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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
The concept was to implant, subcutaneously, breast cancer cells in syngeneic rats, inject immune system stimulators entrapped in a sustained release gel into the tumor and observe a short term and a long term immune response to the tumor. Tumors appeared about 8 days after injecting 1,000,000 cells. As soon as we injected 100 ug of N-formyl-Met-Leu-Phe and 0.1 ug of IL-12 in 0.3 ml of gel, an anti-tumor immune response ensued. Tumor growth halted for about 4 days and then resumed at the rate of untreated control cells. If the tumor was re-injected a second and then a third time, tumor growth halted and resumed. However, 3 consecutive injections was not sufficient to induce a long term response. These results were reproducible and encouraging, considering it was a first attempt. Either stimulant alone or both in combination gave the same results. If continued, we would have to develop a gel formulation that would release the stimulants more slowly than the half-time of about 1 day with the present gel.

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INTRODUCTION

The hypothesis is that the immune system can be trained to respond to and eliminate tumors even at an advanced stage. We proposed to inject immune system stimulators into subcutaeously implanted breast cancer cells and observe local/short term and systemic/long term immune responses. We incorporated into a sustained release gel the peptide, N-formyl-Met-Leu-Phe, which is a chemoattractant for phagocytic cells, and IL-12, which is a stimulator of anti-tumor T-cells. The background (including references), experimental protocols and results are presented in considerable detail in the appended report from Mr. Jianglin Ma. The conclusion is that this area of research deserves further investigation.

BODY

1. Establish a breast cancer animal model.

We obtained MAT IIIB cells from the American Type Culture Collection (ATCC) and syngeneic Fischer 344 rats. Tumor cells were grown in culture and were implanted subcutaneously (SQ) in the rats. It took about 8 days to develop a palpable tumor from an injection of 10^6 cells. In initial experiments, fewer cells were injected due to the use of an improper small needle. These cells took a few weeks to grow into an observable tumor, but they gave interesting results.

2. Prepare a gel implant releasing immunostimulatory factors.

In order to attract and activate Langerhans cells, which are phagocytic, antigen-presenting cells in the skin, we used a proprietary gel formulation that is injected SQ as a liquid and forms a gel within 1 minute (Qiu et al.). We determined the rate of diffusion of fMLF *in vitro* to correspond to a half-time of about 20 hours, as measured by reverse phase chromatography of gel extracts. Based on other studies with this gel system, we know that the more water-soluble analog, fMLFK, should have a faster release rate, while the larger substance, IL-12, should have a slower release rate. Also, the *in vivo* release rate should be similar to the *in vitro* rate.

Control gel (about 0.3 ml) gave no response when injected into rats. Removal of the gel and examination by a veterinary pathologist indicated a typical "foreign body" response but no inflammatory response (Qiu et al.).
In contrast, gel containing f MLF produced a red circle around the gel, indicating an inflammatory response, which was confirmed by examination of stained sections of the gel and surrounding tissue by the pathologist. We examined 3 doses of f MLF, and decided to proceed with 100 ug per injection. We included the T-cell stimulatory factor, IL-12, in our studies. Either separate or in combination, similar results were obtained with f MLF and IL-12. .

3. Treat tumors with injected gel.

Several separate studies were done under slightly varying conditions. Details are presented in the appended document by Mr. Jianglin Ma. The tumor growth curves, as measured using calipers, was relatively consistent for injection of 10^6 cells. When injected into the tumor site, an inflammatory response ensued and in some cases it was easy to discern that the tumor had been partially phagocytosed. In all cases, the growth curve of the tumor remained at a plateau for about 4 days (Figure 1). It should be noted that upon injection, there is a jump in volume due to the injected gel, which is especially noticeable when the tumor is small. When the faster-releasing analog, fMLFK was used, the duration of the plateau was shorter (Figure 2). Injection of control gel did not cause a plateau in the growth curve (data not shown).

Protocol changes were made for the next experiment (Figure 3). Only one tumor was implanted on the back of each rat, so the controls were in separate rats. Instead of receiving no injection, the control tumors were injected with the same volume of blank gel as for the sample gels. After injection on day 9 (Figure 3) the difference in growth rate is small (compare with Figure 1). Reinjection of the immunostimulants on day 13 maintained the plateau, while the control gel had no effect. The third injection on day 19 was 6 days after the second, thereby allowing resumption of cell growth. However, tumor growth was again halted for another 4 days before resuming as in the control rats. Even though the treatment period lasted 2 weeks, there was no long term immunological response capable of eliminating the tumor.

However, in an early experiment, tumors were eliminated in 2 rats (one shown in Figure 4). Due to improper injection, a small amount of tumor cells were transplanted. After failing to get a tumor after 2 weeks, two additional injections of cells were properly made. All 3 tumors then appeared. Although only 1 tumor was given the immunostimulant, all 3 tumors were eliminated (Figure 4). In the other rat, 2 out of 3 tumors were eliminated, the treated tumor from the second implantation and the first transplanted tumor (data not shown). These results suggest a systemic immune response.

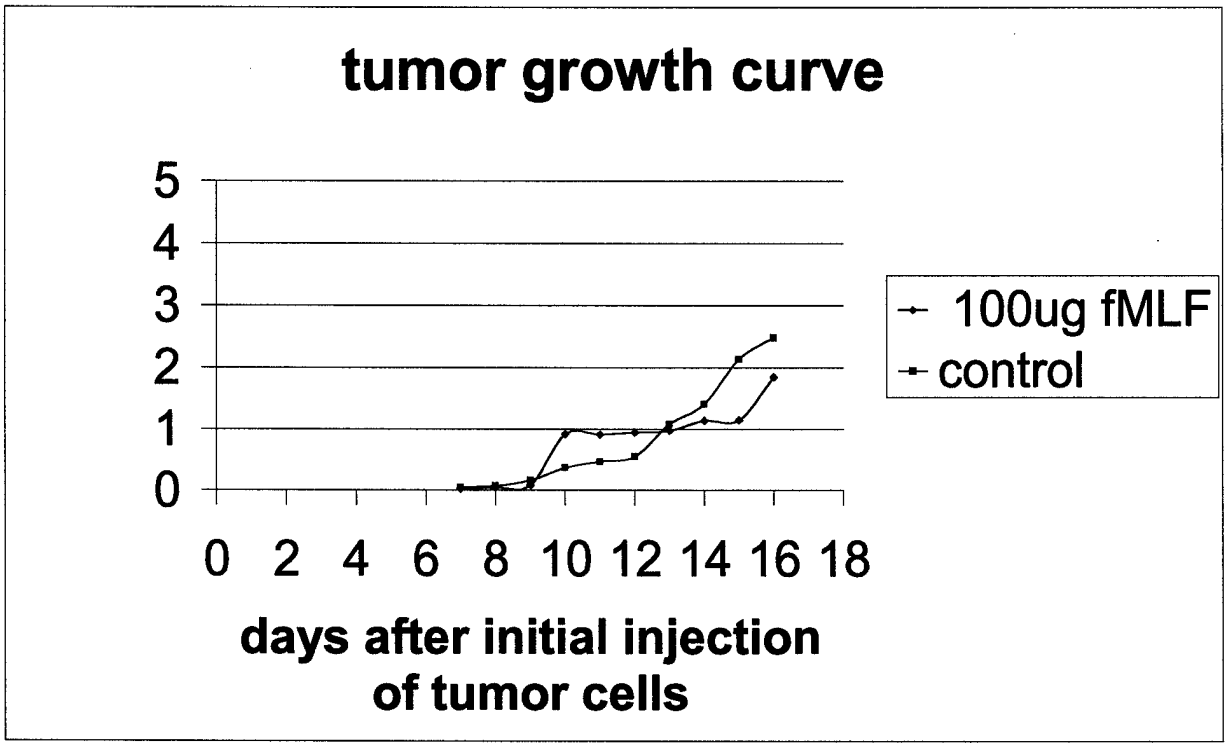

Figure 1. Each group consisted of 4 rats. Each rat was injected with cells on the left side and the right side of the back. One tumor was treated with immunostimulant in gel while the other tumor was not injected. Note the jump in volume in the treated rats is an injection artifact, but the plateau is an anti-tumor response.

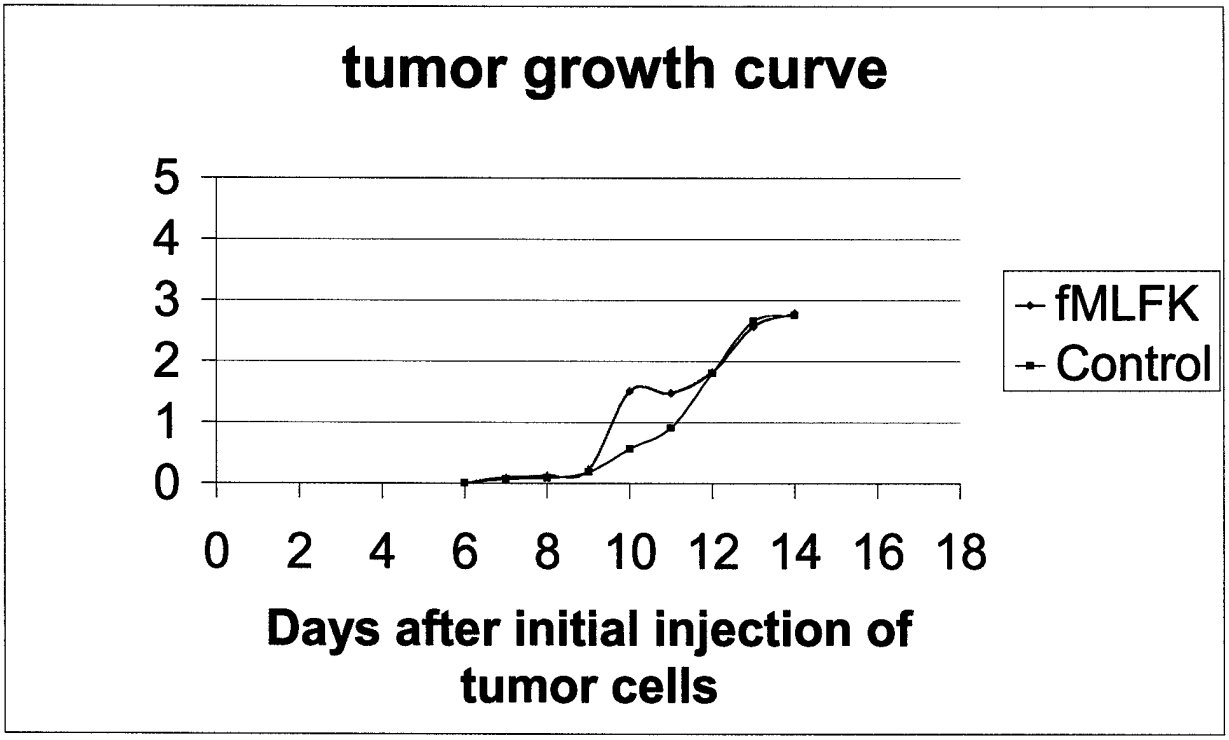

Figure 2. Same legend as Figure 1. This analog releases more quickly from the gel.

