RESEARCH ARTICLE

Prime-boost vaccines encoding an intracellular idiotype/GM-CSF fusion protein induce protective cell-mediated immunity in murine pre-B cell leukemia

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Two vaccines against an intracellularly expressed B cell idiotype were assessed for their ability to induce protective immunity in mice against challenge with a pre-B cell leukemia. One vaccine was based on a plasmid expression vector and the other was a recombinant vaccinia virus; both vaccines expressed a polypeptide derived from the complementarity-determining regions (CDR₂-CDR₃) of the leukemic clone-specific immunoglobulin heavy chain (IgH), as a fusion product with mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF). Mice inoculated with either vaccine showed significantly higher survival rates than controls after challenge with leukemia cells. However, protection

from tumor challenge was optimal when the DNA vaccine was used for priming, followed by a booster immunization with the vaccinia virus recombinant. This vaccination protocol induced resistance not only to the first tumor challenge given shortly afterwards, but also to a second challenge given months later. Both CD4⁺ and CD8⁺ T cells contributed to protection in vaccinated mice. These data suggest that such a vaccine regimen might reduce the incidence of recurrence in patients with minimal residual disease after conventional therapy.

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Introduction

Protein alterations caused by point mutations and genetic rearrangements provide potentially tumor-specific target antigens for active anti-cancer immunotherapy.^{1–3} Although most of those tumor antigens are expressed as intracellular proteins, peptide fragments generated by proteolytic processing are presented on the cell surface in the context of major histocompatibility complex (MHC) class I or class II molecules and, as such, can be recognized by cells of the immune system.^{4–6}

Clonal expansion of cells bearing rearranged antigen receptors is a hallmark of acute lymphocytic leukemias (ALL) of B and T cell lineages. In more than 30% of pre-B ALL, one of the two alleles is productively rearranged, and an in-frame immunoglobulin heavy chain (IgH) polypeptide is expressed intracytoplasmically. In about half of these patients, the rearrangements are stable and therefore represent suitable targets for immunotherapy as demonstrated by the successful induction of protective anti-idiotype B cell response against the IgH expressed on the surface of B cell lymphomas.⁷

Recombinant viral vaccines^{8,9} generally induce potent T and B cell-mediated immune responses against the inserted gene product upon a single injection. More

recently, DNA vaccines have been shown to stimulate production of antibodies, T helper cells, and cytolytic T cells in response to the expressed antigen.^{10–15} DNA vaccines can induce responses to T cell epitopes that are not recognized after immunization with a protein-based immunogen^{16,17} and accordingly can trigger T cell responses in non-responder haplotypes¹⁶ by inducing recognition of cryptic epitopes,^{18,19} presumably as a result of the persistent expression of the antigen by the vector-transfected cells. This response to cryptic epitopes points to a potential advantage of DNA vaccines for induction of a cellular immune response to idiotypes, which are 'self' antigens.

We reasoned that although pre-B ALL do not express surface immunoglobulins, peptides derived from in-frame IgH expressed in the cytoplasm could be presented in the context of MHC. The loss of MHC class I molecules in pre-B leukemia is a rare phenomenon and therefore a strong cellular immune response elicited against IgH-derived peptides could be effective in protecting against leukemia, particularly in a minimal disease setting. In the present study, we show that a recombinant vaccine based on an IgH idiotype expressed intracytoplasmically by pre-B cells can induce protective cellular immunity in mice against leukemic clones from which the idiotype was derived. Granulocyte-macrophage colony-stimulating factor (GM-CSF), which was previously shown to augment the immune response to an idiotype DNA vaccine targeting a B cell lymphoma,⁷ was used as a fusion protein with the specific idiotype. Protection induced by

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priming with a DNA vaccine followed by boosting with a recombinant viral vaccine was higher than that achieved using the DNA or the viral vaccine alone. These results point to the potential benefit of such a protocol in leukemia patients at risk for relapse at the end of chemotherapy.

