Genetic fusion of chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity

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We converted a model, syngeneic, nonimmunogenic tumor antigen into a vaccine by fusing it with a proinflammatory chemokine. Two chemokines, interferon inducible protein 10 and monocyte chemotactic protein 3, were fused to lymphoma Ig variable regions (sFv). The sFv–chemokine fusion proteins elicited chemotactic responses in vitro and induced inflammatory responses in vivo. Furthermore, in two independent models, vaccination with DNA constructs encoding the corresponding fusions generated superior protection against a large tumor challenge (20 times the minimum lethal dose), as compared with the best available protein vaccines. Immunity was not elicited by controls, including fusions with irrelevant sFv; fusions with a truncated chemokine that lacked receptor binding and chemotactic activity; mixtures of free chemokine and sFv proteins; or naked DNA plasmid vaccines encoding unlinked sFv and chemokine. The requirement for linkage of conformationally intact sFv and functionally active chemokine strongly suggested that the mechanism underlying these effects was the novel targeting of antigen presenting cells (APC) for chemokine receptor-mediated uptake of antigen, rather than the simple recruitment of APC to tumor by the chemokine. Finally, in addition to superior potency, these fusions were distinguished from lymphoma Ig fusions with granulocyte-macrophage colony-stimulating factor or other cytokines by their induction of critical effector T cells.

Keywords: interferon inducible protein 10, monocyte chemotactic protein 3, chemokine fusion, antigen presenting cell targeting,idiotypic vaccine

Chemokines are a group of small (7–15 kDa) secreted proteins that induce inflammatory response by orchestrating the selective migration, diapedesis, and activation of blood-borne leukocytes[1–3]. Chemokines act by binding to a specific cell-surface heptahelical G-protein–coupled receptor, which is internalized after binding with the ligand[4–6]. Moreover, chemokines may have potent effects in promoting tumor immunity[7–9]. For example, interferon inducible protein 10 (IP-10) can generate a T-lymphocyte–dependent antitumor response by recruiting other lymphocytes, neutrophils, and monocytes[1–3,7–9]. In addition, monocyte chemotactic protein 3 (MCP-3) is a potent chemoattractant for monocytes and dendritic cells, T lymphocytes, basophils, and eosinophils[1–3,10]. Importantly, most antigen presenting cells (APCs), particularly dendritic cells, express CCR1, CCR2, and CCR3 chemokine receptors, which bind MCP-3 (refs. 1, 11, and 12).

Clonal B-cell malignancies express variable region sequences (idiotypes) on the Ig receptor, which can serve as potential tumor-specific antigens[13]. However, a major obstacle is that syngeneic lymphoma Ig protein is nonimmunogenic and therefore requires conjugation to immunogenic, exogenous carriers (e.g., keyhole-limpet hemocyanin [KLH])[14,15]. Even then, Ig–KLH conjugates elicit primarily antibody responses, with little evidence of T-cell responses against the idiotype. Recent advances in antibody engineering make it possible to produce a refined lymphoma-specific single-chain Ig (sFv), consisting solely of linked V_H and V_L domains that retain the conformation of the native Ig[16,17]. However, KLH crosslinked to sFv failed to induce the anti-idiotypic antibody response usually observed with native Ig–KLH conjugates, due to disruption of the fragile conformation of sFv (data not shown).

We explore a novel strategy for induction of antitumor immunity, in which APCs are specifically targeted in vivo by a fusion protein consisting of chemokine and tumor antigen. We demonstrate that genetic fusion of chemokine moieties to lymphoma-derived sFv, a model nonimmunogenic antigen, converted this "self" tumor antigen into a potent immunogen. In both murine lymphoma models tested, 38C-13 (ref. 18) and A20 (ref. 19), immunization with either chemokine–sFv protein or naked DNA vaccines encoding the fusion elicited a T-cell dependent, protective antitumor immunity.

Results

Chemokine-fused sFv proteins retain conformation of native lymphoma-derived Ig and functional properties of chemokine. Ig variable region fragments were cloned by reverse transcription PCR from two different B-cell lymphomas, 38C-13 (ref. 19) and A20 (ref. 20). The fragments were arranged as sFv and produced as recombinant fusion proteins with either IP-10, MCP-3, control viral epitopes DomA (a 40–amino acid fragment containing major B- and T-cell epitopes of S antigen), or PreS2 of hepatitis B surface antigen (HBsAg) (Fig. 1). Recombinant fusion proteins were purified from *Escherichia coli*[20] and fully characterized. The proper folding of sFv proteins was demonstrated by their ability to inhibit polyclonal or monoclonal anti-idiotypic antibodies binding to 38C-13–derived native Ig protein (Ig38) (Fig. 2). In contrast, control fusions lacking a spacer (IP10dsFv38), fusions with sFv derived from the irrelevant lymphoma, or fusions with viral epitopes DomA and PreS2 failed to inhibit binding to native 38C-13, suggesting that they did not retain proper folding of the antigen. Folding

was not affected by the arrangement of sFv in V_H–V_L or inverse V_L–V_H orientation (INV).

Next, binding experiments were conducted using confocal microscopy analysis of purified T cells and unfractionated murine splenocytes. IP-10 and MCP-3 sFv fusions (Fig. 3A, panels C and E), but not control viral epitope fusions (DomAsFv38; Fig 3A, panel A), were found to bind their respective chemokine receptors. Binding was specific, as it was inhibited by native ligands IP-10 or MCP-3, respectively (Fig. 3A, panels D and F). Moreover, no binding was detected with the truncated IP-10 sFv fusion, IP10TsFv38, which contained a 9–amino acid. deletion of the amino-terminal portion of IP-10 (Fig. 3A, panel B). Similarly, binding of ^{125}I-labeled free chemokine was specifically inhibited by chemokine fusion proteins (data not shown).