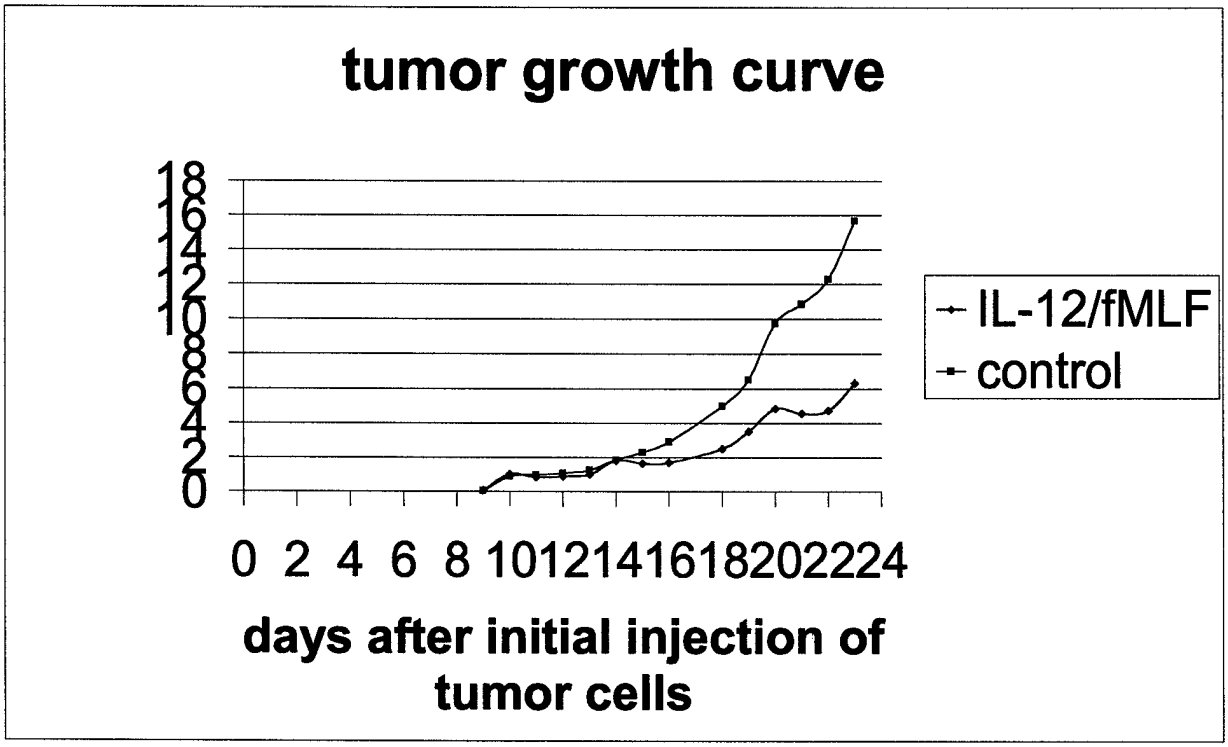

Figure 3. Gels injected on days 9, 13 and 19. Ten rats were each injected once with tumor cells. Five rats received blank gel while the other 5 received gel containing both immunostimulants.

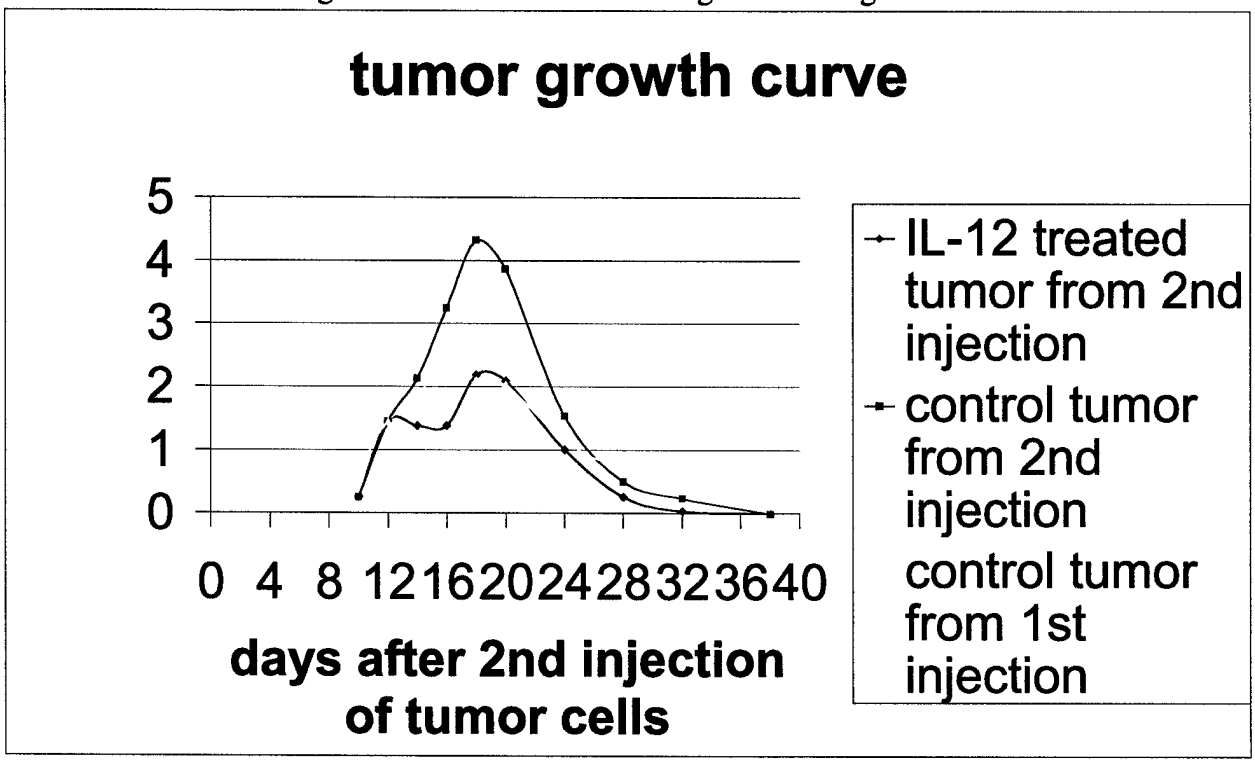

Figure 4. First implantation was with an insufficient number of tumor cells, so two more implantations (one for treatment and the other a control) were made properly. All 3 tumors appeared at about the same day.

KEY RESEARCH ACCOMPLISHMENTS

1. Made advances in gel delivery technology; incorporated into publication by Qiu et al.
2. Achieved localized inflammatory model system that may be useful for other immunological purposes.
3. Established rat breast cancer model system that is convenient and dependable.
4. Completely halted tumor growth by maintaining immunostimulants at the tumor site.
5. Tumor growth inhibition can be repeated even after a lapse in treatment regimen.
6. Observed tumor elimination response in 2 rats.

REPORTABLE OUTCOMES:

1. Contributed to publication by Qiu et al.
2. Thesis for M. S. degree by Mr. Jianglin Ma.
3. Plan to write grant to NIH based on these preliminary results.
4. Unusual findings for an unusual concept. Must discuss with experts in oncology before deciding what else might be needed to publish these findings.

CONCLUSIONS:

There is a possibility that this approach of autologous tumor vaccination can be developed into a clinical product.

REFERENCES

Qiu, B., Stefanos, S., Ma, J., Lalloo, A., Perry, B. A., Leibowitz, M. J., Sinko, P. J. and Stein, S. (2002) A hydrogel prepared by in situ cross-linking of a thiol-containing polyethylene glycol based polymer: A new biomaterial for protein drug delivery. *Biomaterials* in press.

APPENDIX

Thesis for M. S. degree by Mr. Jianglin Ma. This document is a draft. It contains the complete experimental information, but contains some errors and mistatements.The final version should be ready soon and will be sent upon request.

STANLEY STEIN DAMD17-01-1-0657
DRAFT of JULY 19, 2002

In partial fulfillment of the requirements for the M. S. degree.

Autologous tumor vaccination

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Abstract

Langerhans' cells (LC), one type of dendritic cells, reside in the skin in an "immature" state. They are ready to work as phagocytic antigen-presenting cells, responsible for initiation of immune responses. FMet-Leu-Phe (fMLF) is one of the first identified and most potent chemoattractants for polymorphonuclear and mononuclear phagocytes. Interleukin 12 is a multifunctional cytokine and can promote cell-mediated immunity by selectively augmenting Th-1 type immune responses. We will utilize fMLF, IL-12 and Langerhans cells to test our hypothesis that a persistent stimulation of the immune system at the site of the tumor will eventually result in successful autologous tumor vaccination (ATV), regardless of the particular tumor-specific antigens in that patient and overcome any tumor-acquired protective mechanism, Subcutaneously injected fMLF incorporated in a hydrogel was used to promote broadly dispersed Langerhan cells in the skin to migrate, concentrate into the solid tumor site and thus enhance the tumor antigen presentation to T helper cells. Combination therapy of fMLF and IL-12 in a rat model was also tested, considering the ability of IL-12 to produce a distinct sequence of molecular and phenotypic changes in tumors, leading to an anti-tumor immune response, toxicity against tumor cells and an anti-angiogenic effect. Our results showed that either fMLF or IL-12 could transiently suppress the growth of tumor induced by transplanted, in vitro-cultured MATIII B breast cancer cells. The anti-tumor effect lasted as long as the immune stimulator was presesnt, typically 4 days. Multiple consecutive injections of both drugs (fMLF/ IL-12) could still suspend tumor growth. However, even after 3 injections, there was still no sign of an acquired immune response. Still, the dramatic effect on tumor growth warrants further investigation.

Key word:

Langerhans' cells; Antigen-presenting cells; FMet-Leu-Phe (fMLF); Interleukin 12; Autologous tumor vaccination; Hydrogel

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Part I: Introduction

1. Antigen presentation

Antigen presentation cells (APCs) are heterogeneous population of leucocytes with exquisite immunostimulatory capacity. Some have a pivotal role in induction of functional activity of T helper cells, while others communicate with other leucocytes. Cells other than leucocytes, such as endothelial cells can also 'present' antigens when stimulated by cytokines.

APCs are found mainly in the skin, lymph nodes, spleen and thymus. Dendritic cells (DC) are the professional APCs responsible for the initiation of immune responses. Langerhans' cells (LC), one type of DC, are specialized antigen-presenting cells in the skin that reside in the epidermis as sentinels of the immune system [1]. They are different from other DCs in terms of the presence of unique intracellular organelles, known as Birbeck granules (BGs), which are involved in endocytosis. In addition, they differ in terms of the factors that drive their differentiation and the presence of distinct markers [2]. LCs originate from bone-marrow-derived progenitors and acquire their particular characteristics upon localization in the target tissues. TGF-β drives the differentiation of human monocyte and CD34+ precursors into LCs. LCs residing in stead-state tissues are in an "immature" state. They constantly monitor the epidermal microenvironment through taking up antigen by a different repertoire of receptors, such as Fcγ, Fcε receptors, the DEC-205 multilectin (in mice) and langerin (in human and mice) and processing it into fragments that can be recognized by cells of the adaptive immune response. Although low-level migration of LCs from the skin to lymph nodes can occur independently from maturation, only when a certain threshold is reached, LCs become " mature" cells and acquire the ability to migrate as ' veiled cells' via the afferent lymphatics into the lymph node paracortex of the draining lymph nodes, where they interdigitate with many T cells and initiate systemic immune responses. The main function of immature DCs under homeostatic conditions might be to induce and maintain T-cell tolerance.