Results

Surface and cytoplasmic Ig expression in 70Z/3 cells

Analysis of the 320-bp amplicon of the rearranged hyper-variable region of the 70Z/3 IgH chain containing the CDR2 and CDR3 (Figure 1) indicated that the cells have one rearranged allele that is translationally in-frame. They are thus able to generate a stable protein. Staining with fluorescein-conjugated anti-mouse (Ig) antisera to detect IgH expression in 70Z/3 cells revealed cytoplasmic Ig, but not surface Ig (Figure 2), confirming their pre-B developmental stage. 32D(G)GM and OKT3 cells were used as negative and positive controls, respectively.

Efficacy of vaccines against 70Z/3

In initial experiments, B6D2F1 mice were immunized i.m. three times at 21-day intervals with 75 µg of DNA vaccines expressing the idiotype (CDR₂₊₃)-mGM-CSF fusion protein (Figure 3). Protection against disease, and delay in onset of tumors was achieved with this DNA vaccine that give a better survival when compared with the control mice injected with saline (log-rank test, $P = 0.041$) that developed tumors rapidly, only 10% survived over a 160-day observation period (Figure 4). Animals vaccinated with the cytokine-expressing vector or the vaccine expressing the idiotype had tumor growth similar to the control group (log-rank test, $P = 0.723$ and $P = 0.73$, respectively).

In an effort to improve the efficacy of the DNA vaccine, mice were primed with the DNA vaccine and then boosted with the homologous vaccinia virus recombinant. Groups of mice were vaccinated with a single dose of DNA vaccine encoding L-GM-CSF-CDR₂₊₃, L-CDR₂₊₃ or L-GM-CSF. After 5 weeks, mice were boosted with a

70Z/3 VDJ Sequence

Figure 1 Sequence of the CDR₂ and CDR₃ containing regions of the IgH gene rearrangement in the 70Z/3 cell line. RT-PCR using the VH5'2/IgM'2 primers described in Materials and methods amplified a segment of 320 bp. Sequence data identified the CDR2 and CDR3 region of rearranged IgH. The junction with the J segment indicated an in-frame rearranged IgH allele.

Figure 2 Detection of surface and intracytoplasmic immunoglobulins in 70Z/3 cells. Cells were stained with fluorescein-conjugated antiserum to mouse Ig(H+L) to detect cytoplasmic or surface Ig as described in Materials and methods. 32D(G)/GM are myeloid cells used as a negative control; OKT3 is an Ig-secreting B cell hybridoma used as a positive control for surface IgH.

a Chimeric molecules subcloned into pcDNA3 vector

b 70Z constructs subcloned into pSC11/7 vector

Figure 3 Recombinant complementarity-determining regions (CDR) used for DNA and viral vaccination. (a) Chimeric molecules used for DNA immunization: (CDR₂₊₃) alone or in combination with L-GM-CSF protein or a leader sequence (L) were subcloned into pcDNA3 as described in Materials and methods. (b) Chimeric molecules used for viral vaccination: (CDR₂₊₃) in combination with L-GM-CSF proteins or (L) were subcloned into pSC11/7 as described in Materials and methods.

Figure 4 Development of leukemia in DNA vaccinated B6D2F1 mice challenged with 70Z/3 cells. Combined results of three experiments (15 mice/per vaccination condition) are shown. Mice were immunized following the protocol described in Materials and methods.