We further tested the ability of the samples to induce in vitro and in vivo chemotaxis. Both chemokine-sFv proteins (IP10sFv38 and MCP3sFv38) but not control PreS2sFv38, induced in vitro chemotaxis of murine lymphocytes in a dose-dependent manner, with a maximum activity at 100 ng/ml (Fig. 3B). These samples also induced in vivo chemotaxis in syngeneic mice. Histologic evaluation of the site of subcutaneous injection of 10 μg protein showed that injection of IP10sFv38 or MCP3sFv38, but not PreS2sFv38, was associated with substantial numbers of infiltrating mononuclear cells, and fewer polymorphonuclear cells, in the dermis and subcutaneous layers (Table 1). Taken together, these results suggest that IP-10 and MCP-3 fused sFv proteins retained both the conformation of native antigen and functional properties of their respective chemokine moieties.

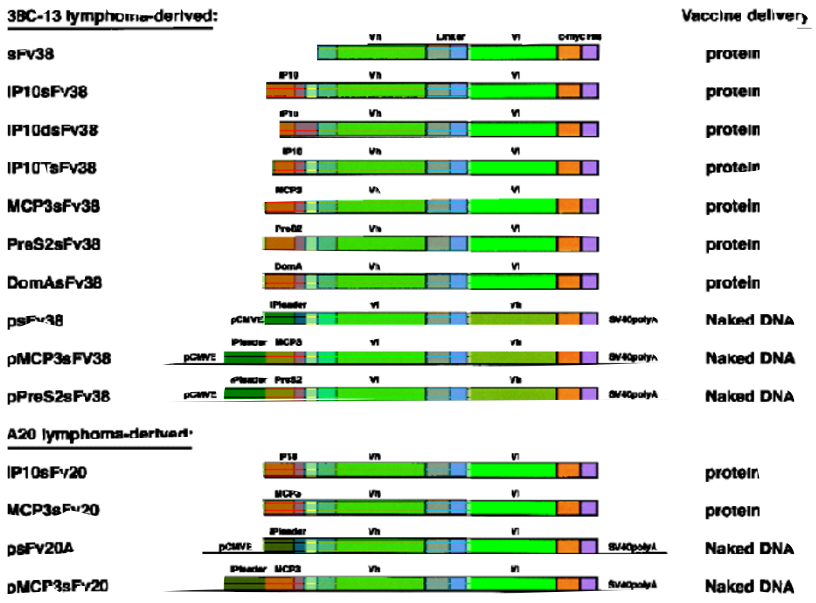

Figure 1. Lymphoma fusion protein and DNA genetic constructs. Expression vectors both for *E. coli*-derived proteins and naked DNA vaccines for 38C-13 (ref. 18) and A20 (ref. 19) lymphomas are shown. DNA fragments encoding IP-10, MCP-3, or control viral epitopes DomA (a 40–amino acid fragment containing major B- and T-cell epitopes of S antigen) or PreS2 of HBsAg, were fused in frame with DNA encoding sFv. IPl0TsFv38 and IP10dsFv38 are identical to IPl0sFv38, except for truncation of the 9 N-terminal residues of IP10 and lack of a spacer, respectively. Protein vaccines were expressed in modified pet11d vector (Stratagene, La Jolla, CA), and naked DNA plasmid vaccines were cloned in pCMVE/AB.

Chemokine fusion renders sFv immunogenic and induces antitumor immunity. C3H/HeN mice were immunized twice biweekly with 100 μg IP10sFv38 or MCP3sFv38 fusion proteins in phosphate-buffered saline (PBS). Their sera were then assayed for anti-idiotypic antibody response using Ig38. Both IP-10 and MCP-3 sFv fusions elicited significant anti-idiotypic antibody responses (mean ± standard deviation, 77 ± 41 and 13 ± 3 μg/ml, respectively; Fig. 4A). This result was in distinct contrast to the lack of any anti-idiotypic antibody response observed in mice injected with a truncated chemokine sFv fusion that did not retain chemokine activity (IP10TsFv38), sFv38 alone, or fusions that did not retain the native antigen conformation (IP10dsFv38 or Pres2sFv38). The highest antibody levels (286 ± 78 μg/ml) were induced by immunization with chemically conjugated Ig38–KLH.

Ten mice per group were immunized with either IP10sFv38 or with control A20 sFv fused to IP-10 (IP10sFv20) as above (same mice in Fig. 4A). Two weeks after immunization they were challenged with 38C-13 tumor cells at 20 times the minimum lethal dose. The survival of controls immunized with IP10sFv20 was indistinguishable from animals receiving free sFv38 or PBS, pointing to the absence of any nonspecific effects of IP-10 on antilymphoma immunity (Fig. 5A). Similarly, no survival increase was detected in mice immunized with the truncated IP-10 fusion, IP10TsFv38 (data not shown). In contrast, significantly prolonged survival was observed in mice immunized with IP10sFv38 (median survival, 22 days vs 12 days for IP10sFv20, log rank $p<0.001$). Moreover, these results suggested that linkage of Ig variable regions in either orientation, V_H–V_L or V_L–V_H, resulted in equivalent antigen processing and presentation to the immune system (compare IP10sFv38 with IP10sFv38[INV]; Fig. 5A).

