^6$ CI498-P cells alone (no animals surviving past day 42). However, mice injected with the same 10:1 mixture of cells and then treated with 5-FC, did not demonstrate enhanced survival, but rather resembled animals injected with $10^6$ CI498-P alone. As shown in Figure 11, animals injected with 1:1 and 1:10 ratios of CD-expressing and parental CI498 cells did not show the markedly reduced survival of mice receiving the 10:1 ratio. However, the 1:1 mixture did show a modest long-term survivor rate, comparable with the survival rate of mice injected with $10^6$ CI498-CD cells alone. Once again, 5-FC treatment of animals receiving the 1:1 cell mixture abrogated any benefit in survival. The effects of 5-FC treatment were not examined at the 1:10 cell ratio. Taken together, these data suggest that total vaccine dose
relative to the parental cell burden (or possibly, the duration of vaccine exposure) is a critical factor in the induction of resistance to C1498 cells. While mice that survived challenge with as few as $10^6$ C1498-CD cells could resist subsequent rechallenge with $10^6$ C1498-P cells (Figure 9), mice that were challenged simultaneously with C1498-CD cells and $10^6$ C1498-P cells required a dose of $10^7$ C1498-CD cells for the induction of protection to the parental cells. Consistent with this notion, mice were able to survive the coinjection of $10^7$ C1498-CD and $10^6$ C1498-P cells only when they were not subsequently treated with 5-FC. Treatment with 5-FC likely reduced the effective vaccine dose, or the duration of vaccine exposure, to a subthreshold level for T-cell activation that could not be overcome by any putative 5-FC cell killing-mediated immunogenicity effect [71].

To elucidate further the mechanisms involved in leukemia cell clearance, and the potential roles of vaccination and 5-FC cell killing in enhancing this process, we analyzed tumour-specific CTL activity in mice that had been immunized with CD-expressing cells. The initial CTL assays were performed using splenocytes from the mice that had survived C1498-CD challenge of either: $10^7$, $5 \times 10^6$ or $10^6$ cells, and also a later rechallenge with $10^6$ C1498-P cells. The splenocytes were cocultured with C1498-P cells. As would be expected, splenocytes of all surviving animals lysed C1498-P cells. No lysis of C1498-P targets was observed using the control naïve mice at the lower E:T ratios (3:1, 11:1 and 33:1), but a low level of lysis was seen at the 100:1 E:T ratio. Mice were then injected with CD-expressing cells and either treated with 5-FC or not, in order to obtain spleens for further CTL assays. In particular, we were interested in determining whether CTL mediated lysis was directed primarily towards the CD protein, or towards
other determinants on the C1498 cell. We therefore varied the coculture step of the CTL assay to examine whether CTL clones specific for the CD antigen or for other AML cell antigens could be differentiated *in vitro*. Splenocytes from CD-expressing cell vaccinated mice were cocultured with either C1498-parental or C1498-CD cells. We anticipated that coculture with parental cells would result in the outgrowth of CTLs specific for leukemia antigens other than CD, while coculture with CD-expressing cells would additionally (or possibly preferentially) result in the outgrowth of CTLs specific for the CD antigen. In addition, we harvested splenocytes both from vaccinated mice that had subsequently received 5-FC treatment as well as from those that had not. We anticipated that the CTL assays might demonstrate differences in CTL activity based on the presence or absence of 5-FC treatment. All mice that had been injected with C1498-CD cells yielded splenocytes that lysed C1498-P cells at a high level, regardless of whether the splenocytes were cocultured with C1498-P or C1498-CD cells, or whether the mice had received 5-FC treatment or not (Figure 17). These results demonstrate that injection of mice with CD-expressing C1498 cells results in the formation of cytotoxic lymphocytes that are directed against the parental C1498 cells, a direct demonstration of epitope spreading (see below) in our mouse model. As expected, C1498-CD cells were also lysed, under both coculture conditions and both with and without 5-FC treatment.

A control murine AML cell line was used to determine if the immunogenic effect observed was cell specific. Lysis of the control MMB-P targets was low by all mice splenocytes, which was not surprising, as C1498 and MMB cells would not be expected to have many tumour restricted antigens in common (Figure 19). Lysis of MMB-CD cells was absent or low when splenocytes were cocultured with C1498-P cells. Coculture
with C1498-CD raised the level of lysis, suggesting that coculture could preferentially amplify CD-specific CTLs, and that such cells could induce lysis of CD-expressing cells, regardless of the cellular background (Figure 20). When splenocytes of mice that had undergone 5-FC treatment and had been cocultured with C1498-CD were used, lysis of MMB-CD was even higher (Figure 20). Taken together, these observations suggest that 5-FC killing of vaccine cells does result in the enhanced priming of CD-specific CTLs, and possibly of other effectors recognizing non-CD epitopes shared by C1498 and MMB cells. Moreover, the lysis of MMB-CD cells, but not MMB-P cells by spleen cells from mice that had never been exposed to MMB cells, suggests that this lysis is mediated through the CD protein alone. In addition, the requirement for 5-FC treatment to raise lysis of MMB-CD but not of C1498-CD targets to a high level indicates that 5-FC treatment increases the activity of T-cells specific for the CD antigen and that C1498-CD cell lysis is not mediated solely through the CD antigen, but through other tumour restricted antigens as well.