single dose of the recombinant vaccinia virus encoding the homologous proteins. They were challenged 2 weeks later with 2×10^7 70Z/3 cells. Control groups were injected once with a single dose of DNA vaccine and challenged 7 weeks later. Other groups were immunized with a single dose of the vaccinia virus recombinants and challenged 2 weeks later. One control group of mice was injected with saline alone. After a 180-day observation time, mice that remained tumor-free were inoculated with a second dose of tumor cells to determine whether the vaccine had induced long-term protection. As shown in Figure 5, a single dose of the DNA vaccine was not sufficient to induce a specific and long-lasting protective immune response. Although a fairly large percentage (35%) of mice inoculated once with the plasmid encoding mGM-CSF only remained tumor-free, they rapidly succumbed to progressive tumor growth upon second challenge. This suggests that the cytokine had activated protective innate effector cells to the first tumor challenge without inducing a long-lasting, antigen-specific memory immune response. Mice immunized with the recombinant viruses showed both antigen-specific and non-specific protection (Figure 5). All control mice immunized with saline developed tumors within approximately 6 weeks. Although tumor development was delayed in mice immunized with the vaccinia virus L-CDR₂₊₃ recombinant, all of the mice died within 5 weeks after challenge. Mice inoculated with the vaccinia virus recombinant expressing GM-CSF only, or with the recombinant encoding a fusion protein of GM-CSF and the CDR₂₊₃ of the 70Z/3 IgH, showed a similar degree of protection after the initial tumor challenge; although mice vaccinated with the antigen-encoding construct showed a slight delay in tumor onset during the early phase after challenge. However, after the second challenge most of the mice injected with the mGM-CSF-expressing construct rapidly developed tumors, while all of the mice immunized with the vaccinia recombinant expressing the fusion construct and surviving the first challenge remained completely tumor-free. These data again demonstrate that mGM-CSF in this system can elicit transient immunity to tumor challenge, but co-vaccination with antigen is required for long-lasting protection. The highest survival rates (log-rank test, $P = 0.002$) were observed in mice that received a combination vaccine regimen composed of DNA vaccine priming followed by vaccinia virus recombinant booster immunization (Figure 5). More than 60% of mice immunized with the combination vaccine expressing the GM-CSF-CDR₂₊₃ fusion protein survived the initial challenge and most of these mice remained tumor-free after the second challenge. Control mice injected with saline or the DNA/vaccinia virus recombinant combination expressing only mGM-CSF developed tumors within less than 6 weeks. The small number of mice vaccinated with the cytokine-expressing vectors that had remained tumor-free after the first challenge developed disease after the second inoculation of tumor cells, underscoring the importance of the antigen component of the vaccine for induction of long-term protective immunity.

In several experiments, vaccinated animals showed tumor-related signs several months after challenge. To determine whether these tumor cells were escape mutants that had altered or lost expression of the CDR2 and CDR3 regions of the IgH used in the vaccine con-

DNA Vaccine

Vaccinia Vaccine

DNA +Vaccinia Vaccines

Figure 5 Tumour immunity induced by sequential administration of DNA vaccine and vaccinia virus-based vaccine. B6D2F1 mice were divided into three cohorts and immunized as described in Materials and methods: cohort 1 received a DNA vaccine encoding different recombinant antigens L-CDR₂₊₃ (eight mice), L-GM-CSF+CDR₂₊₃ (eight mice), or the genetic adjuvant L-GM-CSF (eight mice); cohort 2 received a vaccinia-based vector encoding the same antigens, and the third cohort received the combination of both therapies. Mice were challenged with 70Z leukemia cells at the end of vaccination, and survivors were challenged again at day 180. The experiments have been repeated three times.

structs, several vaccinated mice were autopsied and splenocytes were tested for the presence of spontaneous somatic mutations in the CDR sequence of the 70Z/3 IgH chain. Sequencing of PCR-amplified mRNA from these splenocytes revealed the original VDJ sequence of the

70Z/3 cells (data not shown) suggesting that the late tumor progression in some of the vaccinated mice did not reflect escape from immunosurveillance due to mutations of the tumor cells.