Naked DNA immunization with plasmid encoding MCP-3 sFv fusion elicits potent protective antitumor immunity. To further

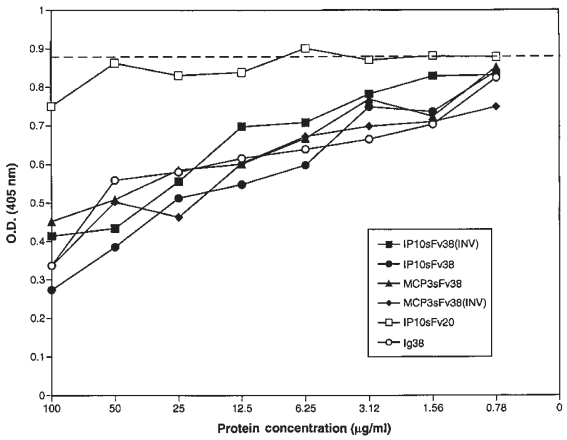

Figure 2. Effect of chemokine fusions on folding of sFv antigen. sFv-chemokine fusion proteins were tested for their ability to inhibit binding of anti-idiotypic sera from mice immunized with Ig38–KLH to native IgM38 protein. The broken line refers to no inhibitor. Ig38 and IP10sFv20 (IP-10 fused to irrelevant sFv) were used as positive and negative controls, respectively. Similar results were obtained with inhibition of an anti-idiotype Mab (S1C5, provided by M. Kaminski, University of Michigan, Ann Arbor, MI).

improve the potency of the antitumor immunity, we constructed expression vectors for particle-mediated DNA vaccine delivery by cloning fusion genes under the promoter-enhanced sequence from the cytomegalovirus (CMV) early gene (Fig. 1). Mice immunized intradermally with control plasmids psFv38 alone, pPreS2sFv38, or MCP-3 fused to sFv from an irrelevant lymphoma (pMCP3sFv20) failed to generate significant amounts of anti-idiotypic antibody. In contrast, immunization with MCP-3 or with IP-10 fused to 38c-13 sFv elicited high levels of specific anti-idiotypic antibody (909 ± 625 and 752 ± 660 μg/ml, respectively; Fig. 4B). Importantly, these levels

of antibody were comparable to those elicited by Ig38–KLH, the most effective hapten-carrier immunogen (576 ± 104 μg/ml). Immunization with chemokine-sFv plasmids or Ig–KLH elicited mostly IgG1 subclass antibodies with insignificant amounts of IgG2a and IgG2b (data not shown).