This migration provides an efficient mechanism for carrying antigen from skin to T helper cells located in lymph nodes. These APCs are rich in class II MHC, important for presenting antigen to T helper cells. For Exogenous antigens, APCs will engulf them by endocytosis, degrade them into fragments (e.g. break an antigenic protein into short peptides), display these peptides at the surface of the cell nestled within a class II histocompatibility molecule, as will be recognized by CD4+ T cells. In contrast, Endogenous antigens, which are generated within a cell (e.g., viral proteins in an infected cell), will be processed and presented to T cells by quite different mechanisms. They will be degraded into fragments (e.g., peptides) within the cell and displayed at the surface of the cell nestled within a class I histocompatibility molecule, as will be recognized by CD8+ cytotoxic T cells.

Another specialized population of APCs is follicular dendritic cells, found in the secondary follicles of the B cells areas of the lymph nodes or spleen. They lack class II MHC molecules but can present antigen to B cells due to high expression of FCγR and the complement receptors CRI (CD35) and CR29 (CD21). B cells engulf antigen by receptor-mediated endocytosis. The B cell receptors for antigen (**BCR**s) are antibodies

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anchored in the plasma membrane. The affinity of these for an epitope on an antigen may be so high that the B cell can bind and internalize the antigen when it is present in body fluids in concentrations thousands of times smaller than a macrophage would need. The remaining steps of antigen processing occur by the same class II pathway described above.

2. fMLF as a chemoattractant

FMet-Leu-Phe (fMLF), released by invasive bacteria or lysed cells, is one of the first identified and most potent chemoattractants for polymorphonuclear and mononuclear phagocytes [3]. It can induce and modulates various cellular response linked to inflammation. For instance, fMLF can induce the secretion of Il-1 alpha, IL-1 beta and IL-6 in human peripheral blood mononuclear cells via a Gi protein [4]. It functions to selectively activate the high-affinity fMLF receptor FPR and its low-affinity variant FPRL1, both of which belong to the seven-transmembrane, G protein-coupled receptor (GPCR) superfamily. Despite the importance of this receptor class to immune function, relatively little is known about the molecular mechanisms involved in their activation. Prossnitz et al [5] demonstrated that multiple activation steps (ligand-induced phosphorylation, internalization etc.) are required for activation of the wild-type FPR. Activation of FPR results in increased cell migration, phagocytosis, and release of proinflammatory mediators. The main responses elicited by fMLF in granulocytes are cell polarization, the generation of full reactive oxygen species, the production of arachidonic acid metabolites and the release of lysosomal enzymes [6].

The human FPR1 structural gene is single-copy gene located on chromosome 19q13.3 and approximately 7.5kb in length [7]. . It is comprised of two exons separated by an approximately 5.0kb intron. The FPR1 is encoded primarily by a 1.6kb mRNA in differentiated HL-60 cells and U937 cells. It shows 53% amino acid similarity to complement C5a. The transmembrane and the cytoplasmic domains are highly conserved, while the highest divergence corresponds to extracellular loops involved in ligand binding [8]. The most likely positioning of fMLF in the binding pocket of FPR is approximately parallel to the fifth transmembrane helix with the formamide group of fMLF hydrogen-bonded to both Asp-106 and Arg-201, the leucine side chain pointing toward the second transmembrane region, and the COOH-terminal carboxyl group of fMLF ion-paired with Arg-205 [9].

The presence of structural determinants in the first intracellular loop and its adjacent transmembrane domains are essential for high affinity fMLF binding [10]. Ligand-receptor binding is proposed not to depend on defined structure but rather that those charged moieties (such as Arg-84 and Lys-85) might function as important " contacts"[11].

Leukocytes can express multiple chemoattractant receptors that trigger adhesion or direct their migration. Regulation of such proadhesive and migratory responses must often occur in a complex cytokine environment in vivo, in which multiple receptors may be engaged simultaneously or sequentially. Interplay between IL-8 receptor and FPR stimulation and its consequences for leukocyte adhesion and chemotactic responses show that fMLF can totally abrogate proadhesive and chemoattactant responses to IL-8. The dominance of FPR may ensure that signals from these terminal phagocytes target can override host-derived recruitment signaling through IL-8 and other chemokine receptors

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[12]. This crosstalk between chemoattractant receptors and their signaling pathways may help target leukocyte migration in the context of complex chemoattractant arrays in vivo.

3. Cell migration and chemotaxis

Under normal condition, leucocytes migrate through all the tissues of the body-the cells present in the blood are the ones that are in transit between different tissues. Each population of cells may have a particular pattern of migration, which also depends on the cells' state of differentiation and activation. For example, phagocytes (neutrophils, monocytes etc.) leave the bone marrow and migrate into peripheral tissues, particularly at sites of inflammation or infection. Virgin lymphocytes migrate from thymus and bone marrow to the secondary lymphoid tissues. For circulating leucocytes, there are two main stages for their migration [13].

First, they attached to the vascular endothelium, followed by a movement between or through endothelial cells. The process of leucocytes migration across endothelium requires several consecutive steps:

1) Tether--mediated primarily by selectins interacting with carbohydrate. Leucocytes are slowed as they pass through a venule and roll on the surface of the endothelium before being halted.

2) Trigger-the tethered cells have a chance to respond to cytokines, chemotactic agents and surface molecules on the endothelium or ECM.

3) Latching and activation---the triggering upregulates integrins (CR3 and LFA-1) that bind to intercellular adhesion molecules (ICAM-1) induced on endothelium and start to migrate.

Second, cells migrate towards the site of infection or inflammation, under the guidance of chemotactic molecules. It has two steps.

1) Migration--cells contact the basement membrane using new sets of adhesion molecules, and migrate beneath the endothelium.

2) Digestion--enzymes are released to digest the collagen and other components of basement membrane, allowing cells to migrate into the tissue. The whole process is partially controlled by cell surface molecules on the migrating cells, partially by a variety of soluble signaling molecules.

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We propose that a concentration gradient of fMLF at or near a tumor site can attract Langerhans cells of the skin to the tumor site. After phagocytosis of the malignant cells and taking up the tumor-related antigens, they can migrate into local lymph nodes to present these antigens to CD4+ T cells. One propose of this migration is to give the small numbers of lymphocytes which are specific for any particular antigen the chance to meet that antigen. Lymphatic drainage pathways and the migration of cells ensure that lymphocytes, APC, and tumor-related antigen converge on the lymph node. Different patterns of movement occur at different stages of a lymphocyte's life span. Normally resting T cells tend to migrate across high endothelial venules into secondary lymphoid tissues. In the secondary lymphoid tissues the antigen-specific activated lymphocytes are clonally expanded, released into efferent lymphatics, the circulation and then the site of inflammation. Once leucocytes have arrived at a site of infection or inflammation, they release mediators, which control the later accumulation and activation of the other cells. However, in inflammatory reactions initiated by the immune system, the ultimate goal is an effective systemic immune response to tumor-specific antigens.

4. IL-12 as an immunostimulatory

Interleukin 12 is a multifunctional cytokine secreted by a wide variety of cells including macrophages and B cells. It promotes cell-mediated immunity by its ability to selectively augment Th-1 type immune responses. This pleiotropic cytokine exerts numerous effects on T and NK cells-a critical effector [14], resulting in an enhancement of cytolytic activity and direct stimulation of IFN-gamma production [15-16]. This cytokine plays an important role in the promotion of the host resistance to infection by bacterial, fungal and protozoan pathogens. Expression of Fas (CD95, APO-1), a cell surface receptor is involved in IL-12 induced apoptosis of tumor cells and elimination of targeted cells by natural killer and T cells. Corruption of this pathway, such as reduced Fas expression, can allow tumor cells to escape elimination and promote metastatic potential. Treatment of IL-12 resulted in a dose-dependent up-regulation of FAS, as is independently of IFN-gamma [17]. IL-12 has also been shown to cause tumor regression and reduce metastasis in animal models, due to the promotion of antitumor immunity and the significant inhibition of angiogenesis [16] and up-regulation of E-cadherin, a metastasis suppressor. Il-12-treated tumors had an abundant cellular infiltrate, consisting mainly of CD8+ T cells. mRNA for granzyme B and perforin also could be detected in these cells, suggesting that they were activated. Significant reduction in vasculature after one week of daily therapy and increased apoptotic tumor cells were observed in IL-12 treated tumors [18]. In contrast with anti-tumor effects induced by IL-12, IFN-gamma caused a lesser cellular infiltrate, a minor anti-angiogenic effect and a transient apoptotic effect. The success of IL-12 may be ascribed to its ability to produce a distinct sequence of molecular and phenotypic changes in tumors, leading to an anti-tumor immune response, toxicity against tumor cells and an anti-angiogenic effect. At least two mechanisms are involved in IL-12 regulation of angiogenesis, removing the proangiogenic stimulus and blocking the release and activity of MMP2 [19]. Sustained induction of IP-10 and activation of a resulting cellular infiltrate are thought to be key changes in regressing tumors for IL-12 treatment. Interleukin-12 can facilitate the generation of autologous tumor-reactive CD8+ cytotoxic T lymphocytes from tumor-infiltrating lymphocytes [20].

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5. Tumor vaccination

Vaccination is the best-known and the most successful application of immunological principles to human health. The first vaccination pioneered by Jenner 200 years ago was named after vaccinia, the cowpox virus. The general principle governing vaccination is—altered preparations of microbes can be used to generate enhanced immunity against the fully virulent organism. Non-specific tumor vaccination can date back almost a century to the work of Coley, who used bacterial filtrates with considerable success, possible through the induction of cytokines such as TNF and IFN. However, attempts to equal his results with purified cytokines or immunostimulants (e.g.BCG) have been successful only in a restricted range of tumors. Current efforts are directed at the induction of specific immunity, which is encouraged by the evidence that tumors may sometimes be spontaneously rejected as if they were foreign grafts. The following describes these efforts.

1) Recombinant yeast vaccination

This strategy [21] is to use whole recombinant *saccharomyces cerevisiae* yeast expressing tumor or HIV-1 antigens to potentially induce antigen-specific, CTL responses, including those mediating tumor protection in vaccinated animals. Interactions between yeast and dendritic cells (DCs) can lead to DCs maturation, IL-12 production and the efficient priming of MHC class I and II-restricted, antigen-specific T-cell responses. The role of yeast here is to exert a strong adjuvant effect, augmenting DC presentation of exogenous whole-protein antigen to MHC class I and II restricted T cells.

2) Adjuvanted vaccination

Adjuvants are thought to function in several ways, including by increasing the surface area of antigen, prolonging the retention of antigen in the body thus allowing time for the lymphoid system to have access to the antigen, slowing the release of antigen, targeting antigen to macrophages, activating macrophages, or otherwise eliciting non-specific activation of the cells of immune system [22]. Currently alum or aluminum hydroxide is the only adjuvant approved in the United States for human vaccines. It can induce the production of T_H cells. Scott and Trinchieri [23] provided a pharmaceutical composition useful as a vaccine comprising an antigen from pathogen and an effective adjuvanting amount of protein, Interleukin –12(IL-12), the resulting composition capable of eliciting the vaccinated host's cell-mediated immunity for a protective response to the pathogen. The invention also provides a composition comprising one DNA sequence encoding the antigen from a pathogen (a subunit, or a fragment) rather than the protein or peptide itself or one DNA sequence encoding IL-12 (a subunit, or a fragment).