Role of T cells in protective immunity against 70Z/3 leukemia cells

To assess the potential contribution of a cellular immune response to protection against tumor challenge, mice were vaccinated with the combination vaccine (DNA vaccine priming followed after 5 weeks by a booster immunization with the vaccinia virus recombinant) expressing the GM-CSF-CDR₂₊₃ fusion protein. They were then treated three times with anti-CD8, anti-CD4, or both antibodies, or left untreated (Figure 6). Control mice were injected with saline or with empty vector

Figure 6 Tumor immunity induced by combination therapy in T cell subset-depleted mice. B6D2F1 mice (eight mice per group) were immunized with a combined DNA vaccine and recombinant vaccinia as described in Figure 5. After immunization, mice were treated with anti-murine CD4 or anti-CD8 mAb at day -5, -3, -1 before tumor challenge. At day 0, mice were challenged with the tumor cells, and time of death was recorded. An additional control group consisting of mice immunized with an empty plasmid vector and boosted with a vaccinia virus recombinant encoding a rabies virus glycoprotein (VRG) was used as a negative control. The experiment has been repeated twice.

(pcDNA3) followed by a booster immunization with a vaccinia virus recombinant expressing an irrelevant viral antigen (ie the glycoprotein of rabies virus).⁹ Mice were challenged 2 weeks after the boost immunization, immediately after the T cell depletion. Upon challenge with 70Z/3 cells, mice injected with saline or the control vaccine rapidly developed leukemia and died in less than 30 days. Half of the mice immunized with the combination vaccine remained disease-free for 3 months, when several additional mice developed disease and died (not shown). Mice treated with anti-CD8 mAb developed tumors with the same kinetics as unvaccinated mice (log-rank test, $P = 0.593$) or mice vaccinated with the control rabies virus glycoprotein vaccine (log-rank test, $P = 0.98$); whereas mice depleted of CD4⁺ T cells, either by treatment with anti-CD4 mAb alone or in combination with anti-CD8 succumbed to challenge more rapidly than control mice (log-rank test, $P = 0.0013$ and $P = 0.0008$, respectively). These data indicate that both CD8 cytotoxic T cells and CD4⁺ T helper cells are required for protection against 70Z/3, and suggest that CD4⁺ T cells might provide some inherent resistance to 70Z/3 tumor cells even in unvaccinated mice.

Discussion

Many tumors express tumor-associated antigens that are unique to the cancer cells because they are products of point mutations or genetic rearrangements. Although many of these modifications are potentially immunogenic, activation of the immune response is often precluded by the lack of appropriate antigen presentation in the context of co-stimulatory molecules, necessitating reprocessing of tumor antigen by professional antigen-presenting cells. Furthermore, tumor-derived cytokines such as interleukin-10, transforming growth factor- β or prostaglandins can actively suppress the immune system.²⁰ Thus, vaccines for cancer have undergone extensive development and testing in recent years to overcome these blocks in immune responses to tumor-associated antigens.

T and B cell malignancies express clonally rearranged antigen receptors that are unique to the tumor. Pioneering work from Levy's laboratory has shown that the idiotype of B cell lymphoma cells can induce protective immunity, which can be optimized in combination with GM-CSF as an adjuvant.^{7,21} The mouse B cell lymphoma line used in those studies expressed surface Ig which is readily accessible for antibody-dependent immune effector mechanisms. Our present work, showing that vaccines expressing a fusion polypeptide between mGM-CSF and an IgH CDR derived from a pre-B leukemia cell line protect mice against a leukemia challenge, demonstrates the feasibility of immunizing against a malignant cell-specific intracellular antigen.

The vaccines that we developed are known to induce a broad spectrum of immune response, including CD8⁺ T cells, which are generally not activated upon immunization with protein vaccines. Two prototype vaccines were tested: vaccinia virus recombinants and DNA vaccines. Viral recombinant vaccines induce rapid and fairly potent immune responses against the product of the inserted gene, although immune responses to the viral carrier can predominate especially when the target protein is only weakly immunogenic as in the case of