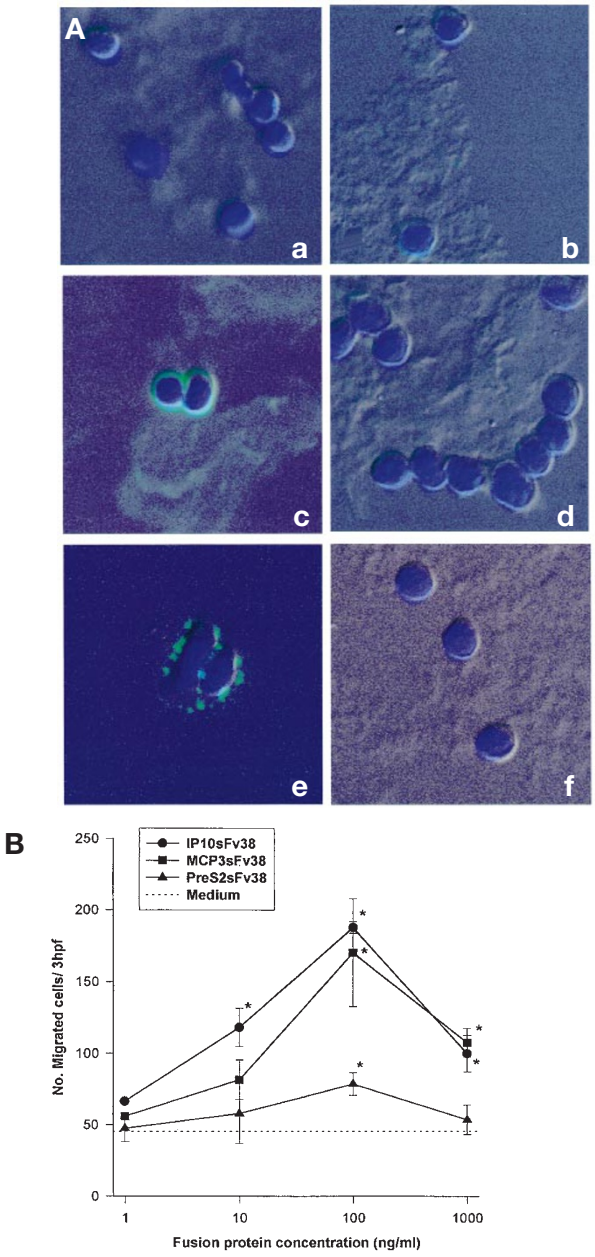

Figure 3. Receptor binding and biologic function of chemokine-sFv fusions. (A) Chemokine binding assay using laser confocal microscopy[5]. 10^6 purified T cells per ml from C3H mice were incubated with 100 nM chemokine-sFv (IP10sFv38, panel C or MCP3sFv38, panel E), control viral epitope-sFv (DomAsFv38, panel A), or truncated IP10 fusion (IP10TsFv38, panel B) for 1 h at 37°C. For ligand competition, 100 nM chemokine-sFv was co-incubated with 500 nM of the corresponding chemokine (IP-10, panel D or MCP-3, panel F). Ligand competition with irrelevant chemokines did not inhibit chemokine-sFv binding (data not shown). (B) T-cell migration in vitro was assessed by 48-well microchemotaxis chamber technique as described[32]. Purified murine splenic T cells were prepared by high-affinity negative selection (R&D System, Minneapolis, MN). The chemotaxis assay was performed at 37°C for 2 h. The number of migrated cells in three high power fields (400X) was counted by light microscopy after coding the samples. The results are expressed as the mean ± standard error of triplicate samples. *p<0.02, compared with medium alone.

We performed an additional experiment to determine whether the chemokine moiety must be linked to sFv to render it immunogenic. Mice were immunized with either pMCP3sFv38, free psFv38, or a mixture of DNA constructs expressing unlinked free sFv38 and MCP-3 (provided by pMCP3sFv20). Again, MCP-3 fused to sFv38 elicited high levels of anti-idiotypic antibody (503 ± 264 μg/ml) at levels comparable to Ig38–KLH (244 ± 37 μg/ml; Fig. 4C). However, unlinked psFv38, alone or in combination with pMCP3sFv20, failed to induce antibody production. Thus, the ability of chemokines to render sFv immunogenic required that the chemokine be physically linked with the antigen

To test for the protective effect of the naked DNA vaccines, groups of 10 mice immunized with pMCP3sFv38 or control plasmids were challenged with tumor 2 weeks after the last of three serial immunizations. The survival of mice receiving pPreS2sFv38, MCP-3 fusion to A20 sFv, or free psFv38 was not significantly different from those receiving PBS (Fig. 5B and C). In contrast, mice immunized with

Table 1. Inflammatory effect of administration of chemokine-sFv in mice.

Animal no.	Treatment 10 μg	Dermis PMN	Dermis MNC	Subcutis PMN	Subcutis MNC
1	IP10sFv38	–	1F	–	3F
2	IP10sFv38	1F	3F	–	1F
3	IP10sFv38	1MF	3MF	1MF	3F
4	MCP3sFv38	1F	3F	1MF	2MF
5	MCP3sFv38	–	2F	–	2MF
6	MCP3sFv38	1F	3F	–	2MF
7	PreS2sFv38	–	–	–	–
8	PreS2sFv38	–	–	–	1MF
9	PreS2sFv38	–	–	–	–
10	PBS	–	–	–	–

C3H/HeN mice were injected subcutaneously with 10 μg of the indicated fusion protein in PBS. After 72 h, the injection site was biopsied, fixed, stained with hematoxylin and eosin, and examined microscopically on coded slides. Numbers of infiltrating polymorphonuclear (PMN) and mononuclear (MNC) cells were scored as follows: –, no significant lesion; 1, mild; 2, moderate; 3, severe; F, focal; mF, multifocal. The endotoxin content of the preparations injected was 0.5–1 U.

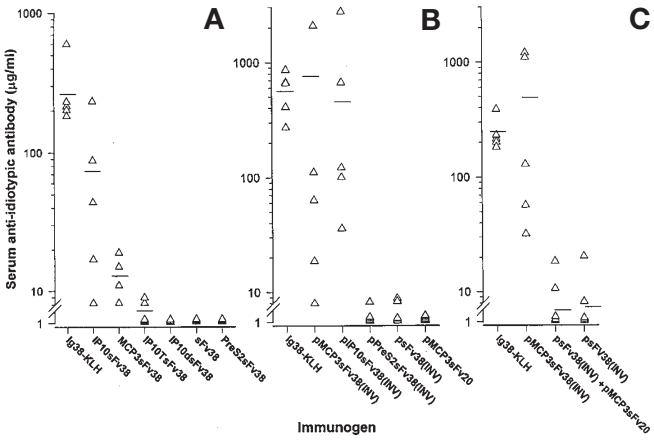

Figure 4. Antibody responses following immunization with chemokine-sFv fusions, but not sFv alone or mixtures of chemokine and sFv. Six- to 9-week old female syngeneic C3H/HeN mice were immunized (A) intraperitoneally with 100 μg fusion protein or Ig38-KLH in PBS, or (B and C) intradermally by Accell gene delivery device (Agracetus) with plasmid DNA[35]. Serum anti-idiotypic antibody levels were determined by ELISA as described[33]. Specificity for idiotype was shown by the lack of binding on a control, isotype-matched IgM (TEPC-183, Sigma, data not shown). Bars indicate the mean levels of five individual mice per group. (C) Mice were immunized either with a plasmid coding for MCP3sFv38 fusion, in which antigen is physically linked with the chemokine moiety, or a mixture of DNA constructs expressing unlinked sFv38 and pMCP3sFv20 (psFv38+pMCP3sFv20).

pMCP3sFv38 demonstrated significant protective immunity (40% survival, log rank $p<0.01$ vs pPreS2sFv38 and pMCP3sFv20, respectively; Fig. 5B–D). Furthermore, for the first time, DNA vaccine formulation of this antigen provided protective immunity exceeding that of Ig38–KLH protein; that is, 11/30 vs 3/30 survivors, respectively ($p<0.008$ by Fisher's exact test; log rank $p = 0.05$; Fig. 5B–D pooled).

