Vaccine site inflammation potentiates idiotype DNA vaccine-induced therapeutic T-cell, and not B-cell, dependent anti-lymphoma immunity

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ABSTRACT

Lymphoma idiotype protein vaccines have shown therapeutic potential in previous clinical studies, and results from a completed pivotal, Phase III controlled trial are promising. However, streamlined production of these patient-specific vaccines is required for eventual clinical application. Here we show that second generation, chemokine-fused idiotype DNA vaccines, when combined with myotoxins which induced sterile inflammation with recruitment of antigen-presenting cells at vaccination sites, were exceptional in their ability to provoke memory antitumor immunity in mice, compared with several TLR agonists. The combined vaccination strategy elicited both antigen-specific T-cell responses and humoral immunity. Unexpectedly, vaccine-induced tumor protection was intact in B-cell deficient mice, but was abrogated completely by T-cell-depletion in vivo, suggesting T-cell dependence. Furthermore, the optimal effect of myotoxins was observed with fusion vaccines which specifically targeted antigen delivery to antigen-presenting cells, and not with vaccines lacking a targeting moiety, suggesting that the rational vaccine design will require combination strategies with novel, pro-inflammatory agents and highly optimized molecular vaccine constructs. These studies also challenge the paradigm that antibody responses are the primary of idiotype-specific antitumor effects and support the optimization of idiotype vaccines designed to induce primarily T-cell immunity.
INTRODUCTION

Vaccine therapy, with the potential for eradicating residual disease and establishing long-term memory immunity to prevent relapses, may be a promising treatment for hematological malignancies. A successful vaccine strategy relies largely on the selection of a tumor-specific antigen, against which immune responses specifically target tumor cells while sparing normal tissues. Idiotype has been identified as the unique sequences embedded in the variable regions of the heavy and light chain of the immunoglobulin molecule expressed on the surface of B cells. Given that malignancies of mature and resting B cells arise from clonal proliferation of cells that express immunoglobulins with a unique variable region sequence, the idiotype of a given B-cell malignancy can serve as a tumor-specific antigen and has been exploited as a target for vaccine therapy \(^1\-^3\). Kwak \textit{et al.} initially evaluated individualized idiotype protein vaccines in human lymphoma patients \(^4\), and several other clinical trials of patients with lymphoma or myeloma have confirmed immunogenicity \(^5\-^7\). Among the most important findings of these studies was the demonstration that in patients with follicular lymphoma in minimal residual disease, as defined by complete remission (CR), autologous idiotype protein can be formulated into an immunogenic antigen when it is conjugated with a carrier protein, keyhole-limpet hemocyanin (KLH), and administrated with granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant \(^5\). In particular, the NCI Phase II clinical trial using this vaccination strategy resulted in molecular remissions in most patients whose lymphoma could be detected by a characteristic Bcl-2 translocation and long-term disease-free survival in first CR \(^5\). The encouraging results from this Phase II study led to pivotal,
Phase III controlled randomized clinical trials, one of which recently reported promising results.\(^8\)

The current technique of producing patient-specific, hybridoma-generated idiotype protein is labor-intensive. Accordingly, the development of 2nd generation idiotype vaccines, which simplifies the manufacturing process of idiotype proteins, is needed. To achieve these goals, efforts have been made to develop DNA vaccines that encode a short idiotype single-chain (sFv) polypeptide containing only the antigenic variable regions of light and heavy chains. Further modification of idiotype sFv for targeted antigen delivery strategies to specialized antigen-presenting cells (APC) was observed to induce protective immunity in mouse models against lymphoma and myeloma,\(^9,10\) which highlighted the potential of plasmid DNA encoding genetically modified idiotype sFv as next generation idiotype vaccines.

However, given that plasmid DNA is an inherently weak immunogenic, rational design of idiotype DNA vaccines should include combination strategies with other agents which can potentiate immunogenicity. The few adjuvants available for use in humans often induce suboptimal protective immunity.\(^11,12\) Recent reports suggest that various cellular elements of the innate immune system such as endogenous ligands of toll-like receptors are released following tissue necrosis.\(^13\) Given that the induction of robust adaptive immune responses requires intact innate immunity, components of the innate immune system are important for shaping the quality of adaptive immune responses. For example, TLR agonists, functioning through pattern recognition receptors on APC, improve the
potency of antigen-specific T-cell responses elicited by vaccines\textsuperscript{14-16}. Thus, we reason that the combination of vaccine therapy with such molecular adjuvants is critical for optimizing adaptive immune responses resulting from idiotype DNA vaccines.