idiotypes. DNA vaccines induce an immune response only to the antigen encoded by the gene inserted in the vector and thus, unlike viral recombinant vaccines, allow repeated booster immunizations. The immune response to DNA vaccines can be enhanced by using 'genetic adjuvants' in the form of expression vectors that encode cytokines such as GM-CSF.^{11,15} Analogous adjuvants can be provided in a separate plasmid injected concomitantly or as a fusion protein with the antigenic polypeptide. Further enhancement of the immune response to DNA vaccines can be achieved with traditional vaccines, such as proteins or with viral recombinants used for booster immunization. Some protection was achieved by DNA vaccines expressing the variable region fragment as a fusion protein with mGM-CSF, which is known to augment immune responses to tumor antigens, presumably by recruiting and activating dendritic cells for antigen presentation. Interestingly, the DNA vaccine expressing only mGM-CSF occasionally induced significant protection, as reflected by both an increased proportion of survivors and a delayed onset of disease. However, this type of protection, derived most likely from innate immune effector mechanisms such as granulocytes, eosinophils, natural killer cells or macrophages was short-lived, and without immunological memory since mice rapidly succumbed to disseminated leukemic disease after a second challenge.

Consistent with predictions based on previous studies in viral models,^{22,23} priming with the DNA vaccine followed by a booster immunization with the vaccinia virus recombinant clearly resulted in the highest degree of protection. Again, the construct expressing only mGM-CSF induced some protection against the initial challenge given shortly after vaccination but, like the DNA construct, failed to protect against a subsequent challenge.

In this study, protection required both CD8⁺ and CD4⁺ T cells, as already shown after anti-idiotypic immunization of patients with lymphoma.²⁴ B cells express MHC class I and II antigens, and thus both T cell subsets might limit tumor growth by direct lysis of idiotype-positive cells. Alternatively, or in addition, cytokines secreted by activated CD4⁺ T cells might have induced other non-antigen-specific immune effector mechanisms that in turn eliminated tumor cells. A role for antibodies in the protection was not investigated, but seems unlikely given the lack of Ig surface expression.

Our studies show that a vaccine regimen consisting of a DNA vaccine expressing antigen together with a cytokine, ie GM-CSF, followed by boosting with a viral recombinant vaccine can induce complete and long-lasting protection against challenge with a B cell leukemia line in a significant percentage of mice. Considering the ease with which DNA vaccines can be modified by altering the pathway of antigen presentation, or by adding cytokine adjuvants, such vaccines can be further optimized. This also allows construction of customized vaccines for individual patients, an endeavor too time-consuming and costly for most traditional vaccines.

Our data point to the potential clinical utility of sequential vaccination protocols with DNA and a viral vaccine expressing a fusion protein between mGM-CSF and the idiotype for those pre B-ALL patients with an in-frame rearranged IgH. Such patients commonly have long-term persistence of minimal residual leukemia cells after undergoing chemotherapy.²⁵ Relapse at the end of

chemotherapy occurs in about 10% of the treated children. An efficient cytotoxic immune response against the CDR₂₊₃ regions might be adequate to reduce the risk of relapse at the end of therapy.

Materials and methods

Cell lines

The mouse pre-B leukemia cell line 70Z/3, established from a C57Bl6xDBA2 (B6D2F1) mouse bearing a chemically induced pre-B leukemia^{26,27} was purchased from the American Type Culture Collection (ATCC No. TIB 158). Cells were maintained in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with penicillin (100 U/ml), streptomycin (0.01%), 2-mercaptoethanol (5×10^{-5} M) and 10% heat-inactivated fetal bovine serum (FBS). HeLa cells, Green monkey kidney CV1 cells, and thymidine kinase negative (Tk⁻) 143B human osteosarcoma cells (ATCC CRL 8303) were grown in Dulbecco's modified medium (DMEM) supplemented with 10% FBS. OKT3 hybridoma cells (ATCC CRL-8001) were grown in supplemented RPMI medium, and 32D (G)/GM cells²⁸ were propagated in DMEM supplemented with 10% FBS and 10% culture supernatant of WEHI cells as described.²⁹

Viruses

Vaccinia virus strain Copenhagen and recombinant viruses (see below) were propagated on HeLa cells as described.⁹

Mice

Six- to 8-week-old B6GD2F1 (C57BL6 × DBA2)F1 female mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA), and housed in a temperature-controlled, light-cycled room at The Animal Facility of The Wistar Institute.