Role of effector T cells in protective antitumor immunity elicited with MCP-3 sFv fusion. Because the superior protective antitumor immunity elicited by pMCP3sFv38, compared with Ig38–KLH, could not be attributed to a difference in magnitudes of antibody responses, we investigated the possibility that pMCPsFv38 generated a protective T-cell response. Two weeks after immunization with pMCP3sFv38, groups of 10 mice were randomly assigned to treatment with specific monoclonal antibody (Mab) to deplete CD8 or CD4 T cells, or with normal rat IgG as a control. Treatments were administered every day for three doses before tumor challenge. Comparison of survival of Mab-treated and control-treated immunized mice revealed a loss of protection for groups receiving either anti-CD8 (0% vs. 40% survival, respectively; Fig. 5C) or anti-CD4 Mab (0% vs. 30% survival, respectively; Fig. 5D). Taken together, these experiments suggest that effector T cells are required for protective antitumor immunity elicited by pMCP3sFv38 ($p<0.004$ by Fisher's exact test, log rank $p = 0.02$, T-cell depleted vs rat IgG–treated).

MCP-3 fusion also elicits protective antitumor immunity in the second lymphoma model. MCP-3 fusion also rendered A20 lymphoma-derived sFv highly immunogenic in syngeneic mice. Specifically, immunizations with both MCP3sFv20 fusion protein (Fig. 6A) and DNA construct, pMCPsFv20 (Fig. 6B), elicited protective immunity against A20 lymphoma challenge. Moreover, in this model, MCP3sFv immunization was also superior to Ig–KLH, which produced no survivors (log rank $p<0.05$). In addition, similar to the 38C-13 tumor model, IP-10 fusion with sFv20, which was previously demonstrated to retain both proper idiotype folding and the biologic activity of IP-10 (data not shown), failed to elicit protective immunity. The failure of IP-10 fusion to render sFv20 immunogenic provided an additional opportunity to evaluate whether simply mixing biologically active MCP-3 with nonimmunogenic sFv was sufficient to trigger systemic immunity to the sFv. Mixing of MCP-3, supplied by MCP3sFv38, together with IP10sFv20 failed to reproduce the protective immunity observed with MCP3sFv20 (0% vs. 20–40% survival, respectively, log rank $p = 0.002$). This result provides additional evidence, in the second model, that the chemokine must be physically linked to the antigen.

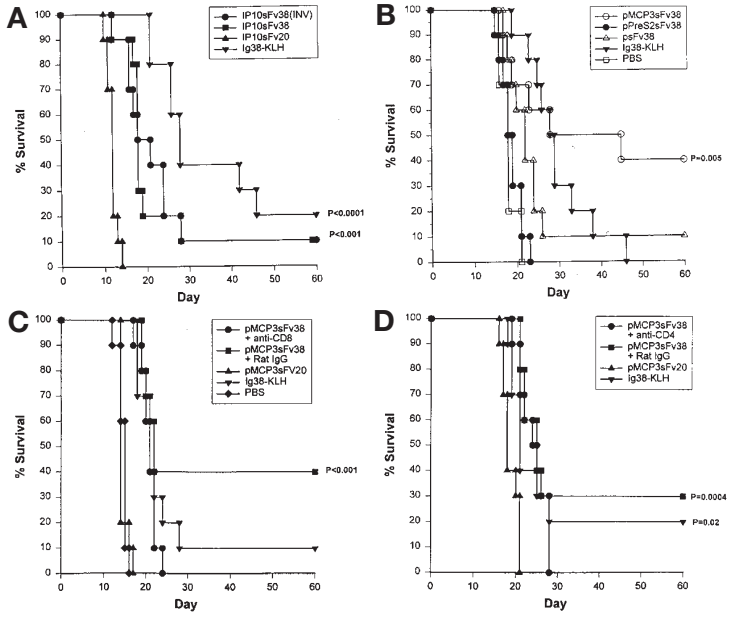

Figure 5. Levels of protective immunity following immunization with chemokine-sFv fusion protein and DNA vaccines. (A) Ten syngeneic C3H/HeN mice per group were immunized intraperitoneally in PBS without use of any adjuvants. Formulations consisted of 100 µg IP10sFv38 or IP10sFv38(INV), Ig38-KLH, or IP10 fused to A20 lymphoma sFv protein (IP10sFv20), administered three times every 2 weeks. Two weeks after the last immunization, mice were challenged intraperitoneally with 2000 38C-13 lymphoma cells from a single preparation of tumor and followed up for survival. Differences in survival between groups was determined by nonparametric log rank test (BMDP statistical software, Los Angeles). *p* values refer to comparison with IP10sFv20. (B) Ten syngeneic C3H/NeN mice per group received three intradermal immunizations of plasmid DNA every other week by gene gun, followed by tumor challenge as above. *p* values refer to comparison with pPres2sFv38. (C and D) Requirement of effector T cells for protective antitumor immunity. In separate experiments, 10 mice per group were immunized with pMCP3sFv38 plasmid, as above, and randomly allocated to treatment with anti-CD8 Mab 53.6.72, anti-CD4 Mab GK1.5, or normal rat IgG (Sigma). Control mice immunized with plasmid expressing MCP3 fused to A20 sFv (pMCP3sFv20) received normal rat IgG. *p* values refer to comparison with pMCP3sFv20. Flow cytometry analysis of splenocytes from normal mice treated with these Mabs in parallel 1 and 2 weeks after treatment confirmed a >90% depletion of the appropriate subset with normal levels of the other subset.