3) Peptide-loaded DC vaccination

The majority of tumor antigens are nonmutated self-antigens, which tend to elicit weak self-reactive T cell responses. A major challenge in cancer vaccines involves finding strategies to break self-tolerance and to generate strong, long-lasting antitumor immunity through manipulation of both the antigen and the delivery system. The half-life of MHC class I/peptide complexes on the DC surface is thought to be critical in determining T cell responses. Enhanced stability of MHC-peptide or MHC-peptide-TCR complexes on the cell surface by substituting favorable key peptide residues has correlated with improved T cell responses both *in vitro* and *in vivo* [24-26].

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Rong-RF et al [27] have developed an approach to enhancing antitumor immunity by prolonging the efficient presentation of antigens on DCs. DCs loaded with a peptide derived from tyrosinase-related protein 2 (TRP2) covalently linked to a CPP1 sequence retained full capacity to stimulate T cells for at least 24 h, completely protected immunized mice from subsequent tumor challenge, and significantly inhibited tumor metastases.

4) In situ langerhans cell vaccination

Normally DC-based vaccinations require several "customizing" processes to (1) isolate and expand DCs from individual patients. (2) Manipulate their maturation state. (3) Load them with tumor-associated antigens (TAAs), and (4) administer TAA-pulsed DCs back to patients. These processes are time-and cost-consuming. Tadashi K et al [28] entrapped migratory LCs by subcutaneous implantation of ethylene–vinyl–acetate (EVA) polymer rods releasing macrophage inflammatory protein (MIP)-3$^{\beta}$ (to create an artificial gradient of an LC-attracting chemokine) and topical application of hapten (to trigger LC emigration from epidermis). The entrapped LCs were antigen-loaded *in situ* by co-implantation of the second EVA rods releasing tumor-associated antigens (TAAs), thus inducing potent cytotoxic T-lymphocyte (CTL) activities and protective immunity against tumors.

5) Heat shock protein vaccination

HSPs are key regulators of immune response to cancers and infectious agents. Platelets can bind gp96, a process that regulates the ability of the HSP to activate dendritic cells. Gp96 appears to bind platelets through at least two receptors, CD91 and CD36 [29]. *Xenopus* tumor-derived HSPs can elicit protective immunity to tumor challenge [30]. Hsp70-peptide complexes isolated from tumor cells confer protection from challenge with live tumor cells. The complexes also induce tumor-specific T-cell responses. Autoantibodies specific for hsp70 are not detected even after repeated vaccination with the complexes [31]. Chaperone of hsp110 to a large protein derived from HER-2 were shown to induce protein-specific CD4, CD8 and antibody response in immunized mice [32]. Heat shock protein gp96 plays a role in trimming peptides to the appropriate size for loading onto MHC class I molecules in the endoplasmic reticulum for subsequent presentation to T cells [33]. Immunocompetent mice reject tumor cells that express gp96 on the cell surface. Further, such tumor cells are able to stimulate the maturation of—and secretion of cytokines by—dendritic cells in a contact-dependent manner [34].

HSPs are present in greater quantities in a variety of human cancers relative to normal cells and that this increased expression correlates with the ability of lysates to cause maturation of dendritic cells [35]. Heat shock proteins were described as the immune system's "Swiss Army Knife"[36]. HSPs complexed to peptides are the immunogenic principle isolated from cancer cells. The HSP is a required component of this principle since immunization with the isolated peptides alone does not confer immunity. The specific immunogenicity of the HSP preparations can be attributed to the unique repertoire of antigenic peptides that exists in different cancers. The peptide repertoire is a product of mutations in cancers, and as mutations arise randomly, this repertoire is never duplicated. Essentially, each cancer harbors a unique "antigenic fingerprint". The heat shock proteins thus confer specific immunity only to the cancer from which they are isolated. One strategy entails purifying HSPs from cells that express antigens capable of

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being recognized by the immune system. This is how Antigenics Inc. manufactures its lead cancer product, Oncophage (HSPPC-96). Another strategy to activate cellular immunity with HSPs is to complex the HSP to defined antigens that are manufactured synthetically. Antigenics' lead product for genital herpes, AG-702, is one example of this type of product.

6) "Naked DNA"-based cancer vaccines

DNA vaccination or genetic immunization is a rapidly developing technology that offers new approaches for the prevention of diseases. It is typically comprised of plasmid DNA molecules that encode an antigen(s) derived from a pathogen or tumor cell [37]. Following introduction into a vaccine, cells take up the DNA, where expression and immune presentation of the encoded antigen(s) takes place. This method of vaccination provides a stable and long-lived source of the protein vaccine, and it is a simple, robust, and effective means of eliciting both antibody- and cell-mediated immune responses. Furthermore, DNA vaccines have a number of potential advantages such as they can address several diseases in one vaccine, they are cheap and easy to produce and have no special cold storage requirement because they are extremely stable. It has proven to be a generally applicable technology in various preclinical animal models of infectious and noninfectious diseases, and several DNA vaccines have now entered phase I/II, human clinical trials. However this technology is still in its infancy. There are several hurdles that need to be overcome on the road to the use of DNA vaccines widely. These include the technical challenges of improving delivery and/or potency so that low doses of DNA can achieve the efficacy of conventional vaccines [38].

7) Dentritic cell-tumor Fusions as a novel cancer immunotherapy

The novel technology combines a patient's dendritic cells with their inactivated tumor cells in a laboratory procedure. The fused cells are injected back into the patient to stimulate an immune response against that patient's cancer. Fusions of murine dendritic cells (DCs) and murine carcinoma cells can reverse unresponsiveness to tumor-associated antigens and induce the rejection of established metastases. Gong J et al [39] generated fusions with primary human breast carcinoma cells and autologous DCs. The resulted fusion cells co-expressed tumor-associated antigens and DC-derived costimulatory molecules. The fusion cells also retained the functional potency of DCs and stimulated autologous T cell proliferation. Significantly, the fusion cells can prime autologous T cells to induce MHC class I-dependent lysis of autologous breast tumor cells. These findings demonstrate that fusions of human breast cancer cells and DCs activate T cell responses against autologous tumors. In contrast with above several methods, the cell fusion process eliminates the need to identify specific antigens to use in a vaccine by automatically incorporating the entire menu of antigens found on the patient's original tumor cells. This enables dendritic cells to present all the patient's cancer antigens to the immune system in anticipation of eliciting a powerful immune response.

6. A polymer matrix (hydrogel) applied in autologous tumor vaccination)

Since a certain percentage of cancer patients undergo spontaneous remission, the clinical trial of any new anti-cancer drug must include a group of patients receiving a placebo. Obviously, these spontaneous remissions must be due to detection and eradication of the cancerous cells by the patient's own immune system. It is our intention

to learn how to take advantage of this phenomenon as a new therapeutic strategy. Thus, when a patient is diagnosed with breast cancer, rather than having radical or even conservative surgery and rather than being given chemotherapy or radiation therapy, she will receive treatment that stimulates her immune system to recognize and eradicate all primary and secondary tumors.

This process, known as autologous tumor vaccination (ATV), is currently being examined clinically in several laboratories (e.g. Rosenberg et al. at NIH and Leong et al. at UCSF). Development of ATV for treating breast cancer has recently been reviewed [40]. A few biotechnology companies, including Genzyme (Framingham, MA), Intracell (Rockville, MD) and Antigenics (New York, NY), have also taken up this cause. Typical therapeutic strategies [41,42] are to (1.) remove the patient's tumor cells, transform them with a gene for an immune system stimulant (IL-2 or GM-CSF) and return these cells to the patient, or (2.) extract irradiated tumor cells and return the extract along with rGM-CSF back to the patient. Since each patient may have a different tumor-specific anigen(s), this individualized therapy can be quite costly.

Our hypothesis is that a persistent stimulation of the immune system at the site of the tumor will eventually result in successful ATV, regardless of the particular tumor-specific antigens in that patient and overcoming any tumor-acquired protective mechanism [43]. To provide a sustained immune system stimulation, we have developed a polymer matrix that can be injected as a liquid into the tumor and will harden into a gel within minutes. The immune stimulator can be covalently linked to the polymer or just physically entrapped within the porous matrix. The rate of biodegradation of the gel can be adjusted to correspond to the rate of regression of the injected tumor. So far, we have injected this gel subcutaneously into normal mice at 3 different doses of degradable covalently-linked chemotactic peptide and have observed a graded response. At the highest dose, the normal skin formed a scab in 2 days. The scab fell off in about 1 week, being replaced by fresh tissue, without any observable distress to the mouse.

Our research plan is to develop a depot formulation that can cause a local immune response resulting in complete elimination of the injected tumor with minimal adverse side effects. Initial immunostimulatory chemicals to be tested, separately and in combination, are N-formyl-Met-Leu-Phe (covalently linked or physically trapped) and IL-12 (physically entrapped and slowly released). Other factors, such as a monoclonal antibody [43] against CTL-4, a protein that functions to protect tumor (and normal) cells from T-cell-mediated destruction can also be included in the future.

Experimentally, a rat breast cancer cell line will be purchased from ATCC and grown in culture. Cells will be harvested and then transplanted at one or two sites into the genetically closest rat strain available. When the tumors are easily palpable, they will be injected with various formulations of the gel containing the chemotactic peptide, N-formyl-Met-Leu-Phe or N-formyl-Met-Leu-Phe-Lys-OH (fMLFK), which will attract cells of the monocyte/macrophage lineage. The macrophages will recognize and become activated by this bacterially derived peptide, and in turn secrete other immunostimulatory factors that will attract and stimulate other types of inflammatory cells. The result is an attack and digestion of the gel, which may occur over an extended period of time (days or weeks). Meanwhile, the cells of the surrounding tumor tissue will be exposed to the degradative enzymes, free radicals, etc. secreted by the activated macrophages, which will also assume their role as professional antigen presenting cells. The rate of regression

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of the injected tumor will be determined from the estimated tumor size. The rat will be sacrificed to confirm disappearance of the tumor. The rate of depot biodegradation will be monitored similarly and can be adjusted, if necessary.

Part II: EXPERIMENTS

1. Materials and methods:

Materials:

Female syngeneic Fischer 344 rat (50-125g); Rat breast cancer cells MATIII B from ATCC; Argon gas; Sectra/Por® membrane; Φ24/40 glass column; Bio-Rad 2000 Alfred Nobel Drive column; micron 3(amicon); TLC aluminium sheets silica gel(DC-Alufolien Kieselgel 60 F254); VYDAC™ Reverse Phase C_4 Column

Reagents:

Mercaptosuccinic acid; triphenylmethyl chloride; dichloromethane (DCM); N, N-Dimethyl-formamide (DMF); silica gel (70-230^m, 60A); α,ω-diamino-poly (ethylene glycol); 4-(dimethyl amino)-pyridine; p-toluenesulfonic acid monhydrate; 1,3-diisopropylcarbodiimide; Trifluoroacetic acid (TFA); Thioanisol; EDT; Anisol; BOP; HOBT; FLURAM (fluorescamine) assay kit; N-succinimidyl-3- (2 pyridylthio) propronate (SPDP); DTT; RPMI 1640; PEG-VS-2000; DMSO; methanol; K_2HPO_4; KH_2PO4; NaCl; Acetonitrile; 2-Propanol

Methods:

1) Preparation of Trityl-thiomalic Acid for protected sulfur group

To a 25ml flask assembled with argon balloon, one equivalent of 2-Mercaptosuccinic acid (1g, 6.67nmol) and three equivalents of triphenylmethyl chloride (5.56g, 20nmol) was charged and dissolved in 15-20 ml degassed DMF. The reaction was carried out at room temperature with magnetic stirring overnight and monitored by thin layer chromatography (5% methanol: DCM). 100ml water was added into the reaction mixture and the reaction mixture was extracted with DCM (v: v=1:1) using 250ml separation funnel. The organic phase (lower layer) was collected, washed with saturated NaCl (v: v=1:1) and concentrated in vacuo to about 1-2ml. The product, trityl-mercaptosuccinic acid was purified by flash chromatography on a silica gel (70-230^m, 60A) column. The product was recovered, concentrated in vacuo first and vacuumed to full dryness later.