Interestingly, the lysis of MMB-CD cells invariably exceeded that of C1498-CD cells (Figures 20 and 18), even though the splenocytes were derived from animals that had been immunized with C1498-CD cells. Possibly, MMB cells process or present CD epitopes more efficiently than do C1498 cells. Alternatively, MMB-CD targets – by virtue of increased surface MHC and B7.1/B7.2 expression – may enhance the ongoing generation of CTLs in vitro more efficiently than do C1498 cells. Finally, it is likely that C1498-CD vaccination results in a polyclonal CTL response directed against a spectrum of C1498- restricted epitopes. It is possible that some such C1498 tumour antigen-restricted primed spleen cells may inhibit the activity in vitro of CTLs directed against
other antigens, a phenomenon known as *in vitro* T-cell competition [73]. Such Cl498-specific effector cell competition may be abrogated when MMB-CD cells are used as CTL targets, resulting in enhanced lysis.

**Epitope Spreading – An Explanation**

We initially showed that the survival of mice challenged with CD-expressing Cl498 cell variant was prolonged relative to that of animals challenged with parental cells. Enhanced clearance of CD-expressing cells was immune-mediated, as animals that had survived challenge with CD-expressing cells were able to survive a subsequent rechallenge with doses of parental cells that would have been universally lethal in naïve animals. Consistent with this observation, while experiments in nude mice indicated that additional T-cell independent mechanisms participated in leukemia clearance, our *in vitro* analyses revealed that animals challenged with CD-expressing cells (but not naïve animals) contained not only CTLs directed against CD epitopes, but also CTLs directed against other non-CD tumour associated epitopes. Thus, expression of a foreign protein - CD – can also lead to enhance immune reactivity to other antigens, which had previously not been immunogenic. This transference of specificity may be explained either by the action of cross-reactive T-cells [74] that were activated by CD epitopes, but can also recognize non-CD tumour epitopes or by the phenomenon of epitope spreading. As it is difficult to reconcile a number of our observations - the differential effects of parental and CD-expressing cell coculture during *in vitro* CTL assays, or the disparate effects on survival of altered parental/CD-expressing cell ratios when modified and parental cells
were coinjected, for example - with the existence of a predominant population of cross-reactive T-cells, we favour the latter explanation.

Epitope spreading has been studied extensively in autoimmune disease. It is a process whereby epitopes distinct from and non-cross-reactive with an inducing epitope become major targets of an ongoing immune response [75]. This same general phenomenon has been observed when engineered tumour cells that express foreign antigens enhance tumour vaccine potency (often termed xenogenization in this context [20] [72, 76]). It is unclear exactly by what mechanism the xenogenized tumour cell enhances the systemic immune response. Vanderlugt et al. [75] explain epitope spreading as a cascade resulting from recognition of a single epitope. APCs first present the administered or transgenic epitope to CD4+ helper cells. These T-cells activate the APCs to upregulate costimulatory signals, and at the same time initiate an inflammatory response. This results in the destruction of cells carrying these epitopes. However, following tissue destruction, cellular debris is taken up by the activated APCs, and this in turn can lead to the priming of T-cells against other peptides that initially were presented at a level below the threshold for T-cell activation. Epitope spreading has been shown to be both intramolecular (other determinants of the same peptide) or, more important for tumour immunology, intermolecular (different antigens present in the same target organ) [77]. In the cases of our leukemia model, therefore, we propose that C1498 parental cells present tumour antigens at subthreshold levels to antigen-specific T-cells, resulting in the inability of the mice to clear injected cells. However, as a result of the immune response to CD-protein expressing C1498 cells, and the resultant enhanced APC
activation and processing of cellular debris, such tumour antigens become stimulatory as well.

We noted, in addition, that mice that had survived C1498-CD challenge with 5-FC treatment both appeared to be more able to resist parental challenge than were mice that had not received 5-FC, and also demonstrated enhanced anti-tumour CTL activity in vitro. A similar effect was observed by Mullen et al., who noted increased CTL response observed against the influenza nucleoprotein (NP) peptide, in animals that had been injected with tumour cells expressing NP and HSV-TK followed by cell killing with GCV [72]. Thus, the death of the tumour cells by prodrug administration increases the stimulatory capabilities of the self-antigen to an even greater degree. Indeed, in vivo prodrug treatment of suicide gene-modified tumour cells is known to produce tumour necrosis and an inflammatory response [72]. It has also been suggested that increased levels of heat shock chaperone protein expression associated with HSV-TK/GCV or CD/5-FC cell killing may lead to enhanced antigen presentation [71].