Severe tissue necrosis and inflammation have long been noted in victims surviving from a viper bite. Cardiotoxin, a small myotoxic polypeptide present in viper venom has been recognized as the principal component responsible for tissue necrosis and inflammation. Although its name implies that it is toxic to the heart, intramuscular injection of mice with cardiotoxin was shown to induce a local tissue necrosis - muscle regeneration cycle without cardiotoxic effects\textsuperscript{17}. In this study, we demonstrated that idiotype DNA vaccine-triggered therapeutic effects were significantly enhanced by pretreating the vaccination sites with a low dose of cardiotoxin. Cardiotoxin causes inflammatory infiltration, triggering the recruitment of antigen-presenting cells, which in turn facilitates the activation of idiotype-specific adaptive immunity, including memory. Using B-cell deficient mice, we demonstrated the first time that anti-idiotype antibody response was not essential for the eradication of lymphoma cells; instead, T-cell immunity played the critical role in vaccine-induced tumor protection.

\textbf{METHODS}

\textbf{Cell line and animals:} A20 murine lymphoma cells were expanded in large scale, and aliquots were frozen down from the common passage used for the animal experiments described thereafter. A reproducible tumor titration on this passage of A20 cells was performed using groups of 10 Balb/c mice to identify the minimal lethal dose which was
found to be $2 \times 10^5$ cells/mice. Balb/c mice were obtained from the National Cancer Institute. Jh (Balb/c background) mice were purchased from Taconic Farms. All mice were maintained in a pathogen-free mouse facility according to institutional guidelines. All animal studies were approved by the Institutional Animal Care and Use Committee at The University of Texas M. D. Anderson Cancer Center.

**Adjuvants and Immunostimulants:** Cardiotoxin and crotoxin was kindly provided by Dr. Paul Reid (Receptopharm, Inc, Plantation, FL). For treatment, 6.8 μg of cardiotoxin or 0.1 μg of crotoxin (dose recommended by the manufacturer) was injected into quadriceps 5 days earlier before vaccination. At these doses, the myotoxins induced muscle degeneration-regeneration without causing significant systemic toxicity in mice. The Toll-like receptor agonists Poly I:C (TLR3 agonist), MPL (TLR4 agonist) (InvivoGen, San Diego, CA), M001 (TLR7 agonist) and M003 (TLR7/8 agonist) (3 M company, St. Paul, MN) were given respectively on the next day following vaccination at doses of 50 μg.

**Constructs and vaccination:** Plasmid DNA constructs encoding MCP3-sFv, MIP3α-sFv and Defensin2β-sFv, respectively, were generated in our early studies. Ova full-length cDNA was cloned from Ova-expressing B16 melanoma cells by RT-PCR, and genetically fused with MCP3. In prophylactic vaccination studies, groups of 10 mice were anesthetized and intramuscularly injected with 50 μg of plasmid DNA. A total of three vaccinations were given on days 0, 14, and 28. Cardiotoxin or crotoxin was given during the first 2 rounds of vaccination. Two weeks after final vaccination, a lethal dose
of $2 \times 10^5$ A20 lymphoma cells was given by i.p. Tumor-free mice surviving the prophylactic studies were re-challenged with $2 \times 10^5$ A20 cells, and followed for survival for 80 days. In therapeutic studies, mice were challenged with $2 \times 10^5$ A20 tumor cells on day 0 followed by vaccinations on days 1, 4, 8, and 18. Cardiototoxin was given 5 days earlier before the first and final vaccinations. In all animal studies, data were statistically analyzed by using the Kaplan-Meier method with a log-rank $P$ value.

**In vivo T-cell depletion:** T-cell depletion was performed by intraperitoneal injection of 200 μg of monoclonal antibody against CD8 (clone 2.43) and/or CD4 (clone GK1.5) as the schedule shows in Figure 4. The efficiency of T-cell depletion was assessed by staining PBMC with CD4-PE, CD8-FITC, and CD3-APC (BD Biosciences).