Discussion

Taken together, these data demonstrate that fusion of chemokines MCP-3 and IP-10 to a model self tumor antigen can convert nonimmunogenic sFv into a potent immunogen. The precise mechanism of the "carrier" activity of IP-10 and MCP-3 remains to be elucidated. Delivery of chemokines to tumor cells, resulting in nonspecific recruitment of effector cells, has been reported[7–9]. However, we propose a novel mechanism for the triggering of antitumor immunity: that the chemokine moiety targets APCs for efficient receptor-mediated uptake and processing of sFv.

The most compelling data supporting this hypothesis demonstrated that chemokine alone failed to elicit antitumor immunity. This was shown by the absence of humoral or protective immunity in mice immunized

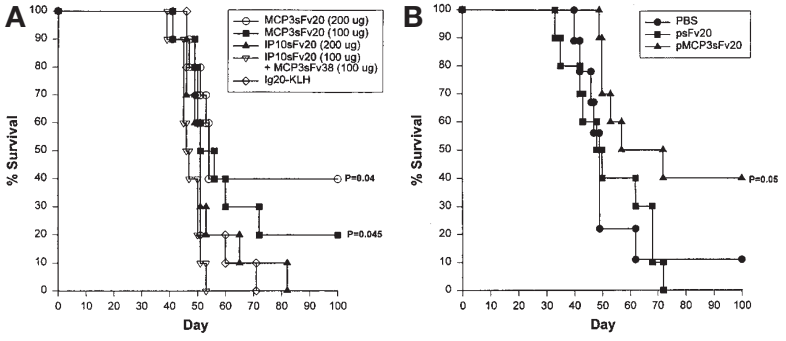

Figure 6. Comparison of chemokine-sFv fusions derived from the A20 lymphoma with Ig–KLH, and requirement for physical linkage between chemokine and antigen. (A) Ten syngeneic Balb/C mice per group were immunized intraperitoneally twice at 2-week intervals with 100–200 µg IP-10 or MCP-3 fused to sFv20 protein in PBS, and then challenged intraperitoneally with 10^5 A20 tumor cells 2 weeks after the last immunization. *p* values refer to comparison with Ig20–KLH. To determine role of free vs linked chemokine, 100 mg IP10sFv20 (which failed to elicit protection) was co-injected with 100 µg MCP-3 fused to irrelevant sFv38 (IP10sFv20 + MCP3sFv38). (B) Similarly, survival data of mice immunized with plasmids encoding sFv20 or MCP3 sFv20 fusion. *p* values refer to comparison with psFv20.

with either a mixture of free, unlinked chemokine and sFv (Figs. 4C and 6A), or with chemokine fused to irrelevant sFv. Moreover, immunity was observed only when the antigen was correctly folded and linked with chemokine that retained its full biologic activity. Finally, our data suggest that interaction with the chemokine receptor was required, because sFv fusion with a truncated IP-10 (without the receptor binding site) failed to elicit significant humoral response (Fig. 4A). CXC- and CC-chemokine receptors are known to be internalized after binding to ligand[4–6].

In vitro studies in our laboratory are in progress to obtain direct evidence of targeting the APC. The only other similar example was reported during this manuscript preparation by Boyle et al.[21], who targeted processing of human Ig Fc portion by DNA immunization with plasmids expressing fusions of this model antigen with L-selectin or CTLA4 in mice. However, the efficacy of this approach was limited by the fact that human Fc (xenogeneic) was immunogenic by itself, and its immunogenicity was simply enhanced by fusion. In contrast, our results may provide a more general strategy for targeting otherwise nonimmunogenic antigens for efficient processing and presentation.

With regard to vaccine development, the sFv–chemokine fusions are novel for several reasons. First, the levels of protective antitumor immunity achieved by MCP3-sFv fusion DNA vaccines were superior in magnitude to those of the prototype Ig–KLH protein, the best available formulation currently in clinical testing[15,22]. In fact, in the second tumor model, A20, vaccinations with MCP-3-sFv fusion protein (without adjuvants) or with DNA were the only formulations to elicit protective antitumor immunity, while all other approaches tested previously failed, including prototype Ig–KLH (Fig. 4D). It should be acknowledged, however, that the potency of Ig–KLH protein can be increased by administration with immunologic adjuvants in some models.

Second, the generation of $CD8^+$ T-cell immunity distinguishes these fusions from previously reported fusions of lymphoma idiotype with granulocyte-macrophage colony-stimulating factor[23] and from other DNA vaccines that elicited exclusively antibody responses[24,25]. In general, in the 38C-13 model, humoral responses were required, but not sufficient, for protection, and antibody titers did not correlate with survival times of individual mice. This discrepancy is exemplified by the observation that mice immunized with pIP10sFv38, despite eliciting high levels of anti-idiotypic antibody (Fig. 4B) comparable to pMCP3sFv38 vaccine, were not protected from tumor challenge (data not shown). Another recent report suggested that in murine models A31 lymphoma and 5T33 myeloma, vaccination with DNA encoding sFv fused with fragment C of tetanus toxin elicited a $CD4^+$ cell–mediated protective immune response. However, there was no clear evidence for the induction of $CD8^+$ T cells[26].