2) Synthesis of Thiol-containing PEG Coploymers

To a flask, equivalent molar of α,ω-diamino-poly (ethylene glycol) (0.9714g, MW 3,400 Dalton), Trityl-mercaptosuccinic acid (0.112g, MW 392.5Dalton), 0.5 equivalent 4-(dimethyl amino)-pyridine (DMAP, 0.0183g) and 0.5 equivalent p-toluenesulfonic acid monhydrate (PTSA, 0.0285g) were dissolved in 15-20ml DCM by stirring with magnetic bead and cooled down to 0°c with ice-water bath. 3.5 equivalents of 1,3-diisopropylcarbodiimide (DIPC, 234ul) was added, the flask was assembled with argon balloon and kept in ice-bath for stirring for about one hour. The

reaction mixture was warmed to room temperature to carry out a direct polycondensation for 48-96 hours monitored by increased viscosity. The product was precipitated with 10 fold ice-cooled diethyl ether, dissolved in water and then dialyzed with 3x1000ml water using spectra/Por® membrane (MWCO: 12-14,000 Dalton). Dialysis product was lyophilized and treated with 6ml TFA (90%TFA: 5% Thioanisol: 3% EDT: 2% Anisol) for 2 hours at room temperature to remove the protecting trityl groups from the polymer pendant chains. The deprotected polymer was precipitated with ice-cooled diethyl ether, washed 5 times and dried under vacuum. The resulting thiol-containing PEG copolymer was stored under argon in the freezer (-20°c).

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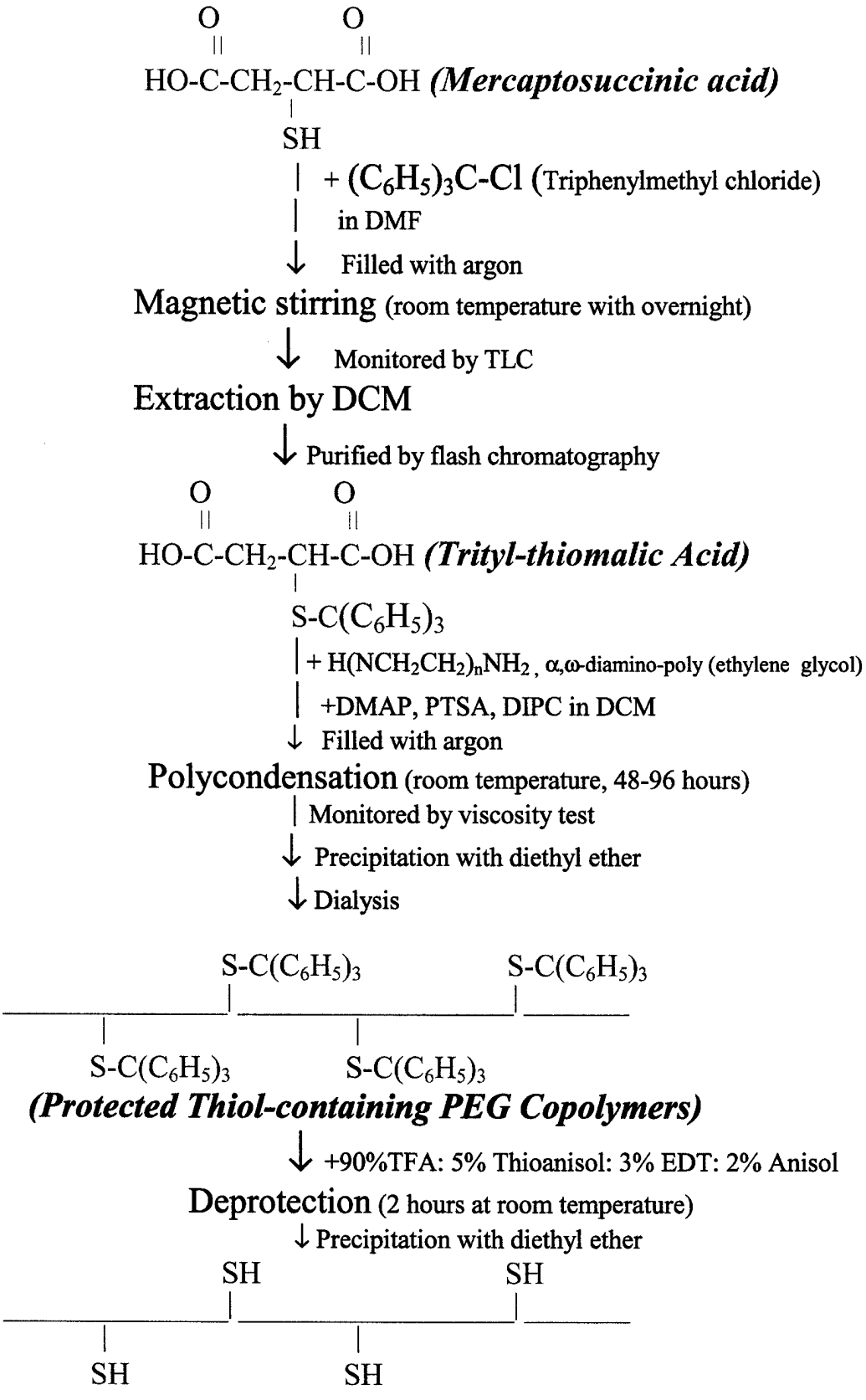
The Reaction scheme:



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(Thiol-containing PEG Copolymers)

3) Attachment of fMLF to 8-arm-PEG and crosslinking

100mg 8-arm PEG (MW 20K): 88.4mg BOP (MW 442.3): 27.04 mg HOBT (MW 135): 27.60mg fMLF (MW 437.6) were dissolved in 500ul DCM and 1000ul DMF for reaction over 48 hours. The reaction was monitored using FLURAM (fluorescamine) assay. The reaction product was dialyzed overnight using spectra/Por® membrane (MWCO: 12-14,000 Dalton). Dialysis product was dried in vacuum. 50mg purified reaction product (8-arm-PEG +fMLF) dissolved in 750ul PBS was reacted with 3 times molar of the bifunctional coupling agent, N-succinimidyl-3-(2 pyridylthio) propronate (SPDP) (Pierce, 2.34mg in 100ul DMSO) overnight. The unreacted SPDP was removed with microconcentration (spin 14,000rpm for 2 hours), using micron 3(amicon). An aliquot (10ul of 800ul) of the reaction was incubated with an excess of DTT (200ul of 26mg/ml in PBS pH=8.0) for 15 minutes to determine the concentration of SPDP reacted polymer by the release of Thiopyridine (TP). The amount of 2-thiopyridine liberated was quantified by Beckman DU-7 spectrometers using a molar extinction coefficient of 8.08×10^3 at 343nm (here have two references). The 8-arm-PEG-fMLF products were stored as 20mg/ml stock solution in PBS (pH=7.0-8.0) for later use.

4) SEC-HPLC analysis of fMLF-containing 8-arm-PEG

Bio-Rad 2000 Alfred Nobel Drive column was washed and balanced by 4-6 fold of column volume PBS (PH 8.0) at a flow rate of 1ml/minute. 100ug(100ul) standard peptide sample and fMLF-containing 8-arm-PEG were loaded to the column respectively, which was monitored by UV at 220nm.

5) Culture &Harvest of rat breast cancer cell line13762 (MATIII B)

To a 250 ml culture bottle, 100-200ul(10^6-10^7) stock MATIII B cells and 25ml fresh RPMI 1640 containing 10%BSA and 1% penicillin & streptomycin were added. Culture was carried out in 5% CO_2 incubator (37^0c). RPMI medium was changed every other day.

To harvest the cell, Old RPMI was removed and 2ml EDTA-Trypsin was added, mixed gently and put in 5% CO_2 incubator for approximate 2 minutes. EDTA-Trypsin was removed and the cells were pipetted using 10 ml fetal serum albumin – free RPMI. Suspended cells were transferred two centrifuge tubes (5ml/each) and spin at 2000rpm for 5 minutes (^{0}c). Cell pellets were washed using the same RPMI 1640 three times. Cells were counted and diluted to desired cell concentration for later subcutaneous injection. Remaining cells were suspended in RPMI containing 5% DMSO and stored in -80^0c first and liquid nitrogen later.

6) Gel formation and injection

A: Polymerization test in vitro

For example, to make a 250ul 4% hydrogel, PEG-VS-2000 (crosslinker) 3mg dissolved in 50ul PBS (pH7.0-8.0) was incubated with 10mg copolymer in 200ul PBS for reaction. Gel could form varying from <1 minute to approximate 3 minutes.

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B: injection

For one subcutaneous injection in one female syngeneic Fischer 344 rat, 200-300ul 3-4% hydrogel could be used. For different experiments, 8-arm-PEG-fMLF, fMLF or IL-12 could be added separately from 20mg/ml stock 8-arm-PEG-fMLF solution in PBS, 20mg/ml stock fMLF solution in DMSO and 0.1mg/ml stock IL-12 in PBS (pH7.0-7.5). In animal room, PEG-vs-2000 was mixed with copolymer using syringe with No.22-23# needle for polymerization and injected subcutaneously. Each rat is marked by punching on the ears.

7) Collection and analysis of tumor growth data

After injection with above syngeneic breast cancer cells, rats were observed daily for tumor and distress. At cell injection or later, rats were injected at the same site with control hydrogel or hydrogel with fMLF and/or IL-12. Rats were monitored for tumor size, skin lesions, distress or ascites and would be sacrificed by carbon dioxide inhalation. The time for euthanasia was empirically determined (2-4 weeks). Tumor size was measured by caliper every day. Tumor volume was calculated by the formula: V (volume)=1/2(length) $(width)^2$.

8) In vitro release experiment for fMLF in hydrogel

A series of 4% 250ul gels with 100ug fMLF were made. 5ml PBS was added into each gel except one (set as day 0). These gels are placed on a rocker shaker at room temperature. At 24, 48, 72, 96,120 hours, PBS was removed from all gel, one gel was put aside in refrigerator and fresh PBS was added into the remaining gels. Finally, All gels are cut into 20-30 slices with razor and put into 1.5ml plastic tube and speed vacuum overnight. 0.5ml of DMSO was added into each tube and incubated on a rocker shaker for a couple hours. The Gel slices were precipitated by centrifuge at 10000rpm for 10 minutes.

VYDAC™ Reverse Phase C_4 Column was completely washed and balanced by solution A (0.1% TFA) at a flow rate of 0.5ml/minute. After each sample was loaded, The HPLC runs in following procedure: 0-5^{th} minute, 100% solution A; 6^{th}-30^{th} , 10% solution B plus 90% solution A was applied to remove DMSO completely; 30^{th}-50^{th}, gradient solution B was used at a rate of 2%/ minutes until it reached 50%. fMLF will be eluted out around 46^{th} –48^{th} minute; 51^{st} –60^{th} , 100% solution B was applied to remove any impure materials; 60^{th} –70^{th} ,100% solution A was applied to balance the column again.