**Idiotype-specific immune responses:** T-cell–mediated immunity was assessed by examining the activation of idiotype-specific and tumor-reactive T cells. The immunized splenocytes were isolated, and *in vitro* activated for 5 days with bone marrow-derived dendritic cells pulsed with 5 μg/mL A20 sFv H-2K$^d$ epitope peptide (A20106-114). The stimulated splenocytes were then seeded in a 96-well ELISPOT plate at $2.5 \times 10^5$ cells/well either with the peptides or irradiated A20 tumor cells at a 5:1 T cell/stimulator ratio for 48 hours. IFN-$\gamma$-producing T cells were detected by using an IFN-$\gamma$ ELISPOT kit (BD Biosciences), and analyzed on a CTL ImmunoSpot® Analyzer (Cellular Technology Ltd.). The Student $t$ test was used for statistical analysis. The antibody response was determined by measuring serum levels of anti-idiotype antibodies by using
ELISA with recombinant A20 idiotype protein (Favrille Biotech, San Diego, CA) as reported previously \(^25\).

**Immunohistochemistry:** Balb/c mice were intramuscularly injected with 6.8 \(\mu\)g cardiotoxin in the quadriceps, and specimens were then collected, cryo-fixed, and stained for hematoxylin and eosin to identify cellular infiltration, and the cryo-sections were immunostained for antibodies for monocytes/macrophages (F4/80) and dendritic cells (CD11c).

**RESULTS**

**Combination therapy with myotoxins potentiated the tumor protection of lymphoma DNA vaccines**

Given that the components involved in tissue inflammation play an important role in triggering immune response, we tested several candidate adjuvants in this category for their potential to improve antitumor effects of a novel DNA vaccine encoding lymphoma idiotype, which was expressed in a single chain format, and genetically fused with monocyte chemotactic protein 3 (MCP3-sFv), as a strategy to target antigen delivery to chemokine receptors on antigen-presenting cells \(^10\). In head-to-head comparisons against conventional adjuvants and TLR agonists, cardiotoxin was the most potent in inducing protective antitumor immunity when combined with the idiotype vaccine. Specifically, syngeneic BALB/c mice which received pre-administration of 6.8 \(\mu\)M cardiotoxin at DNA vaccine injection sites were highly resistant to tumor challenge (A20 lymphoma, 90%) compared with DNA vaccine alone (10%, \(P<0.01\)) (Fig 1A). These results differed
from DNA vaccine combinations with Poly IC (TLR 3 agonist, Fig 1C), MPL (TLR 4 agonist, Fig 1D), M001 (TLR 7 agonist, Fig 1E) and M003 (TLR 7/8 agonist, Fig 1F) respectively, which failed to enhance tumor protection. The potent adjuvant effect of cardiotoxin on protective antitumor immunity was reproduced by crotoxin, another myotoxin with the main component of phospholipase A₂ that induces muscle degradation (Figure 1B). These results were further confirmed with two other chemotactic peptide-fused idiotype DNA vaccines, showing that cardiotoxin significantly enhanced tumor protection when combined with either defensin2β- (Figure 2A) or MIP3α-fused (Figure 2B) antigen. Taken together, these in vivo studies suggest that administering cardiotoxin or crotoxin together with chemokine-fused lymphoma idiotype is an effective strategy to enhance the therapeutic effect of this cancer vaccine.

**Cardiotoxin-combined vaccination elicited memory anti-tumor and therapeutic immunity.**

A unique advantage of active immunotherapy is its potential to establish memory immunity to prevent disease relapse, and thus the rational vaccine design should include strategies of inducing memory anti-tumor response. For this purpose, we examined if the cardiotoxin-combined idiotype vaccine therapy would favor the development of memory immunity against tumor re-challenge. Consistent with the experiment in Figure 1a, mice which received pre-treatment of cardiotoxin at vaccination sites were protected from lethal tumor challenge compared with DNA vaccine alone (Figure 3A). Cardiotoxin alone or together with a DNA vaccine encoding MCP3-fused HIV glycoprotein 120, serving as an irrelevant antigen, elicited no protection (Figure 3A). Tumor-free mice surviving this
primary challenge were then collected, and without any further treatment, the mice were re-challenged with the same lethal dose of tumor cells and followed for survival. More than 80% of mice protected by combination DNA vaccine plus cardiotoxin were resistant to tumor re-challenge, compared with less than 40% of mice protected by DNA vaccine alone (P=0.01), suggesting immune memory (Figure 3B). Moreover, this combined vaccination strategy also demonstrated its potential to eradicate established tumors in the therapeutic setting, as evident by long-term survival in >50% of tumor-bearing mice (Figure 3C).