Characterization of 70Z/3 cells

Immunofluorescent detection of IgH and MHC class I expression: 70Z/3 cells were grown on glass coverslips and fixed with 95% ethanol and 5% glacial acetic acid. Cells untreated or treated with Triton X-100 for 20 min were incubated with goat anti-mouse Ig(H+L)-fluorescein isothiocyanate (FITC) (Southern Biotechnology, Birmingham, AL, USA). After washing and incubating with diluted biotinylated rabbit anti-goat IgG(H+L) (Vector Laboratories, Burlingame, CA, USA), cells were stained with fluorescein-avidin (Vector Laboratories). For detection of MHC class I determinants, FACS analysis was performed using a FITC-labelled anti MHC-class I antibody (Pharmingen, San Diego, CA, USA).

Immunoglobulin (Ig) heavy (H) chain CDR2 and CDR3 regions of 70Z cells: RNA was extracted from the 70Z/3 cells using RNazol (Biotecx Laboratories, Houston, TX, USA), redissolved in DEPC-treated water and used as a template for the reverse transcriptase reaction. RNA was incubated in the presence of IgM-2 3'-constant region primer (5'-CAC CAG ATT CTT ATC AGA-3'), and the cDNA was used as template for the polymerase chain reaction (PCR) to amplify the VDJ region of the IgH chain. The sequence of the 5'-VH2 primer used for amplification was: 5'-CAG GTC CAG TTG CAG CAG (A/T)

C(A/T) GG-3' (Integrated DNA Technologies, Coralville, IA, USA). The reaction mixture contained amplification buffer, dNTP, upstream and downstream primers, template DNA, and Thermus aquaticus (Taq) polymerase DNA, was overlaid with light mineral oil (Perkin Elmer Cetus, Foster City, CA, USA). Amplification was carried out for 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, for 40 cycles, and a final elongation of 5 min at 72°C using the Perkin Elmer Cetus Taq polymerase protocol. The purified PCR product was sequenced using the Sequenase kit (USB, Cleveland, OH, USA) according to the manufacturer's instructions.

Vaccines

Plasmid vectors: The pcDNA3 plasmid (Invitrogen, San Diego, CA, USA) was used throughout this study as an expression vector. Figure 3 shows a schematic representation of the expressed chimeric (GM-CSF-CDR₂-CDR₃) molecules. The mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF) cDNA containing its own leader sequence (L) was excised from the pRJB-GM plasmid³⁰ (gift of Dr M Prystowsky, University of Pennsylvania, Philadelphia, PA, USA) by *Bam*HI-*Xho*I cleavage (Figure 3-A2). The plasmid containing L-GM-CSF+CDR₂₊₃ was generated by fusion of the GM-CSF encoding sequence, from which the stop codon had been eliminated, to the CDR2-CDR3 sequence of the 70Z cells (Figure 3-A1). The CDR₂-CDR₃ region of the 70Z IgH was obtained after RT-PCR amplification using the following primers: upstream primer 5'-AGT GGT AGT ACT AAC TAC AAT GAG-3' (primer 1) and downstream primer 5'-TCA TCA GAC CGT GGT CCC TGT GCC CCA-3' (primer 3) (Figure 1). CDR₂+CDR₃ inserts were also subcloned into *Eco*RI-*Xho*I-cleaved pcDNA3 plasmid, and the mGM-CSF leader sequence was fused in-frame upstream of the idiotype sequence (Figure 3-A3). The mGM-CSF leader was obtained by annealing oligonucleotides encoding the leader sequence of the mGM-CSF: 5' oligo GAT CCA TGT GGC TGC AGA GCC TGC TGC TCT TGG GCA CTG TGG CCT GCA GCA TCT CTG and 3' oligo AAT T GAG ATG CTG CAG GCC ACA GTG CCC AAG AGC AGC AGG CTC TGC AGC CAC ATG.