Studies into the epitope spreading phenomenon in autoimmune diseases have shown that a certain amount of time is required before T-cell responses to a second epitope is seen [75]. Theiler’s murine encephalomyelitis virus (TMEV) is a natural mouse pathogen which induces a life-long persistent central nervous system (CNS) infection that can lead to a chronic demyelinating disease. After infection with TMEV, responses to viral epitopes are seen within 7 days in mice [75]. However, no T-cell responses are seen to a panel of myelin proteins until 3-4 weeks after the disease onset. This observation suggests that the viral epitopes must be present for a period of time in order to effect epitope spreading to the myelin proteins. The requirement for a certain
duration of exposure of CD-expressing cells to the immune system in order for a reaction against parental cells to develop demonstrates that the anti-parental tumour effect we have seen is due to epitope spreading and not due to the cross reactivity of T-cells.

Conclusion

The goal of this research was to develop and optimize in a murine system a suicide gene based tumour cell vaccination strategy for the treatment of AML. Since the cause of death in most AML patients is not the inability to achieve remission but rather the high incidence of relapse [1], a treatment option that would allow for the long-term management of minimal residual disease would be greatly beneficial. Manipulation of the immune system may be an effective method for controlling leukemic minimal residual disease [2].

Strong immune reactions that impair tumour growth have been elicited by vaccination with live tumour cells in many tumour models [24, 55, 78-82]. Such an approach may be successful in aiding the elimination of tumour cells involved in AML minimal residual disease based on several lines of evidence, which have been previously outlined (see introduction). There are likely to be concerns about the use of live tumour cell vaccines in patients that have already achieved remission. In order to eliminate such concerns, whether real or perceived, a mechanism to allow for the elimination of the vaccine cells is desirable. One method that has been studied extensively is the use of suicide genes (which encode for non-mammalian enzymes that convert nontoxic prodrugs to toxic metabolites) to render the cells selectively sensitive to a drug that has no effect on normal mammalian cells. One of these enzymes, CD from E. coli, converts the
prodrug 5-FC to the toxic metabolite 5-FU. As the first step toward a vaccination strategy to the treatment of AML, we engineered a murine AML cell line to express cytosine deaminase for study in a murine model of AML.

Our results provide insights into the uses of CD in the AML setting that are relevant to potential future human studies:

We determined that in our model, \textit{in vivo} 5-FC treatment does not eliminate 100% of the CD-expressing cells. Nevertheless, enhanced survival was seen with CD-expression alone. We have shown that the immunogenicity of the CD protein can elicit an immune reaction against parental cells by epitope spreading. In addition, resistance to parental rechallenge and concurrent challenge was seen with mice immunized with CD-expressing cells.

These observations have implications for human studies as the optimum vaccine dose, timing between vaccination and the beginning of 5-FC treatment, as well as the manner in which 5-FC is delivered will have to be determined before a human vaccine can be developed. Once this is elucidated, this knowledge could be applied to tailor the vaccine dose, and possibly other important factors in vaccination, to the patient based on the characteristics of their leukemia cells.

Our results are encouraging, considering that the murine AML cell line used demonstrated only very low or absent costimulatory molecule expression. One might anticipate that this strategy would be an even more successful using cells that expressed B7 or other costimulatory molecules, or greater levels of MHC I.

The ultimate success of immunotherapy for leukemia may require the use of cells engineered to express a combination of immunostimulants. In a combinatorial vaccine,
suicide gene killing would likely enhance the immunogenicity of some other modification, as has been demonstrated in murine models other cancers [81, 82]. We believe that our experiments provide a useful first step towards developing such combinatorial vaccines for the use in AML patients. In the long run, we hope that these murine experiments will lead to a new strategy for the management of minimal residual disease in humans with AML.
Future Directions

While our research has provided insights into the application of live CD-expressing tumour cell vaccination for the treatment of minimal residual disease in AML, a number of questions remain. As mentioned earlier, one area that we would like elucidated is the optimal timing of 5-FC administration. Further studies comparing various durations of time between vaccination and 5-FC administration, as well as the timing of 5-FC administration are needed to optimize the epitope spreading effect we have seen. Once the optimum schedule of vaccination and 5-FC administration is determined the effect of combinatorial vaccines should be examined.

In addition, there are recently studied methods aimed at improving the efficacy of 5-FC. Using yeast CD as opposed to bacterial CD increases cell killing by 5-FC [59]. Engineering the cell to concamitantly express methioninase which depletes methionine inside the cell, increases 5-FU killing efficiency [37]. Expression of uracil phosphoribosyltransferase is another method, which facilitates the uptake of 5-FC by the cell [61].

On the other hand, in a combinatorial vaccine setting it may not be necessary to eliminate all the CD-expressing vaccine cells by 5-FC treatment as the immune system should be able to eliminate at least some of these cells that have been engineered to be highly immunogenic. Other investigators have shown that double transfectants such as CD and interferon-γ (IFN-γ) [83]; CD, IFN-α and IFN-β [35] as well as CD and IL-2 [81] can elicit antitumour reactions that are greater than achieved with CD expression alone.
As the ultimate goal is to provide a new treatment option for AML patients in minimal residual disease, the next step would be to assess the ability of the optimum combinatorial vaccine as determined by in our murine model, on human AML cells.
References