**Cellular but not humoral immunity served as the immune mechanism for idiotype DNA vaccine-induced tumor protection**

We investigated the relative roles of idiotype-specific T cell and antibody immune responses in vaccine therapy. First, immunologic studies in vaccinated mice revealed the induction of tumor-specific cellular responses (Figure 4A). For example, the mean number of idiotype peptide-specific T cells per $2.5 \times 10^5$ splenocytes was $40 \pm 2.6$ in cardiotoxin-combined mice, compared with $18 \pm 3.8$ in mice receiving vaccine alone (P<0.01) and $33 \pm 8$ compared with $7 \pm 0.9$ for tumor-specific T cells, respectively (P<0.05). Next, depleting CD8+ T cells *in vivo* after vaccination plus cardiotoxin was clearly associated with reduced tumor protection, and depletion of both CD4+ and CD8+ T cell subsets abrogated protection completely (Figure 4b), suggesting a requirement for effector T cells in vaccine-induced antitumor immunity. T cell depletion was confirmed by showing the absence of CD8+ and/or CD4+ T cells in the peripheral blood (Figure 4c and d).
Similarly, vaccination also elicited humoral immunity, as shown by serum titers of antigen-specific antibodies which were substantially increased after combining DNA vaccination with cardiotoxin. High levels of anti-idiotype antibodies were maintained even after tumor challenge (Figures 5a and 5b). However, in contrast with effector T cells, B cells were not required for tumor protection, as DNA vaccine plus cardiotoxin protected both genetically B-cell deficient Jh mice and wild type mice equally from tumor challenge (Figure 5c). More than 80% of tumor-free Jh mice survived from the primary challenge were surprisingly highly resistant to tumor re-challenge, which suggests that anti-idiotype antibodies did not contribute principally to memory anti-tumor immunity (Figure 5d). The memory anti-tumor immunity developed in Jh mice was comparable to that found in vaccinated wild type counterparts (Figure 5d). Together, these data support a critical role for effector T cells activated by cardiotoxin plus idiotype DNA vaccination.

**Myotoxins induced sterile inflammation and recruitment of antigen-presenting cells**

To further elucidate the unexpected immunologic effects of cardiotoxin, we examined muscles at cardiotoxin injection sites histologically and observed marked cellular infiltration (Figure 6a). Further identification revealed the majority of infiltrating cells were dendritic cells and monocytes/macrophages (Figure 6a).

We hypothesized that the recruitment of antigen-presenting cells into vaccination sites by myotoxins might potentiate the action of the chemokine motif in the fusion vaccine to enhance vaccine-triggered anti-tumor immunity. This hypothesis was tested by
demonstrating that even though cardiotoxin administration was able to convert non-immunogenic, unfused antigen into an effective immunogen which induced antitumor immunity (Figure 6b), the optimal adjuvant effect of cardiotoxin was only observed with antigen fused to chemokine, which likely targets DC for a more efficient antigen delivery, as shown by our previous experiments that mixing free antigen and chemokine was not sufficient to induce immunity 10.

Discussion

Although non-Hodgkin’s lymphoma is responsive to chemotherapy and remission is common, the majority of patients eventually relapse. Therefore, novel, streamlined, potent therapeutic approaches are required. Lymphoma idiotype vaccine is a promising approach for eradication of minimal disease. In combination with chemotherapy, its potential to prevent relapses and to prolong disease-free survival has been supported by clinical studies 4,5,8,27. DNA vaccines represent a 2nd generation vaccine approach with the aim of simplifying the manufacturing process and improving the potency of antitumor immunity. The latter can be achieved by genetic modification of idiotype antigen for targeted delivery to antigen-presenting cells 9,10. For example, our previous studies showed that genetic fusion of lymphoma idiotype in single chain format with chemokines enhanced the immunogenicity of the antigen, and induced tumor protection in lymphoma mouse models 10,21.

