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Tumor antigens are autologous antigens and thus are weakly immunogenic. Unresponsiveness appears to be related to suppression of antigen specific helper T cell function which can be overcome by providing heterologous help. Carbohydrates are richly expressed on the surface of many cancers, at frequencies higher than oncogene products. Consequently, tumor associated carbohydrate antigens, are in principle, excellent targets for immunotherapy. However, carbohydrates are generally poor at eliciting effective antibody responses and rarely provide target epitopes for CTL because of their T cell-independent nature. The major objective of this application is to examine ways to maximize the tumor-protective immunity directed to carbohydrate antigens expressed on breast tumors. Towards this end we are developing peptide mimotopes of tumor associated carbohydrate antigens as they are T cell dependent antigens. In our progress to date we have shown the 1.)immunization with peptide mimotope activates a specific cellular response to a model murine tumor cell line; 2.) vaccination of mice with peptide eradicates established tumor; 3.) Immunization with DNA format of the peptide suppresses tumor growth in further challenge; and 4.) Induced immunity has a cellular nature as it is transferred to nude mice by transferring splenocytes from cured mice.

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Introduction

Carbohydrates are the most abundantly expressed self-antigens on tumor cells and consequently they are perceived as viable targets for immunotherapy. Aberrant glycosylation of membrane components due to specific alterations of glycosyltransferase activity is a common feature of carcinoma cells and is usually associated with invasion and metastasis. Examples of tumor-associated carbohydrate antigens include GD2, GD3, fucosyl GM1, Globo H, STn and the neolactoseries antigens sialyl-Lewis x (sLex), sialyl-Lewis a (sLea) and Lewis Y (LeY). While pure carbohydrate antigens elicit diminished immune responses because of their T cell independent nature, conjugate vaccine technology has overcome some of these limitations of carbohydrates as vaccines because of the T-dependent (TD) help conferred by the carrier protein. However conjugation of carbohydrates to a carrier protein that elicit carrier-specific T and B cell responses does not necessarily enhance carbohydrate immunogenicity. Furthermore, as there are many carbohydrate types expressed on a tumor cell it may be impractical to develop multivalent vaccines that target each of the surface expressed carbohydrate antigens. Consequently, new formulations or alternative ways to augment carbohydrate immune responses are being evaluated. One alternative that we are pursuing is the development of peptide mimics of core carbohydrate structures expressed on the tumor cell surface. These core structures would be reflective of conformational similarities among what are other wise considered dissimilar carbohydrate antigens. The identification of such peptides would simplify vaccine development in that it may reduce the complexity of multivalent vaccines. Such peptides would function as surrogates of broad-spectrum antigens.

Our studies have evolved to focus on two aspects. One, to better define carbohydrate targets on breast cancer cells that are important in defining their tumor phenotypes and two to develop new formulations or alternative ways to augment carbohydrate reactive immune responses. During the current funding period we have 1.) further examined the murine 4T1 model as a prototype breast cancer cell line; 2.) showed that immunization with the DNA form of a rationally chosen peptide mimic, using a lectin reactive profile with 4T1 cells as a template to define carbohydrate types, induces a significant increase in survival rate of tumor-bearing animals in this murine mammary tumor model.; and 3.) identified patterns in altered carbohydrate expression that correlate with organ-specific tumor growth characteristics of a human breast tumor line selected with an enhanced bone colonization phenotype.

In the previous year we demonstrated that a peptide mimicking breast associated carbohydrate antigens are capable of activating a carbohydrate specific cellular immune response (manuscript #1 in press) that limit tumor growth in vivo. Thus, carbohydrate-mimicking peptides represent a new and very promising tool to augment immune responses to tumor associated carbohydrate antigens.