2. Results:

To see if fMLF can elicit visible inflammatory response in rats, we first compared the effect of subcutaneous injection of 4% control theragel and theragel including covalently-linked fMLF. In both control and experimental group, we didn't see any visible inflammatory response two weeks after injection. Skin sections were recovered and Formalin-fixed, microscopic pathology report showed both skin sections from 500ug/ml linked fMLF injection and control injection contained similar appearing focally-extensive, well-demarcated granuloma structures within adipose tissue of the subcutaneous layer. The granulomas were formed by a thick outer wall of fibroblast,

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which surrounded and enclosed a moderately thick layer of moderate numbers of macrophages, with lesser numbers of lymphocytes and rare multinucleated giant cells. According to the pathology report of Dr. Stiefel (Taconic Farms, Rockville, MD), the inflammatory cell reaction was considered to be minimal. There is no difference between control and experimental group at all. That means the covalently–linked fMLF didn't mount acute inflammatory response, as expected according to its function. The main possible reason is that covalently–linked fMLF in vivo didn't release at all, i.e. the in vivo environment is not enough to break the amide bond between fMLF and PEG. Therefore, it is trapped inside granuloma not only physically but also chemically.

To address this problem, one is to replace the more stable bond with less stable bond such as ester bond etc. Another alternative way is to directly trap the chemoattractant peptide such as FMLF or immunostimulatory cytokine such as interleukin-12 (IL-12) physically in polyethylene glycol-based hydrogel. This might result in a rapid release of the drugs from the gel. Our result demonstrated that physically-trapped fMLF could elicit a visible inflammatory response (Fig1, Fig2) with varying intensity depending on the concentration of fMLF.

The skin section (43202-2) recovered 14 days after 10ug fMLF injection represented a chronic-active and marked inflammatory response and an incomplete granuloma formation. It is composed of degenerating inflammatory cells, probably neutrophils, amorphous, eosinophilic materials, macrophages etc. In contrast, the skin section (43202-3) recovered 6 days from 100ug fMLF injection was similar to 43202-2, but differ in degree of severity. This section presented with a more acute inflammatory response with less granulomatous response.

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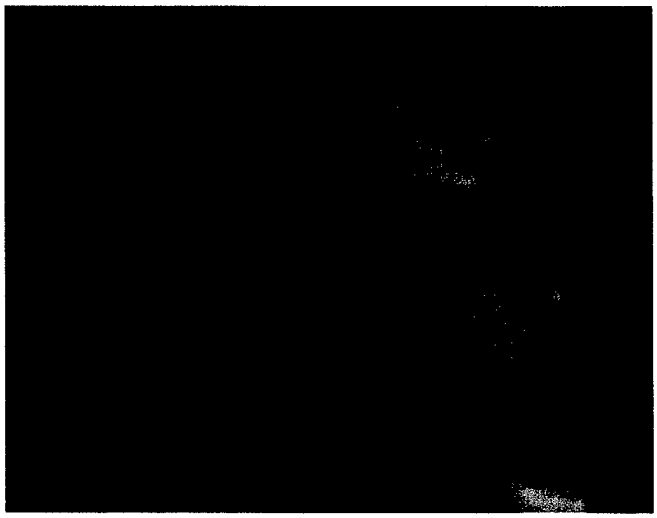

Fig1: Subcutaneous gel containing 10ug fMLF in 0.2 mL depot. Note: circle of inflammatory response

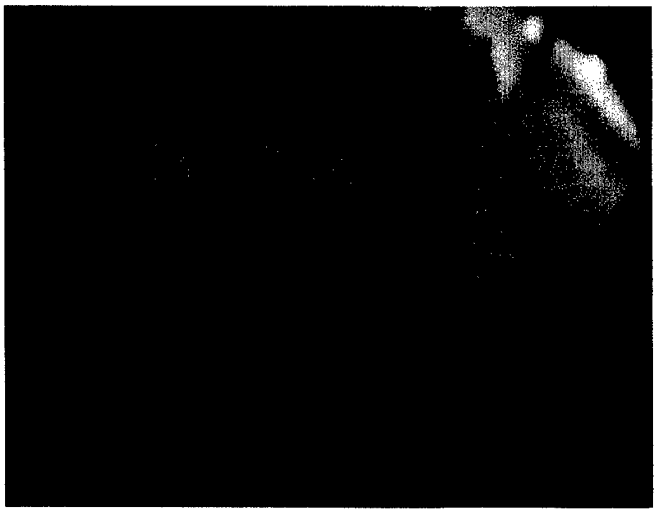

Fig2: subcutaneous control gel in 0.2 mL depot

Direct subcutaneous injection (No.23 needle. OD 0.64mm) of a certain amount of MATIII B breast cancer cells can induce solid tumor in syngeneic Fischer 344 rats. Tumor can be palpable (approximate ϕ0.50*0.50cm) within 8-9 days for injection of one million cells (10^6). Tumor may not be developed within three weeks for injection of 10^4 cells. We also noticed the same difference in the speed of tumor growth among these syngeneic rats even for the same concentration of injected tumor cells.

Based on the above data, we first induced solid tumor in rats by subcutaneous injection of breast cancer cells and then the hydrogel with or wthout fMLF was injected directly into the tumor on the first day it is palpable (see table 1).

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Table 1

Injection	8 rats injected with 10^6 MAIII B cells on day1 4 of them injected with blank gel (300ul) on day 9(untreated), 4 injected with 100ug fMLF gel (300ul) on day 9(treated)
Observation	Tumor can be palpable on day 8-9 for all rats. Gel injection will enlarge the tumor volume (see Fig1). In a couple of days, most treated tumor showed a regression and intense inflammatory reaction, such as red, swell etc.
Conclusion	One injection of fMLF resulted in a transient suppression of tumor cell growth

Figure 3 depicts the average tumor growth curve of treated (gel containing fMLF) group and untreated (blank gel) group.

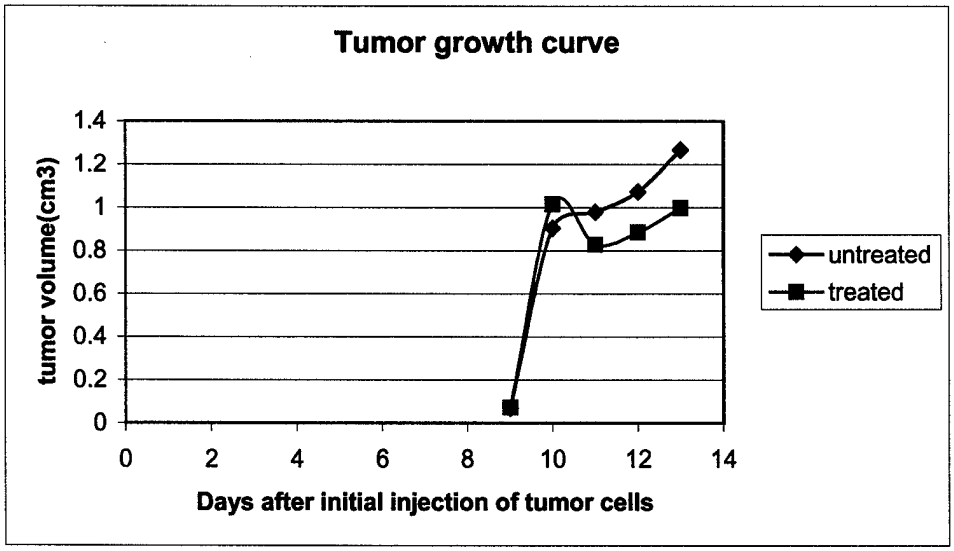

Fig3. Tumor growth curve for treated and untreated groups (4 rats/each group). Hydrogel depot is injected on day 9 after injection of 10^6 tumor cells

Just as above mentioned, the existence of individual difference (data not shown here) in the speed of tumor growth among these syngeneic rats even for same concentration of injected tumor cells may affect severely our analysis of tumor therapy. To counteract this adverse effect, same amount of tumor cells are injected into different subcutaneous sites in one rat, for example, one is injected on left back for control and another is on right back for experiment (table 2). This works very well in individual rats.

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Table 2

Injection	Each of 4 rats injected with tumor cells into left back and right back (10^6 MAIII B cells/per site) on day1 4 Left tumor injected with blank gel (200ul) on day 7 4 right tumor---no injection
Observation	Tumor can be observed on day 6-7 for all rats. Gel injection will enlarge the tumor volume a little bit (see Fig2). There is almost no visible difference in tumor growth curves between injected (left) tumor and non-injected (right) tumor.
Conclusion	This result further confirmed that above anti-tumor effect is from fMLF and hydrogel itself is non-immunogenic. The hydrogel can be degraded and will last for 3 weeks or even years if no sufficient immune response is elicited there.

By adopting this novel method, we first demonstrated that control gel almost has no effect in tumor growth at all, as is reflected from Fig4.

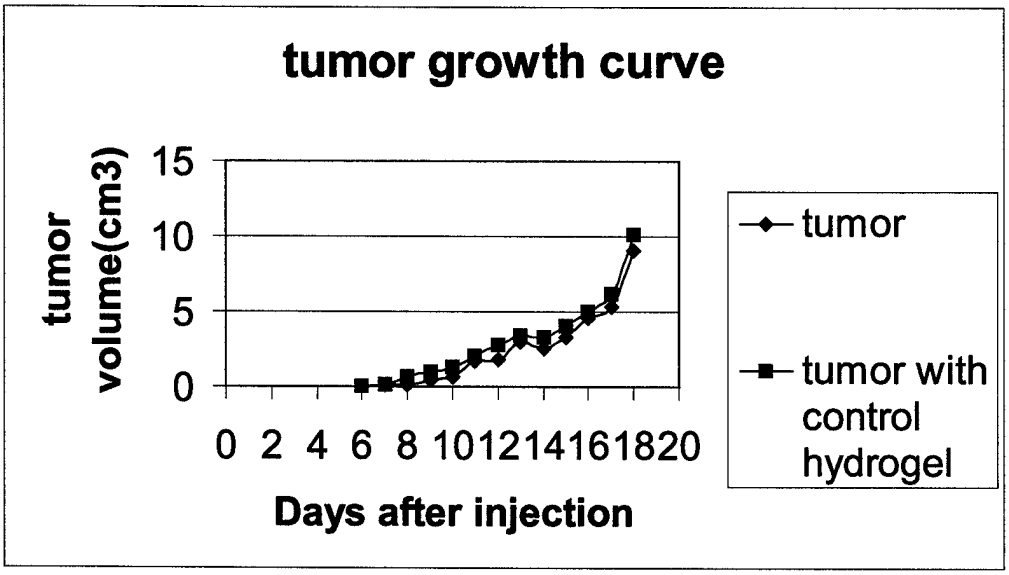

Fig4. Tumor growth curve for tumor with control hydrogel and tumor itself. hydrogel is injected on day 7.

However, we found that hydrogel can block the initial formation of visible tumor. We incubated 10^6 MATIII B breast cancer cells with 250ul hydrogel and injected them together into subcutaneous tissues of 6 rats. None of them developed visible tumors in one month. In this case the hydrogel may completely surround and entrap those tumor cells, cut off the supply of nutrients by complete inhibition of angiogenicity.

To test therapy effect of different drugs in tumor growth, we divided 12 rats into 3 groups (4 rats/each group). One group is for fMLF, one for IL-12, one for both (see table 3).