However, most controlled trials of cancer vaccines, when used as monotherapy, have failed. It is clear that successful therapeutic vaccination will require combination
strategies, either with additional pro-inflammatory agents which can trigger the induction of specific immune responses, or by agents which provide release from immune suppression at the effector phase of the immune response. In our studies, when combined with idiotype DNA vaccines, myotoxins elicited potent antitumor immunity. Importantly, a desirable memory T-cell response was also elicited by these vaccine and cardiotoxin combinations. Myotoxins have been used previously to induce muscle regeneration and enhance uptake of DNA vaccines, with limited success. However, our data suggest an additional effect of cardiotxin and crototoxins inducing sterile inflammation (Fig. 6a). Future mechanistic studies are warranted to fully elucidate this previously undescribed immune effect on the microenvironment.

The potency of this combined vaccine therapy was also evident by the ability of cardiotoxin to convert an otherwise non-immunogenic antigen (sFv alone, Fig. 6B) into a protective immunogen. This feature is highly desirable in designing cancer vaccines as most candidate tumor-associated antigens are self proteins. The therapeutic efficacy of cancer vaccine is largely determined by its potential to break immune tolerance to these self-antigens. Thus, myotoxins may represent a new class of adjuvants which may be generally applied to the development of active immunotherapy against other types of malignancies.

Adaptive immunity includes cellular and humoral responses. As shown in current studies, both idiotype-specific T cells and antibodies were induced by combining idiotype DNA vaccines with myotoxins. However, the exact roles of effector T cells and antibodies in
vaccine-induced eradication of tumor cells are not fully elucidated. Antibodies have generally been thought to be the primary cellular mechanism underlying the antitumor effects of vaccines against lymphoma idiotypes. Serum level of anti-idiotype antibodies has been frequently used for monitoring the efficacy of vaccination in patients. Using antibody-deficient Jh mice, for the first time, we have shown that the antibody response was not required for idiotype-induced antitumor effects, even though the combination of vaccines with cardiotoxin strikingly enhanced serum anti-idiotype antibody titers. Prior studies in a different mouse lymphoma model, in which depletion of effector T cells in vivo abrogated tumor protection, also suggested a role for T-cell immunity. This novel finding suggests a focus on induction of T-cell immunity in designing future idiotype-based immunotherapy strategies. The significance of enhancing T cell-responses is apparent with the widespread use of rituximab in the treatment of B-cell NHLs, due to impaired humoral immunity as the result of B-cell depletion by rituximab.

Optimal antitumor immunity induced by cardiotoxin combinations required fusion vaccines which targeted receptors on DC for antigen delivery, exemplified by genetic fusion of antigen to chemokine receptor ligands (Fig. 6B). Our working hypothesis is that chemokine motif in the de novo synthesized fusion proteins facilitates APC to capture antigens via chemokine receptor-mediated endocytosis, and subsequently triggers antigen “cross-presentation”, loading antigens to MHC Class I pathway for T cell priming. This principle was shown in studies with a MIP3a-fused melanoma antigen gp100 protein, which when pulsed onto DC, activated the antigen-specific CD8+ T cells, and this effect was abrogated by blockage of the MHC Class I pathway. We have
previously shown that chemokine receptor ligands fused to antigen, such as defensin2β, can also function to induce DC maturation \cite{18}. Using this data collectively with our current results, we conclude that the ideal, fully optimized vaccine strategies should simultaneously create a favorable immune microenvironment to recruit and activate APC, and target antigen delivery to APC.

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AUTHORSHIP

Contribution: H.Q, S.C and S.S.N designed studies, performed studies, conducted data analysis and prepared the manuscript. Y.L and J.W conducted animal experiments. L.W.K developed the project, supervised research and wrote the manuscript together with Y.J.L.