Task 1. Establish murine breast tumor model (months 1-3). Our initial focus for generating anti-carbohydrate immune responses to tumor cells was based on the murine fibrosarcoma cell line Meth A which reacted with the lectin WGA and sorted for FH-6 reactive epitope which is an extended form of sLex (manuscript #1 in press). WGA reactivity has been shown to be associated with processed glycopetidep as T cell targets and FH6 reactivity has been associated with complement dependent cytotoxicity (CDC)

killing of tumor cells. In addition, sLex and slea forms are associated with cell adhesion and metastatic properties. We observed that Meth A expressed FH6 reactive sLex forms while 4T1 cells did not (Table 1). Therefore, Meth A cell targeting provided proof of principle concepts to be explored while we were developing the more appropriate 4T1 model. The purpose of this task was to develop a model cell line expressing carbohydrate antigens relevant to breast cancer. We chose the 4T1 tumor model based on its tumorgenecity, high metastatic potential and similar characteristics with human breast tumors in that 4T1 can metastasize to the lung, brain, liver and bone. However, using monoclonal antibodies FH-6, BR-55 (LeY specific), CA19.9 (sLea specific) and Cslex (sLex specific) in FACS, we established that 4T1 cells do not express these epitopes. In order to use 4T1 cells as a relevant breast model for evaluating our mimotopes, we planed to transfect the 4T1 cell line with fucosyltranferases required to generate constituents of neolactoseries antigens in order to express these antigens on the cell surface. We postponed this task in the first year as we explained in the first-year report. Consequently we pursued this task during the current funding year.

The gene fucosyltransferase III (FTIII) was cloned into the pCDNA3 vector (Invitrogen). In our first attempt to transfect 4T1 cells we used liposomal transfection reagents Genfect and Maxfect (Mediatech, Herndon, VA) based on the manufucturer's instructions. Transient expression was assessed by FACS using a panel of anti-carbohydrate antibodies (BR55-2, FH6, CA19-9, Cslex and the ganglioside specific monoclonal antibody ME361). In none of the experiments could we detect gene expression and we could not establish a stably transfected cell line. It looked like the efficiency of transfection was too low for selection. We changed the transfection reagent to DOTAP (Roche) but still the efficiency seemed very low. We used a GFP construct in co-transfection to assess our transfection efficiency for each transfection, It confirmed that the efficiency of transfection in the 4T1 line was very low.

To overcome difficulties regarding the low efficiency of transfection we decided to use GFP as a selective marker and cloned the FTIII gene in a special construct, which is supposed to express both GFP and the FTIII. Then, using FACS, cells were sorted based on the GFP expression. Using the following primers (5' CGAGAATTCTCAGGTGAACCAAGCCGCTATG 3' and 5' CGACTCGAGATGGATCCCCTGGGTGCA 3') we cloned the FTIII gene between EcoRI and Xho cloning sites of plasmid pIRES2-EGFP (BD Biosciences). pIERS2-EGFP contains the internal ribosome entry site (IRES; 1, 2) of the ECM virus between MCS and the GFPgene. This allows both the gene of interest (here FTIII, cloned into MCS) and the GFP gene to be translated from a single bicistronic mRNA. This plasmid is designed for the efficient selection of transiently transfeceted mammalian cells and can be used to obtain stably transfected cell lines without time-consuming drug and clonal selection. We cloned the gene and transfected 4T1 cells using lipofectamine^{TM} 2000 (Invitrogen). Cells were propagated in selective medium for a week and then checked for the GFP expression by FACS (Fig 1). Having a good GFP-positive cell percentage we looked at carbohydrate epitopes using the above mentioned antibodies plus KM-93. KM-93 (against sLex) was found to be highly reactive with 4T1 cells and was recently added to our array of anti-carbohydrate antibodies. What we saw was an increase in the

percentage of KM-93 reactive cells (Fig 2). It seems that the product of the gene expression mostly contributes to the formation of KM-93 reactive sLex epitope, which is present on the majority of 4T1 cells.