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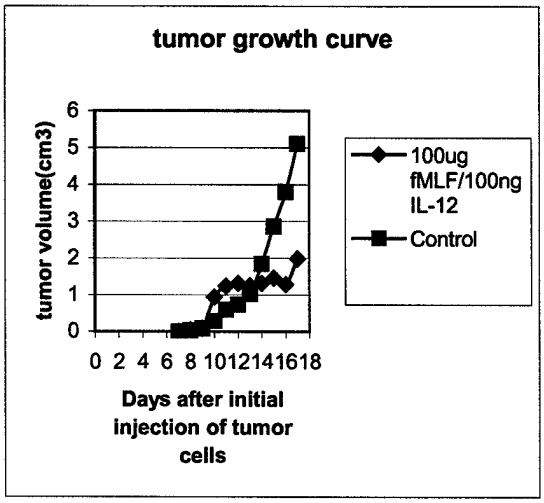
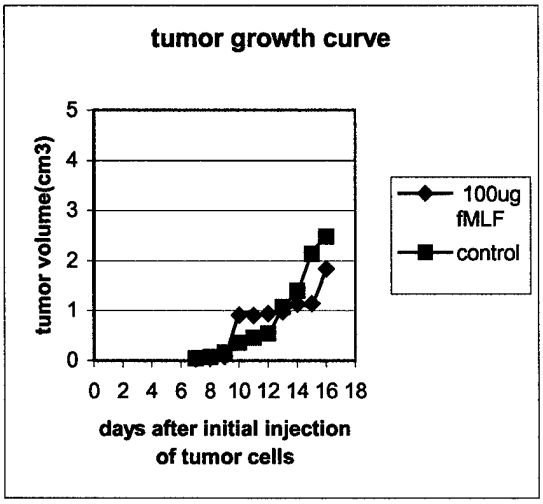
Table 3

Injection	Each of 12 rats injected with tumor cells into left back and right back (10^6 MAIII B cells/per site) on day 1. On Day 9 Group1 (4 rats): left –100ug fMLF, right—no injection for control Group2 (4 rats): left –100ng IL-12, right—no injection for control Group3 (4 rats): left--100ugfMLF/100ng IL-12, right-control
Observation	Tumor can be observed on day 7 for all rats. Tumor volume reached to approximate $1cm^3$ (see Fig5) one day after 250ul hydrogel was injected. Acute inflammatory response (red, swell) in those treated tumors was observed. Treated tumors have an initial increase due to the volume of gel, followed by a 4-5 days of growth-inhibition (a plateau)
Conclusion	It seems that once fMLF or IL-12 was injected, the initial high release of drug could inhibit tumor growth to the maximum degree.

Our results shown that either fMLF or IL-12, or both could transiently inhibit tumor growth or caused tumor shrink to certain extent (Fig5A-C).



(A)



(B)

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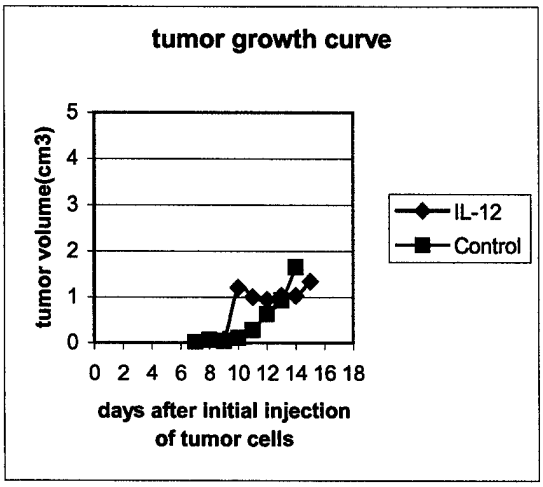

(C)

Fig5A-C. fMLF, IL-12 can retard or suppress tumor growth. 10^6 MATIII B breast cancer cells are injected subcutaneously into left and right back of each rat for inducing tumor. 100ug fMLF and/or 100ng IL-12 are applied on Day 9 for experimental group.

Our previous data shown that after the initial burst, the physically entrapped drugs can be released at a certain rate. They can last from several hours to several weeks for different drugs. Our in vitro release experiments for fMLF have shown that FMLF can last 48 hours to 72 hours (Fig 6).

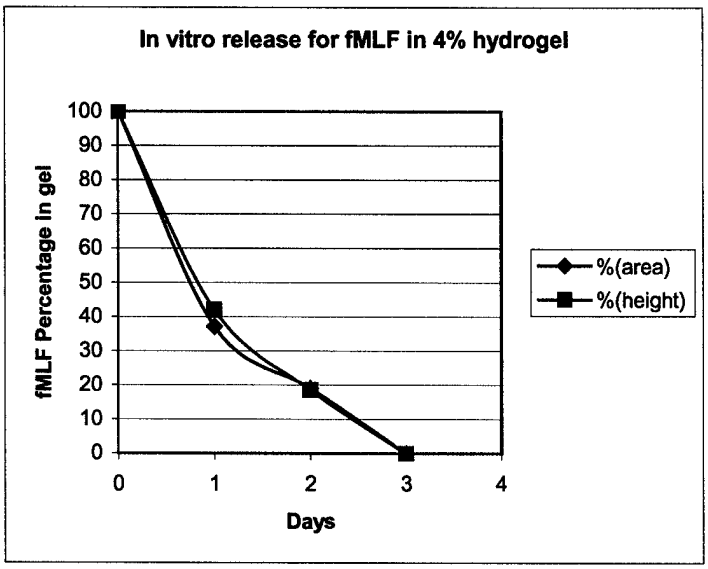

Fig6. In vitro release curve for fMLF in 4% hydrogel

fMLF is a poorly soluble small molecule drug, while IL-12 has a high molecular mass. Therefore, they may have the similar in vitro release curve and both can last for 3-5 days in vivo. This may justify our tumor growth curve in case of IL-12 and fMLF.

In contrast, another water-soluble fMLF analog—fMLFK can release much faster. It may last only 24-48 hours, as is confirmed by our data (Fig7).

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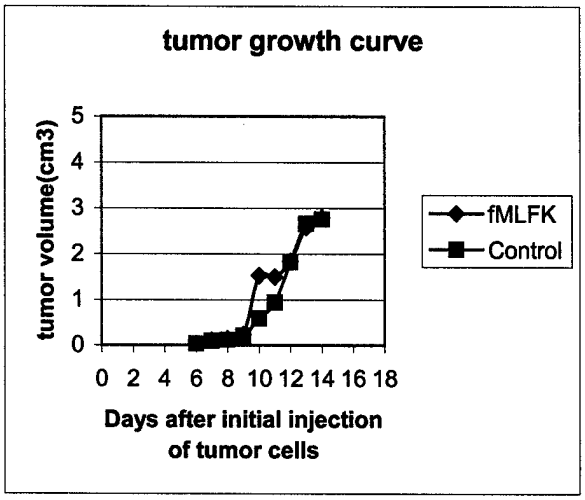

Fig7. Comparison of tumor growth curve in presence and absence of fMLFK. 10^6 MATIII B breast cancer cells are injected subcutaneously into left and right back of each rat for inducing tumor. 100ug fMLFK are applied on Day 9 for experimental group.

It seems that in our case, either one injection of fMLF or IL-12 could promote macrophages or monocytes to aggregate in tumor site and engulf tumor cells. Since this anti-tumor might be dose-dependent, We tried to use multiple injections to see if we can completely cure tumor by mounting an immune response to the tumor. We demonstrated that multiple injections (table 4) could significantly retard tumor growth (Fig8). Tumor growth returned when we stopped injection of drugs, but tumor growth stopped each time we re-injected the drug/gel depot.

Table 4

Injection	Each of 10 rats injected with 10^6 MAIII B cells tumor cells on day 1. On Day 9^{th} ,13^{th},19^{th} Group1 (5 rats): injected with 100ug fMLF/100ng IL-12 in 250ul gel Group2 (5 rats): injected with 250ul blank gel.
Observation	Each time both drugs were injected, they could slow down the tumor growth or caused regression for 3-4 days. Finally, average untreated tumor was almost 3 times treated tumor at day 23. Some treated tumors even formed a hole (Φ0.3-0.5cm) in them.
Conclusion	Although multiple injections can significantly reduce tumor growth, they finally fail to induce system or even local immune response when injections are stopped.

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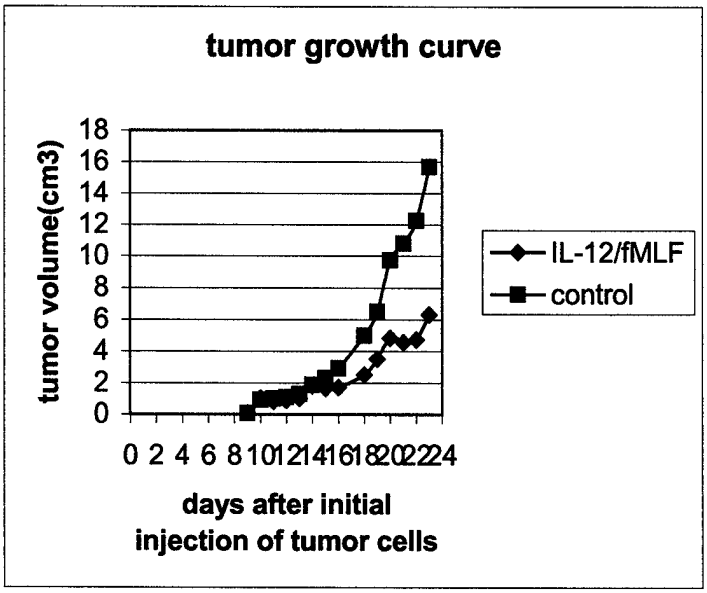

Fig8. Effect of multiple injections on tumor growth. 100ng IL-12/100ug fMLF is injected at day 9th, 13th ,19th separately.

The presence of transaction (Fig9) may demonstrate somewhat enhancing recognition of the tumor cells by immune system. For those rats with two tumors of same size on left and right back, IL-12 and/or FMLF were applied to only one tumor (for example left side) for experimental group and blank hydrogel was used for control group. Surprisingly, another tumor (for example right side) in experimental group also showed a little bit slow tumor growth compared with control group.

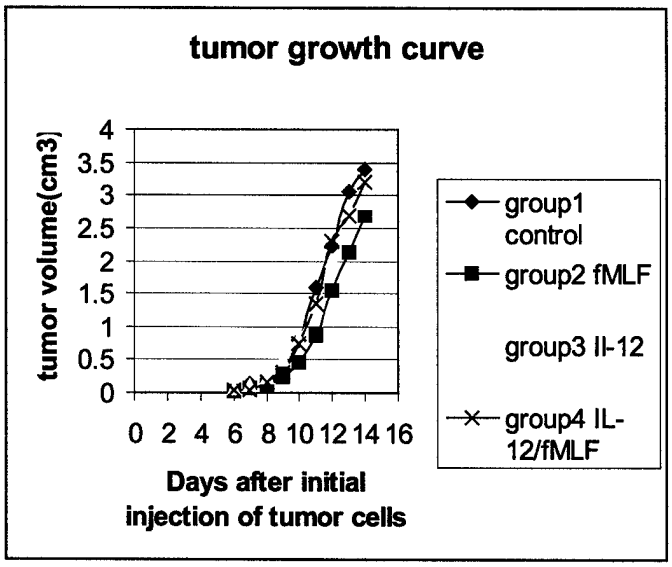

Fig9. Transaction. Injection of IL-12 and/or fMLF on one side could affect the tumor on another side to certain degree.