CONFLICT OF INTEREST DISCLOSURE

All the authors declare no competing financial interests.
REFERENCES


Figure legends

Figure 1: Administration of myotoxins at vaccination sites significantly enhanced idiotype DNA vaccine-induced tumor protection. Ten BALB/c mice per group were injected i.m. with 6.8 μM cardiotoxin (a) or 0.1 μg crotoxin (b) followed by i.m. vaccination at the same site 5 days later with 50 μg plasmid DNA encoding MCP3 chemokine-fused A20 lymphoma-derived idiotype antigen (MCP3-sFv). TLR agonists including TLR3 agonist Poly I:C (c), TLR4 agonist MPL (d), TLR7 agonist (M001) (e) and TLR7/8 agonist (M003) (f) were given respectively on the next day of vaccination at a dose of 50 μg. A total of 3 vaccinations were given with an interval of 14 days. Two weeks after final vaccination, all mice were challenged with a lethal dose of $2 \times 10^5$ A20 lymphoma cells by i.p., and followed for survival for 80 days. Control mice were injected with plasmid DNA without candidate adjuvants, or PBS. Survival differences between groups were analyzed by logrank test. The data shown are from a single experiment, with results presented in multiple panels for clarity.

Figure 2: Prophylactic antitumor effects of additional DNA vaccines were significantly improved by the combined vaccination therapy. In prophylactic studies, 10 BALB/c mice per group were immunized with two DNA vaccines encoding the idiotype single chain antigen fused to either defensin2β (a) or MIP3α (b), respectively, with or without cardiotoxin, and then challenged with A20 tumor cells as in Figure 1 and followed for survival.
Figure 3: The combined vaccination therapy elicited memory and therapeutic anti-tumor immunity. (a) For primary challenge, 10 BALB/c mice per group received were vaccinated 50 μg MCP3-sFv plasmid DNA together with cardiotoxin as Figure 1. Control mice were injected with plasmid DNA without cardiotoxin pretreatment, PBS or cardiotoxin alone, or with plasmid DNA encoding an irrelevant, HIV gp120 antigen, fused with MCP3. Following 3 vaccinations, all mice were challenged with a lethal dose of 2×10⁵ A20 lymphoma cells by i.p., and followed for survival for 80 days. (b). Tumor-free mice collected from the primary challenge experiments were re-challenged with 2×10⁵ A20 tumor cells i.p. and followed for survival. Data represent combined results from 3 independent experiments. Survival differences between groups were analyzed using the logrank test. (c) In therapeutic studies, 10 BALB/c mice per group were first inoculated with 2×10⁵ A20 tumor cells i.p. on day 0. On days 1, 4, 8 and 18 the mice were then vaccinated with plasmid DNA encoding MCP3-fused idiotype sFv w/ or w/o cardiotoxin pre-treatment 5 days before the 1st and last vaccination. Control mice were injected with cardiotoxin or PBS alone. Data represent combined results from 2 independent experiments.

Figure 4: Combined cardiotoxin and DNA vaccine elicited potent, T-cell dependent, tumor antigen-specific immunity. (a) Splenocytes pooled from 5 BALB/c mice injected i.m. with MCP3-sFv plasmid DNA w/ or w/o cardiotoxin as in Figure 1, or cardiotoxin alone, 10 days earlier were stimulated in vitro with bone marrow-derived DC pulsed with 5 μg/ml MHC Class I-binding A20 idiotype epitope peptide²³ for 5 days. The stimulated cells (2×10⁵ per well) were plated with either the peptide or irradiated A20 tumor cells at
a 5:1 T cell/stimulator ratio\textsuperscript{24}, respectively, and analyzed for INF\textgamma production by ELISPOT after 48 h. Differences between groups were analyzed using the Student $t$-test (** $P<0.01$; *$P<0.05$). (b-d) \textit{In vivo} T cell depletion was achieved by i.p. injection of 200$\mu$g anti-CD8 mAb (clone 2.43 alone, CD8 depletion) or with 200$\mu$g anti-CD4 mAb (clone GK1.5, CD4/CD8 depletion) according to the schedule in (d). T cell depletion was performed on mice vaccinated with cardiotoxin plus MCP3-sFv DNA vaccine as in Figure 1 (10 BALB/c mice per group). Controls received rat IgG instead of T cell depletion antibodies. After confirming the efficiency of T cell depletion, which was determined by the presence of CD8 and CD4 T cells in the peripheral blood samples (c), all the mice were challenged with $2\times10^5$ A20 tumor cells shown in Figure 1 and followed for survival for 80 days. The data represent 2 independent experiments.