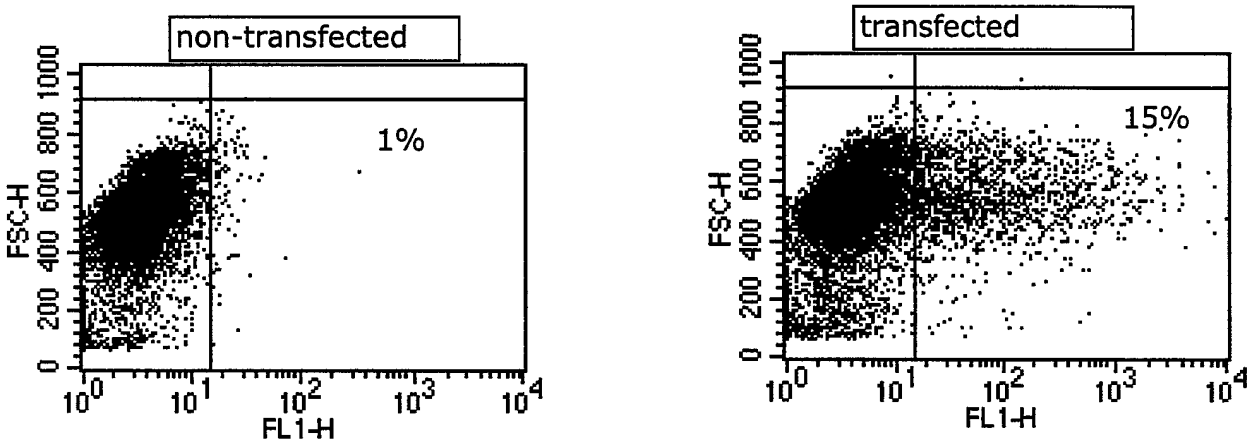


Figure 1. *4T1 cells were transfected with FTIII-EGFP construct and transfected cells were kept on selective medium for one week then green fluorescence was measured. 15% is the percentage of GFP-positive population.*

As the carbohydrate profile is an endpoint of many cellular interactions, which depends on the cell type, we probably ended up with more of the KM-93 epitope. FH6 reactivity was also increased (Figure 3).

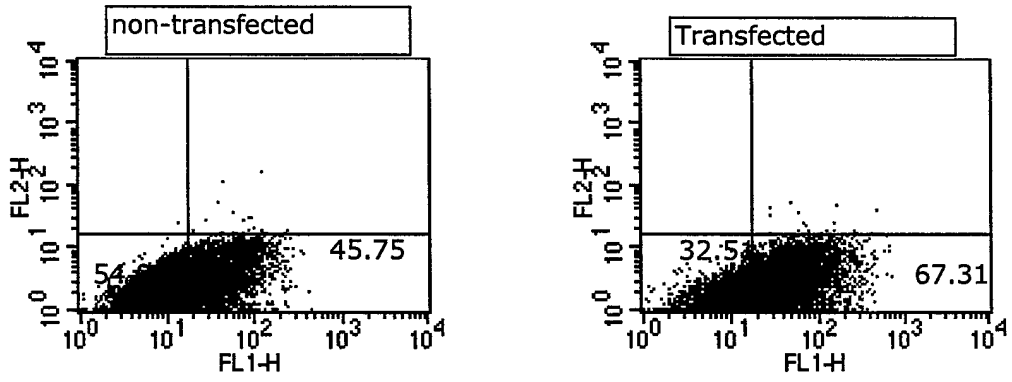


Figure 2. *4T1 cells were transfected as in fig. 1 and their reactivity with KM93 was measured by FACS after culturing in selective medium for a week. FH6 reactivity was observed on 67% of the cells after transfection with FTIII.*

Based on this data, we decided to further characterized the cell surface carbohydrates of the original 4T1 cells and explore the potential of other mimotopes to be used to target those carbohydrates. This constituted a deviation in our objectives for this Task but falls

within the stated objective of the proposal, which is exploring ways to maximize responses to carbohydrate antigens on breast tumors.

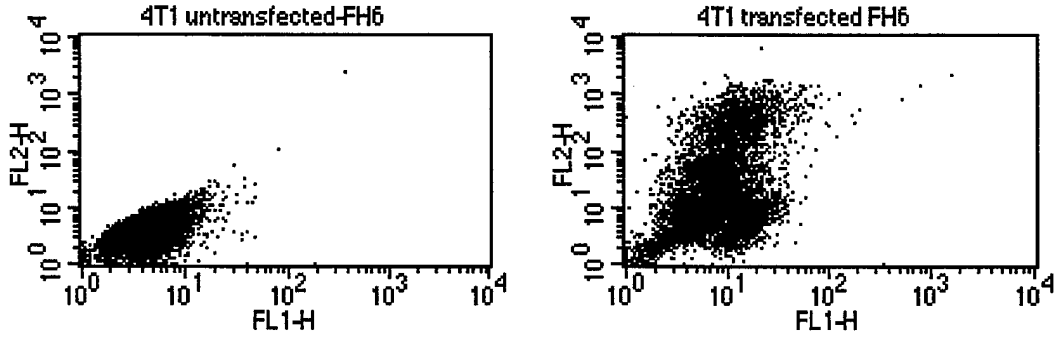


Figure 3. *Cells were transfected with a vector containing both GFP anf Fuc-TIII encoding genes. Cells were kept for two weeks in selective medium and sorted for green fluorescence activity. After propagating sorted cells in selective medium, they were sorted once with FH6 antibody. Cells, then, were propageted and a FACS analysis revealed the FH6 reactivity as shown above. FL1 is GFP activity and FL2 is FH6 binding by PE-conjugated antibody.*