Plasmid DNAs were purified from transformed DH5 α -competent bacteria cells (Gibco BRL, Gaithersburg, MD, USA) using the Qiagen Mega Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's specifications.

Recombinant vaccinia viruses: Vaccinia virus recombinants were generated as described.^{31,32} Briefly, the sequences encoding the fusion protein L-mGM-CSF-CDR₂₊₃, L-CDR₂₊₃ and the cytokine L-mGM-CSF (Figure 3-B) were cloned into the pSC11/7 coinfection transfer vector, using the *Kpn*I and *Not*I sites. Wild-type vaccinia virus (strain Copenhagen) was used to infect CV-1 cells. The transfer vector, expanded and purified from transformed DH5 α bacteria, was precipitated using the Pharmacia CellPect Kit (Pharmacia, Uppsala, Sweden). The vaccinia virus-infected CV-1 cells were transfected with vector DNA. After 48-h incubation, cells were frozen, thawed and vortexed for three rounds and sonicated. Virus-containing supernatants were plated on to confluent TK⁻ human osteosarcoma cell line (143B) in the presence of BUDr, and overlaid with Nobel Agar (Gibco BRL) containing X-gal. Blue plaques were selected, and sub-

cloned three times on Tk⁻ cells. The final lysates were assessed either for GM-CSF biological activity by measuring proliferation of 32D (G)/GM cells (for constructs containing the GM-CSF gene sequence), or by PCR reaction. Recombinant vaccinia viruses (VV) were propagated on confluent monolayers of HeLa cells.³³ Viral titers were determined by a plaque assay on HeLa cells using serial 10-fold dilutions. Infected cell monolayers were washed several times with PBS and stained with crystal violet solution to visualize viral plaques.

Immunization and challenge of mice

Genetic immunization: B6D2F1 mice (eight mice per group) were injected intramuscularly (i.m.) into the quadriceps with 75 μ g of DNA diluted in 150 μ l of phosphate buffer saline (PBS), using a 1-ml syringe with a 27-gauge needle. Mice were injected three times at 3-week intervals, and challenged intraperitoneally (i.p.) with 2×10^7 tumor cells 1 week after the last booster. Mice immunized with the vector expressing L-GM-CSF were used as a control. An additional negative control group was inoculated with PBS.

Viral immunization: B6D2F1 mice were injected subcutaneously (s.c.) once with 4×10^7 p.f.u. of virus using a 1-ml syringe with a 27-gauge needle. Two weeks after immunization, mice were challenged as described above.

Prime-boost vaccination: B6D2F1 mice were injected i.m. with 75 μ g of plasmid DNA, and 5 weeks later were boosted s.c. with a single dose of 4×10^7 p.f.u. of the vaccinia virus recombinant. Two weeks after the boost, mice were challenged with 70Z/3 cells. Control mice were immunized with either empty vector or just with vehicle PBS on the two occasions.

T cell depletion studies

Mice were depleted of specific T cell subsets by injection with rat monoclonal antibodies (mAb) to mouse CD4 (GK1.5, ATCC TIB 207) or CD8 (53-6.72, ATCC TIB 105). Each mouse was injected i.p. with 200 μ l of ascitic fluid (1:10 dilution), at a pre-determined dose shown to induce >95% depletion of the appropriate cell subset, as assessed by indirect immunofluorescence and cell sorting. The mAbs were injected at days -5, -3, and -1 before tumor challenge (day 0).

Statistical analysis

The statistical analysis was performed using Stata6 (Stata Corporation, College Station, TX, USA). The Log-rank test was used for the survival studies.

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