Because fusions with previously described cytokine moieties have been shown to induce anticytokine antibodies, we have initiated studies to determine whether antibodies are induced to chemokines or to junctions of our fusions. If present, they are probably of little significance, based on our observations that there is no significant difference in survival between control sFv-chemokine immunized and PBS-treated mice, or any overt side effects in such mice monitored for more than 100 days (data not shown).

Protein and DNA formulations of the same chemokine-sFv fusions generated comparable levels of protection (e.g., Fig. 6A vs. B). DNA vaccines delivered only 1–3 μg DNA, which suggests that the levels and duration of protein expression resulting from DNA vaccination were sufficient to elicit the same levels of antitumor immunity generated by 100–200 μg bolus protein. The mechanism underlying these differences remains to be elucidated, however. We can only speculate that in addition to targeting of skin Langerhans' cells by chemokine fusions, CpG motifs present in DNA may have acted as an adjuvant. Investigators using similar gene-gun vaccinations reported that DNA expression lasted about 2 weeks until the skin shed[27]. Further studies directly comparing protein vs DNA vaccine delivery to determine the optimal method are warranted.

DNA vaccine delivery is also of considerable appeal for its potential to generate $CD8^+$ T cells to model heterologous antigens[28–30]. As the induction of protective antitumor immunity required both $CD8^+$ and $CD4^+$ effector T cells (Fig. 5C and D), our results provide the first demonstration of T-cell immunity elicited by DNA vaccination against a syngeneic antigen. Thus, the approach we have developed is simple and versatile and did not require the use of immunological adjuvants. In combination with naked DNA delivery, it may contribute to the development of individual idiotypic vaccines and potent vaccines against other clinically relevant antigens.

Experimental protocol

Fusion gene cloning and plasmid constructions. Lymphoma-specific V_H and V_L fragments were cloned by reverse transcription PCR techniques from total RNA of 38C-13 (ref. 18) and A20 (ref. 19) and designated sFv38 and sFv20A, respectively. The following PCR primers were used: PRV_H38-5′: CTCGAGGTGAAGCTG GTGGA GTCTGGA; PRV_H38-3′: AGAGGAGACTGTGAGAGTGGTGCCTT; PRV_L38-5′: GACAT CCAGATGACACAGTCTCCA; PRV_L38-3′: GGATCCTTTTATTTCCAGCT TGGTC CCCCCTCCGAA; PRV_H20A-5′: CCATGGTCCAACTGCAGCAGTCAGGGCCTGAC; PRV_H20A-3′: TGAGGAGACTGTGAGTTCGGTACCTTGGCC; PRV_L 20A-5′: GATGTTGTGATGACGCAGACTCCACTC; and PRV_L20A-3′: GGATCCTTTGACTTC CAGCTTTGTGCCTCCA. V_H and V_L were arranged into sFv, containing a 17-amino acid linker $(Gly_3Ser)_3GlySer$[16]. The C terminus of sFv was fused in frame with a tag sequence coding *c-myc* peptide AEEQKLISEEDLA and six histidines, respectively. Genes for the mature sequence of murine IP-10 and MCP-3 were cloned by reverse transcription PCR from RNA of lipopolysaccharide-induced murine monocyte cell line ANA-1 (ref. 31) using the following primers: PRIP10-5′: CCATGGCCATCCCTC TCGCAAGGACGGTCCGC; PRIP10-3′: GAATTCAGGAGCCCTTTTAGACCTTTTTTG; PRMMCP3-5′: ACCATGGCCCAACCAGATGGGCCCAATGCA; and PrmMCP3-3′: GAATTC AGGCTTTGGAGTTGGGGTTTTCAT. Fusions containing IP-10, MCP-3, PreS2, and DomA were made by fusing them to the N terminus of sFv through a spacer sequence NDAQAPKS. All constructs were verified by DNA dideoxy-sequencing method, using a T7 sequenase kit (Amersham, Arlington Heights, IL). Constructs for DNA vaccination were fused in frame to a leader sequence of IP-10 in pCMVE/AB (provided by A. Biragyn).

Expression and purification of fusion proteins from *E. coli* and assay for correct folding of sFv. Recombinant fusion proteins were expressed in BL21(DE3) cells (Invitrogen, Milford, MA) as inclusion bodies, after 8 h of induction in Super-Broth (Digene Diagnostics, Beltsville, MD) with 0.8 mM isopropylthiogalactoside, in the presence of 150 μg/ml carbenicillin and 50 μg/ml ampicillin at 30°C. IP10sFv38, MCP3sFv38, and sFv38 were purified from the inclusion bodies and refolded according to Buchner et al.[20] with modifications. The refolded fusion proteins were purified by heparin-sepharose chromatography (Pharmacia Biotech, Uppsala, Sweden). The integrity and purity of recombinant proteins were tested by SDS-PAGE under reducing conditions and by western blot hybridization with 9E10 anti-*c-myc* Mab. Purification usually yielded soluble protein with greater than 90% purity. Correct folding of purified sFv38 proteins was determined by the ability to bind to anti-idiotype Mab S1C5 (provided by M. Kaminski, University of Michigan, Ann Arbor, MI), as detected by ELISA and by inhibition assay (Fig. 2). Briefly, serially diluted sFvs were added to microtiter plates coated with 10 μg/ml anti-*c-myc* Mab 9E10. After washing, plates were incubated with a 1:300 dilution of biotinylated S1C5. Plates were washed again, incubated with streptavidin-HRP (1:5000, Jackson Immunoresearch Lab, Bar Harbor, ME) and developed with ABTS peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Absorbance at 405 nm was measured.