Carbohydrate profiling of 4T1 cell surface

High reactivity of KM-93 with 4T1 cells directed us to use the reactive epitope for targeting. The KM-93 epitope is present on a majority of carcinomas including breast. To extend our pool of probes for carbohydrate identification we also used relevant lectins to further characterize 4T1 cell surface carbohydrates. The data are summarized in figure 4 and Table 1.

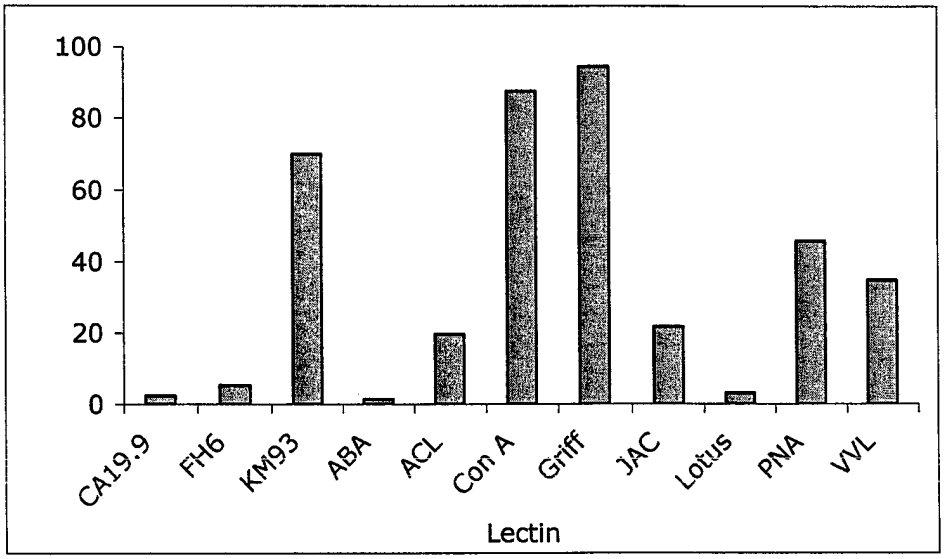
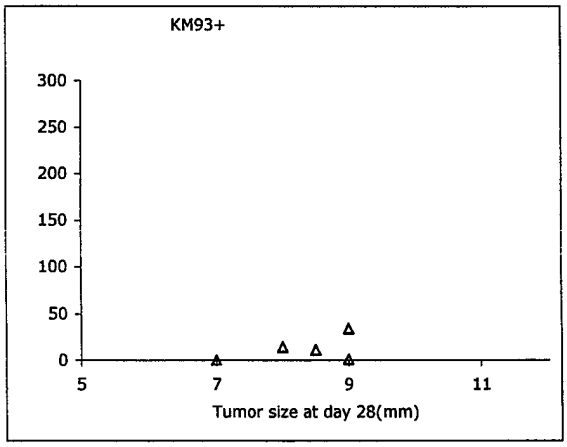


Figure 4. *Percentage of positive 4T1 cells for indicated lectins.*

Table 1. *Summary of reactivity profiles of lectins and antibodies for tumor cell lines based upon strength of binding as the respective probes in FACS analysis. The number of +'s indicate relative reactivity.*

number	Lectin	Cell line					
		Meth A	P815	4T1	MCF7	MDA231	
1	WGA	+++	++	+++	+++	+++	
2	ABA	-		-	-	-	
3	ACL	+++	++	+	+++	++	
4	PNA	+-	+	+	+++	++	
5	ConA	+++	+++	+++	+++	+++	
6	GS-I	+++	++	++	++	-	
7	VVL	+	+	++	++	+-	
8	Lotus	-	-	-	++	-	
9	MPL	++	++	+++	+++	++	
10	Jac	+++	+++	++	+++	+++	
11	SNA (EBL)			++	++	-	
12	ECL			++	+++	++	
13	HPA			+-	++	++	
14	PHA			+++	+++	++	
15	KM93		+++	+++		+++	
16	FH6	+++	+	-		+	
17	BR55		-	-	+++	-	
19	CA19 .9		++	-		+	