In our experiment, we also observed such an interesting phenomenon: when we first injected 10^6 cells using small needles (#25 OD 0.51mm), within 10-12 days only one of 15 rats developed visible tumor (ϕ0.70cm). If we injected another 10^6 cells into different sites using bigger needles (#20) at day 16th, all rats could develop tumor from 2nd injection within 10 days (day 26th). Another 4 of all rats also developed tumor from 1st

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injection at that time. Then we treated these rats with IL-12 and/or FMLF randomly. For one of three rats with three tumors (two from 2nd injection, one from 1st injection), when we treated only one tumor with 100ng IL-12 for about three weeks, it was completely cured. The IL-12 treated tumors from 2nd injection disappeared first and the controls from 1st and 2nd injection disappeared several days later (Fig10). Based on that, we think that systemic tumor immunity was induced in this rat.

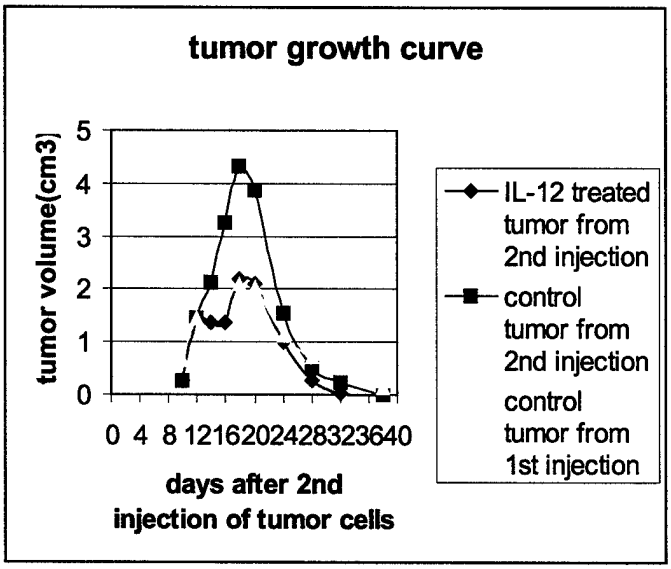

Fig10. Systemic tumor immunity

At the same time, we also noticed that for another rat with three tumors, when we treated one of two tumors from 2nd injection with the combination of IL-12 and fMLF, the control tumor from 1st injection disappeared at day 28 and the IL-12/FMLF treated tumor from 2nd injection showed a regression until day 28, while control tumor from 2nd injection showed no regression at all (Fig11). Therefore, we think that local tumor immunity was induced in this rat.

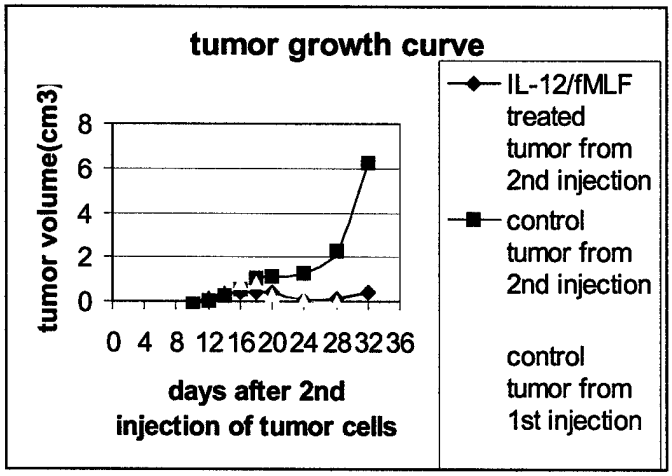

Fig11. Local tumor immunity

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3. Discussion:

Tumor resistance mediated by immune cells, was directed at tumor-associated transplantantation antigens (TATAs) of two types. The first are T antigens of viral origin, which are nuclear antigen shared by many tumors, even though these may not be even of the same tissue of origin. The second are antigens, which are specific to an individual tumor (tumor specific transplantation antigens-TSTAs). Tumors may express both specific and shared antigens.

However, tumors have multiple mechanisms for evading immune responses. The most obvious is that the tumor is non-immunogenic. This is not because potential tumor antigens are lacking but because the tumor cells are poor antigen-presenting cells. One possible reason for poor immunogenicity of tumors is the induction of peripheral tolerance by tumor cells that fail to deliver costimulatory signals [44], therefore, Costimulation may be necessary for tumor cells to function as alloantigen-presenting cells. Cell-surface molecules or cytokines secreted by APC can work as co-stimuli signal. Tumor antigen may be presented to T cells in a number of ways: 1.An absence of necessary co-stimuli might induce a negative signal resulting in T-cell anergy to tumor [45]. 2. Directly by a tumor that expresses co-stimulatory molecules, resulting in T cytotoxic cell activation. 3. Directly by tumor cells and indirectly by specialized APCs (such as langerhan's cell), resulting in activation of both T_C and T_H cells. The B7 molecule on specialized APCs, is now known to be the key co-stimulus acting via its counter-receptor CD28 on the T-cell surface. In addition, if the tumor lacks MHC class II, initiation of T_H responses will depend on processing of tumor antigen by specialized APC.

Tumor cells may also lack other molecules (such as LFA-1 and –3 or ICAM-1) required for adhesion of lymphocytes, or they may express molecules such as mucins, which can be anti-adhesive. Some tumors may also secret immunosuppressive cytokines such as TGF-beta. One particularly important escape mechanism is loss of MHC antigens [46-47], leading to inability to present tumor antigen peptides. More than 50% tumors (such as breast cancer cells) may lose one or more MHC class I alleles, and sometimes all class I is lost.

Just as above mentioned, dendritic cells are bone marrow derived leukocytes whose capacity for stimulation of the immune system through T-cell-mediated responses are the most effective of all known antigen-presenting cells (APCs). They possess an abundance of both classes of MHC molecules and present antigen for a prolonged period [48] accompanied by a range of costimulatory and adhesive accessory signals, including B7-1, B7-2, ICAM-1, ICAM-3, CD-40 [49]. The ability of highly motile dendritic cells to transport antigen to lymph nodes and activate effector T-cells has led to their proposal as the ideal candidate for the induction of specific anti-tumor immunity.

As we summarized above in Part I, DCs can be externally loaded with tumor antigens by competitive displacement of endogenous peptides normally bound to class I MHC or transduced with genetic constructs coding for the tumor antigen, as well as with cytokines that amplify immune responses. Unfortunately, the full array of tumor antigens is almost invariably not known, limiting the breadth of immune reactions induced by these approaches. What is more, these vaccination strategies of cell therapy require complex and costly ex vivo manipulation of patient's cells.

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To produce broader anti-tumor responses against a spectrum of tumor antigens, malignant tumor cells can be hybridized with DCs, yielding tetraploid cells [50] capable of production, processing and presentation of tumor antigens to CD8 T cells. However, this approach also remains technically challenging. A simple method for providing large numbers of DCs endogenously enormous chance to meet all available tumor antigens would be advantageous. That is the role of fMLF in our experiments: to promote broadly dispersed langerhan cells in skin to migrate and concentrate into solid tumor site and thus enhance the tumor antigen presentation to T helper cells. This did work well and the expected cytotoxic T lymphocyte response (CTL), local or systematic immune response can be observed in some rats.

There are currently several significant problems that need to be addressed.

1) Most but not all physically trapped fMLF in subcutaneous hydrogel can elicit acute or chronic inflammatory response in subcutaneous layer. We observed that only one of two rats with 100ug fMLF injection have inflammatory response. Our pathologic report from Dr. Stiefel shown that the formation of granuloma has something to do with inflammatory response. In the rat, which didn't have obvious inflammatory response, the granuloma shown a well-demarcated structure, while granuloma was formed incompletely in another rat, which had an acute response. We still don't know why this happened. We proposed that one well-decarmated granuloma might completely or partially block the release of drugs and inhibit the formation of concentration gradient of FMLF, which may be essential for chemotaxis. Another possibility is that incomplete granuloma may be the result of digestion of langerhan cells attracted by fMLF, which was supported by our findings that all tissues for control rats have well-decarmated granulomas. It has yet to be demonstrated further. If the second possibility is true, we have to yet find which factors are involved in inflammatory response and what caused such difference even for same concentration of applied fMLF.

2) Considering that anti-tumor effect may be dose-dependent for IL-12 and fMLF in most rats. Multiple injections (i.e. every 4 or 5 days) are proved to be effective to significantly reduce tumor growth (2-3X). However, this method has obvious disadvantages such as trivial, high volume of hydrogel needed, some unnecessary pain, interference of tumor volume etc. In the future, we mean to use different depot formulations such as the excipients, degradable chemically-linked drug etc to adjust or slow down our drug release speed to desired status. In this case, for only one injection the sustained release amount drug can be enough to function for several weeks or even months instead of 4-5 days.

3) Multiple injection didn't cure tumor in our experiment. Although the 4 of 5 (80%) treated rats shown significantly reduced growth curve compared with control rats (Fig7), the progression of these tumors finally still killed these rats. That means these treated rats themselves did not mount enough immune response to circumvent tumor but depended on exogenously applied cytokines (fMLF or IL-12) to temporarily retard tumor progression. Some data [51] showed that the most effective in situ activation of syngeneic tumor specific CTL can be generated within no more than 9 days by primary immunization. In our case, tumor-specific CTL response wasn't observed as long as two weeks after initial administration of fMLF and IL-12. One possible reason is that although the attracted immature langerhan cells can engulf the tumor cells and take up

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antigen, they didn't reach certain threshold, which is required for their maturation and migration into lymph node. Therefore, they can not interact with T helper cells there and initiate systemic immune response and tumor tolerance is resulted in. It is still a mystery whether there are any other factors involved in such maturation of langerhan cells besides the prolonging contact between langerhan cells and tumor antigen.

4) To our surprise, one occasional phenomenon attracted our attention. Two injections of tumor cells in different sites followed by administration of IL-12 alone (Fig9) or IL-12 with fMLF(Fig10) but not fMLF alone can elicit CTL response in 9-10 days after treatment of drugs. The mechanism is still unknown. But there is one point that we can confirm. The first injected MATIII B breast cancer cells seem to be less active because it took 26 days for some of them to develop visible tumors. In contrast, it only took only 9-10 days for the second injected breast cancer cells of same concentration. What was function of first injected less active tumor cells? Did they work as irradiated (inactive) autologous tumor vaccine [52]? Why did only 5 of 15 rats first injected with less active tumor cells of same concentration develop tumor? These problems are yet to be unveiled.

5) We wonder why IL-12 can elicit systematic immune response while combination of IL-12 and fMLF just result in local immune response. Does the existence of fMLF partially counteract the role of IL-12? Just as above mentioned, fMLF can totally abrogate proadhesive and chemoattactant responses to IL-8. The dominance of FPR may ensure that signals from these terminal phagocytes target can override host-derived recruitment signaling through IL-8 and other chemokine receptors [12]. Can we get some hints from this point? Combination therapy didn't show any better anti-tumor effect than therapy using IL-12 alone or fMLF alone, or even a little worse than IL-12 alone (Fig 5A,C). Is there also any crosstalk between IL-12 and fMLF function?

A typical immune response usually culminates in a state of specific T and B memory where secondary responses are more vigorous and effective than primary response. Generation of memory T and B cells is the end result of a highly destructive process in which most of the responding lymphocytes are rapidly eliminated, and only a small proportion survive to become long-lived memory cells. If we make better use of that cured rat and inject it with same line of tumor cells of higher concentration several weeks or months later, we can know if it did develop immune memory response.

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