\textbf{Figure 5: Ant-idiotype antibody response was not required for the vaccine-induced tumor protection.} (a-b) Serum samples obtained from mice vaccinated as in Figure 1 were examined for anti-idiotype antibodies by ELISA using plates coated with recombinant A20 idiomotype protein (gift from D Gold, Favrille, San Diego, CA)\textsuperscript{25}. Bound antibodies were detected by HRP-conjugated anti-mouse IgG1. Anti-idiotype antibodies were primarily observed after the 1\textsuperscript{st} boost dose, peaking after the 2\textsuperscript{nd} boost, and persisting after tumor challenge. (c) Ten wild type BALB/c (WT) or antibody-deficient J\textsubscript{h} mice per group were vaccinated with MCP3-sFv DNA plus cardiotoxin as shown in Figure 1, or saline, then challenged with tumor and followed for survival. (d) Tumor-free J\textsubscript{h} and WT mice from vaccinated groups in (c) were re-challenged with tumor and followed for survival (J\textsubscript{h} n=8; WT n=7).
Figure 6: Cardiotoxin administration recruits APC, and converts non-immunogenic, unfused antigen into a protective vaccine. Cardiotoxin alone-treated (6.8 μM by i.m. injection) quadriceps were collected, cryo-fixed, sectioned and stained for H&E. Further identification of infiltrated cells was performed by immunostaining tissue sections with cell-specific markers for DC (CD11c) and monocytes/macrophages (F4/80) (representative of six quadriceps analyzed). (b) 10 syngeneic BALB/c mice per group were immunized with DNA vaccines encoding chemokine-fused (MCP3-sFv) or free antigen (sFv) plus cardiotoxin and challenged with lethal tumor as in Figure 1. The data are representative of two identical experiments.
Figure 1

A

Days after tumor challenge

Overall survival rate (%)

P<0.01 vs MCP3-sFv
P<0.01 vs Saline

P<0.05 vs Saline

Crototoxin + MCP3-sFv
MCP3-sFv alone
Saline

B

Days after tumor challenge

Overall survival rate (%)

P<0.01 vs MCP3-sFv
P<0.01 vs Saline

Poly IC+MCP3-sFv
MCP3-sFv alone
Saline

C

Days after tumor challenge

Overall survival rate (%)

P<0.01 vs MCP3-sFv
P<0.01 vs Saline

MPL+MCP3-sFv
MCP3-sFv alone
Saline

D

Days after tumor challenge

Overall survival rate (%)

P=0.01 vs MCP3-sFv
P<0.01 vs Saline

M001+MCP3-sFv
MCP3-sFv alone
Saline

E

Days after tumor challenge

Overall survival rate (%)

M003+MCP3-sFv
MCP3-sFv alone
Saline

F

Days after tumor challenge

Overall survival rate (%)

M003+MCP3-sFv
MCP3-sFv alone
Saline
Figure 2

A)

Days after tumor challenge

Overall Survival (%)

P<0.01 vs DF2b-sFv
P<0.01 vs Saline
P<0.05 vs Saline

B)

Days after tumor challenge

Overall survival rate (%)

P<0.01 vs MIP3a-sFv
P<0.01 vs Saline
P<0.01 vs Saline
Figure 3

A. Overall survival rate (%)

B. Tumor free (%)

C. Overall Survival
Figure 4

A

Number of spots/2.5x10^5 cells

Peptide  MCP3-sFv+Cardiotoxin  MCP3-sFv  Cardiotoxin

Days after tumor challenge

Overall survival rate (%)

B

Vaccination: MCP3-sFv+Cardiotoxin

C

untreated  Control IgG  CD8 depletion  CD4/CD8 depletion

D

Check peripheral blood T cell population
Figure 5

A. MCP3-sFv+cardiotoxin vs MCP3-sFv alone

B. MCP3-sFv+cardiotoxin

C. Primary challenge

D. Secondary (re-)challenge

Vaccination: MCP3-sFv+Cardiotoxin
Figure 6

[A] Control

Cardiotoxin-treated

CD11c

F4/80

[B] Overall survival rate

Days after tumor challenge

- MCP3-sFv+Cardiotoxin
- sFv+Cardiotoxin
- sFv
- Saline

P = 0.015 vs sFv+Cardiotoxin
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