However we observe that negative selection of 4T1 variant cells for expression of KM93-reactive sLex significantly enhanced metastatic potential of the cells (Manuscript # 2 in preparation). Our findings indicate that indirect positive selection for fucosylated structures on the cell surface might have caused extensive enhancement of metastatic spread. 4T1 cell line was sorted to produce two sub-populations of KM93-Positive (KM93-Pos, high expression of the epitope) and negative (KM93-Neg) variants. We observed that all mice inoculated with both negative and positive variants produce lung metastasis (Figure 5). However, the number of clonogenic lung metastasis increased significantly in the group inoculated with the KM93-negative variant ($p = 0.018$). Carbohydrate profiling of tumor cell surface revealed that negative selection of tumor cells considerably increased the frequency of Lotus-reactive cells.



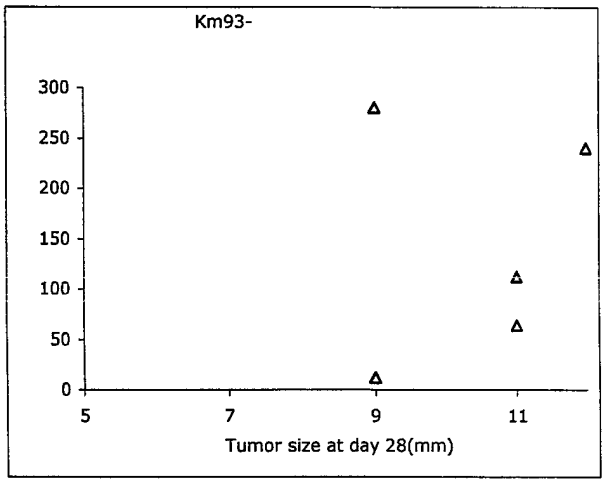


Figure 5. *Triangles represent individual mice.*

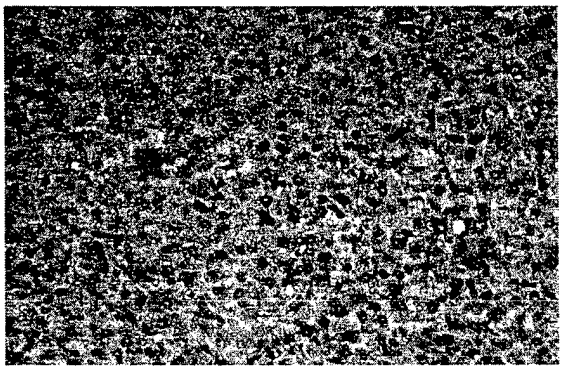
Lotus lectin reactivity for the original 4T1 population or positively selected variant was negative or slightly positive as assessed by FACS (Figure 6). Lectin histochemistry on organs harvested from a tumor-bearing mouse inoculated with original 4T1 cells demonstrates positive patches in primary mass and that tumor cells in liver and lung are also positive for Lotus (data not shown) (manuscript # 2 in preparation). While emphasis has been placed on sialylated Lewis antigens based upon their reactively with selectins and subsequent tissue dissemination, these data propose a major role for the involvement of fucosylated structures in metastasis of the 4T1 cell line.

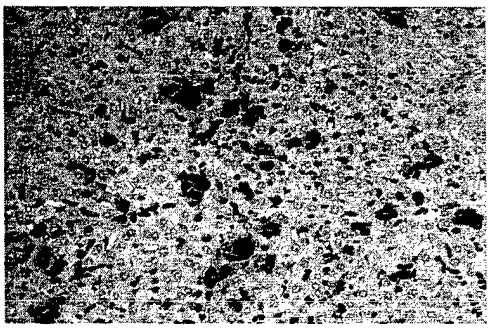
Lectin binding to metastasized tumor cells