Chemokine receptor binding assay using laser confocal microscopy[5]. Ten million purified T cells per ml from C3H mice were incubated with 100 nM chemokine-sFv or control viral epitope-sFv for 1 h at 37°C. For ligand competition, 100 nM chemokine-sFv was co-incubated with 500 nM of the corresponding chemokine. The samples were then washed twice with PBS and fixed with 2% paraformaldehyde. Slides were then incubated with 9E10 anti-*c-myc* Mab antibody (Sigma, St. Louis, MO) at a 1:50 dilution in wash buffer (0.25% gelatin, 0.15% saponin, 1% goat serum in PBS), followed by goat antimouse IgG F(ab′)2-FITC (Boehringer Mannheim, Indianapolis, IN) at a 1:50 dilution for 30 min at room temperature in a humidified chamber. Slides

were washed three times with 0.25% gelatin and 0.15% saponin in PBS. Finally, they were incubated 10 min with a 1:100 dilution of DAPI, washed twice in Tris-buffered saline, then once in dH_20, air dried, and mounted using aqueous mounting medium (Gel.Mount, Biomeda, Foster City, CA).

In vitro chemotaxis assays. T-cell migration in vitro was assessed by 48-well microchemotaxis chamber technique as described[32]. Briefly, 26 µl aliquots of sFv fusion protein, serially diluted in chemotaxis medium (RPMI 1640, 1% bovine serum albumin, 25 mM HEPES), were placed in the lower compartment, and 50 µl of 5×10^6 cells/ml suspension (purified murine splenic T cells by high-affinity negative selection (R&D System, Minneapolis, MN) was placed in the upper compartment of the chamber. The two compartments were separated by polycarbonate filter (5 µm pore size; Neuroprobe, Cabin John, MD) coated with 10 µg/ml of fibronectin (Sigma). The chemotaxis assay was performed at 37°C for 2 h. The number of migrated cells in three high-power fields (400×) was counted by light microscopy after coding the samples. The results are expressed as the mean ± standard error of triplicate samples.

Tumor cell lines and mice. The carcinogen-induced, C3H 38C-13 B-cell lymphoma[18] secretes and expresses IgM (k) on the cell surface and was a kind gift from R. Levy (Stanford University Medical Center, Palo Alto, CA). Inoculation of as few as 10^2 38C-13 tumor cells intraperitoneally into normal syngeneic mice results in progressive tumor growth and death of the host with a median survival time of only 20 days. Mice surviving past 60 days from tumor challenge are long-term survivors. The BALB/c A20 lymphoma[19], which expresses IgGk, was obtained from the American Type Culture Collection (Rockville, MD). 38C-13 and A20 cells from a common frozen stock were passaged in vitro 3 days before plating in RPMI 1640 supplemented with 100 U/ml of penicillin and streptomycin, 2×10^{-5} M β-ME, and heat inactivated 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD).

Serum anti-idiotypic antibody levels were determined by ELISA as described[33]. Microtiter plates coated with 10 µg/ml Ig38 were incubated with anti-idiotypic sera (1:500) and various amounts of sFv in 2% bovine serum albumin/PBS. Bound serum antibodies were detected with goat anti-mouse IgG1-horseradish peroxidase Mab (Jackson Immuno Research Lab, Bar Harbor, ME; Caltag, San Francisco, CA). The reaction was developed with ABTS peroxidase substrate (KPL, Gaithersburg, MD), and absorbance at 405 nm was measured. Serum antibody levels were determined by comparing sera titration curves with a standard curve obtained with a known concentration of a purified monoclonal anti-idiotype (SIC5). Specificity for idiotype was shown by the absence of binding on a control, isotype-matched IgM (TEPC-183, Sigma; data not shown).

In vivo immunizations. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals[34]. Six- to 9-week old female C3H/HeNCrlBR or BALB/c mice (Charles River Laboratories, Frederick, MD) were used. Syngeneic C3H/HeN or Balb/C mice (10 per group) were immunized intraperitoneally with either 100 µg sFv fusion protein or 50 µg Ig–KLH, or PBS three times every 2 weeks. Similarly, for naked DNA vaccination, mice were immunized intradermally by Accell gene delivery device (Agracetus, Middleton, WI) with plasmid DNA three times every 2 weeks[35]. The abdominal area of mice was shaved, and 1 µm gold particles (Bio-Rad, Hercules, CA) carrying 1–3 µg DNA were injected at 400 psi. Two weeks after the last immunization, mice were challenged intraperitoneally with 2000 38C-13 lymphoma cells from a single preparation of tumor and followed for survival. Differences in survival between groups was determined by nonparametric log rank test (BMDP statistical software, Los Angeles). *p* values refer to comparison with IP10sFv20.

In vivo T-cell subset depletions. In vivo antibody depletions started 2 weeks after vaccination by treatment with three intraperitoneal doses of 400 µg anti-CD8 Mab 53.6.72, anti-CD4 Mab GK1.5 (both ammonium sulfate–purified ascites, Biological Resources Branch, National Cancer Institute-FCRDC, Frederick, MD), or normal rat IgG (Sigma). Treatments were administered every other day, starting 2 weeks after the last immunization, and prior to tumor challenge. Depletion of lymphocyte subsets was assessed 1 and 2 weeks after final treatment, using flow cytometry analysis. Splenocytes from normal mice treated with these Mabs were assessed in parallel[33,36].

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