To investigate the expression profile of tumor cells in lung and liver, we decided to first focus on GS-1 binding epitope. GS-1 displays multiple carbohydrate reactivies that include leb, leY and the Gal epitope. Mice were transplanted with 4T1, sacrificed, organs were harvested and immune staining was performed on lung, liver and primary tumor mass. As demonstrated in figure 7, GS-1 binds to a percentage of tumor cells in the primary mass and lung, while it binds to all tumor cells in liver. Data indicate that the carbohydrate moiety is present on metastatic cells in both liver and lung but the percentage of GS-1-reactive tumor cells in lung are much less than liver. Data imply that tumor cells do somehow need GS-1-reactive structure to grow in the liver.

To further investigate GS-1-reactive specificity for lung and liver, tumor cells were isolated from the liver of tumor-bearing mice and returned to 5 mice after in vitro culture and propagation. Then cell migration to lung and liver was compared. Metastasis to both lung and liver was observed in all mice. Tumor cells were recovered from each organ and cultured in vitro. A FACS analysis of GS-1 binding to propagated cells revealed that the lectin binds very well to cell harvested from the liver and not to cells from the lung. This results confirms the histology data and implies that the carbohydrate structure do not participate in lung metastasis. While there are no differences between individual metastasis to the lung and the liver, compare to previous experiments, it seems that in vivo enrichment increase the rate of metastasis to the liver.

A) **B)**





C)

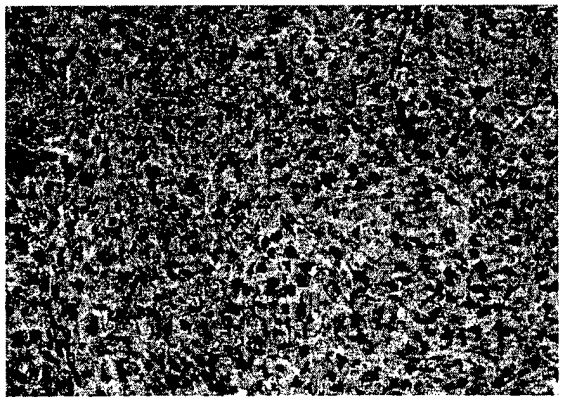


Figure 7. Tumor was inoculated and 45 days later animals were sacrificed, organs harvested, cuts made and slides were prepared. Slides taken from different organs were stained with GS-1 at 2.5 ug/ml concentration. A, B and C correspond to sections from primary tumor, Liver and Lung, respectively.

Mimotopes reactive with GS-1

Peptide mimotopes shown in Table 2 were screened for reactivity against Con A, WGA and GS-1 to identify a peptide reactive with all three of these lectins. Initially starting with GS-1 we observed that GS-1 binds predominately to peptides 107 and 105 as shown in figure 8A (manuscript # 3 in preparation). As shown in figure 8B, GS-1, but not lotus, binds to peptide 107 in a dose-dependent manner. In an inhibition assay, the 107 peptide significantly inhibited GS-1 lectin binding to the cells (Fig. 8C). Therefore, we selected a peptide that parallels lectin reactivity profile with the 4T1 cell surface. Peptides 104 and 109, which were isolated from library screening against BR55-2, used as a negative control as we expect that they both target LeY antigen, which is absent from the cell membrane.

Table 2. Peptides used in the study

Peptide	Sequence
104	GGIMILLIFSLLWFGGA
105	GGIYYPYDIYYPYDIYYPYD
106	GGIYWRYDIYWRYDIYWRYD
107	GGIYYRYDIYYRYDIYYRYD
109	GGARVSFWRYSSFAPTY

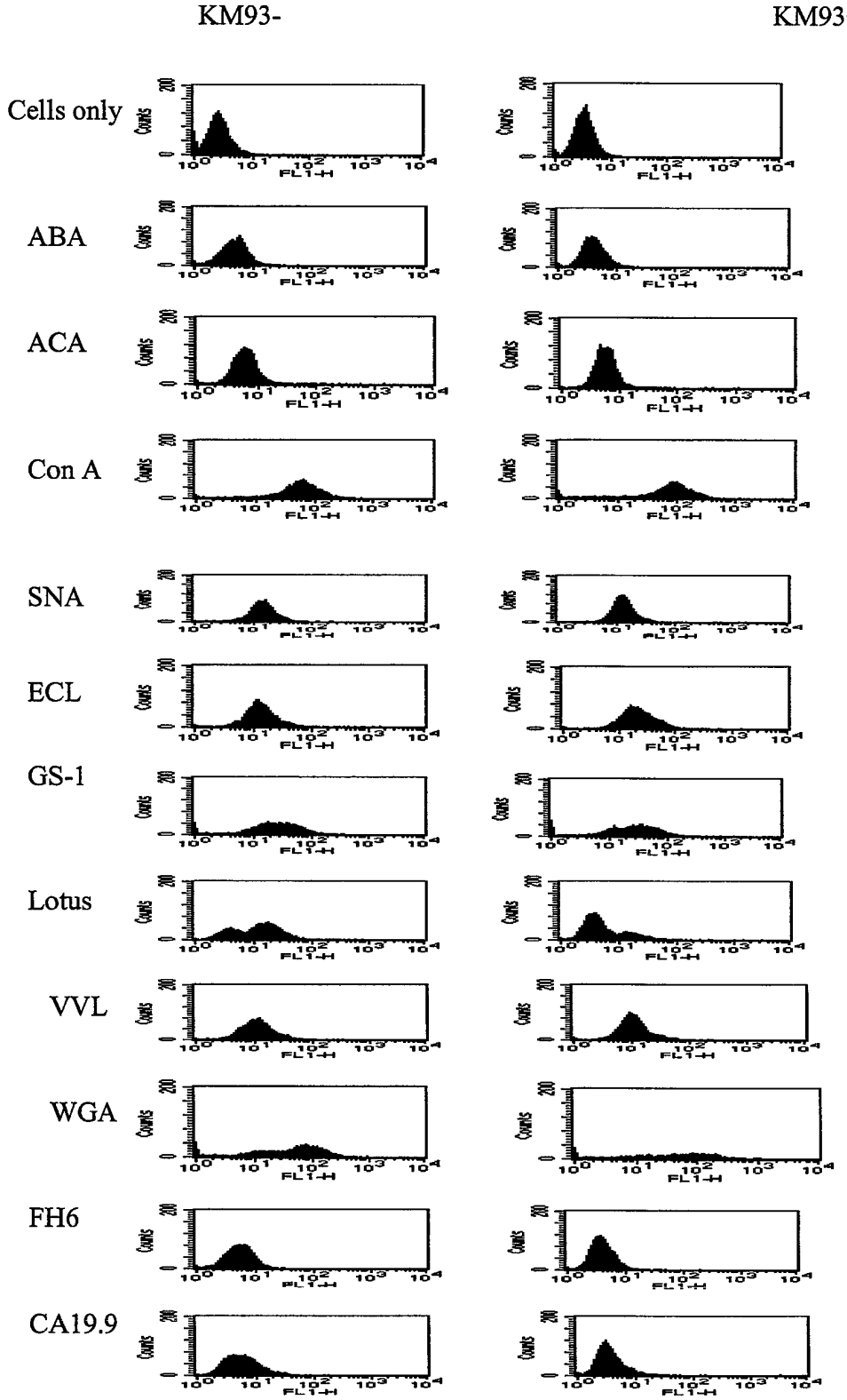


Figure 6. *Lotus distinguished between KM93Neg and KM93Pos cells.*

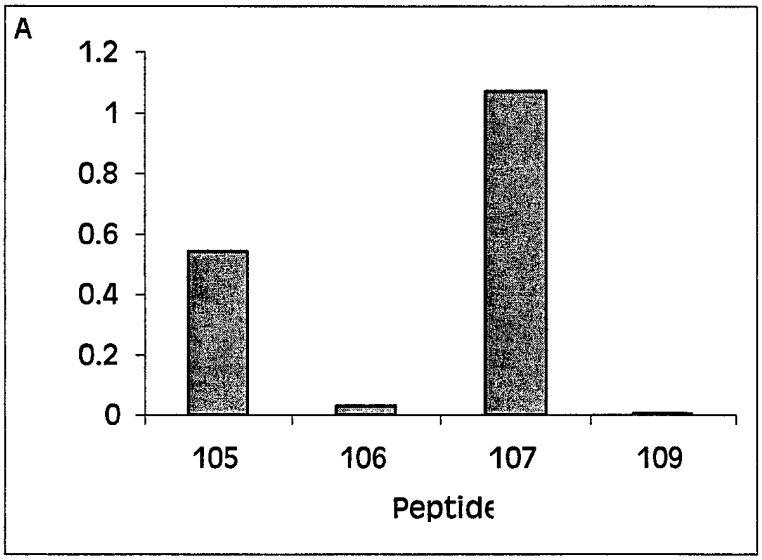


Figure 8A. GS-1 specifically binds strongly to the 107 peptide. A) ELISA plates were coated with indicated peptides and GS-1 binding was detected using biotin/streptavidin system.

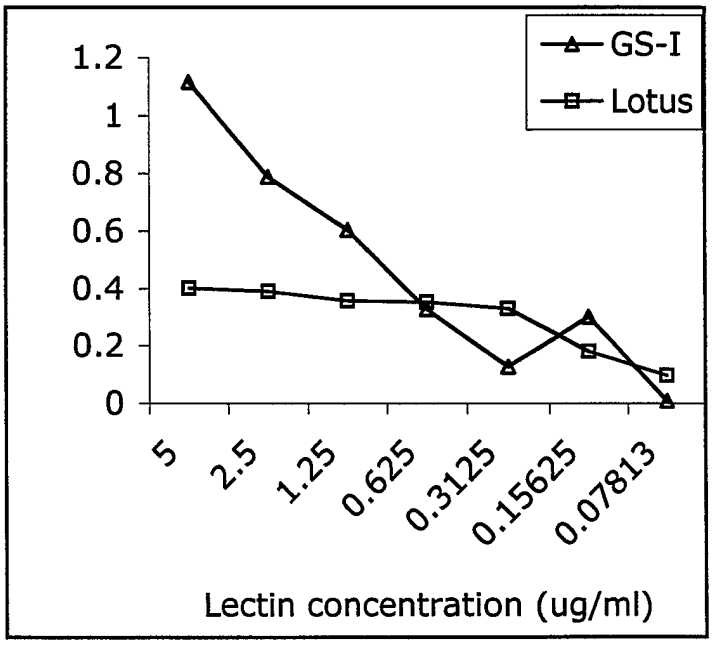


Figure 8b. Plates were coated with 107 peptide and dose-dependent reactivity of GS-1 and lotus lectins were assessed.

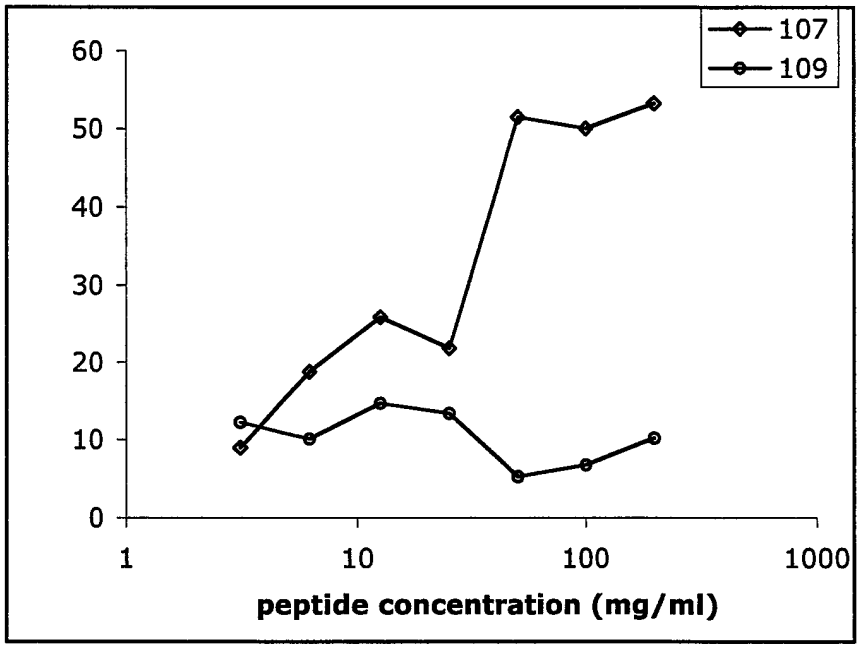


Figure 8C. GS-1 binding to 4T1 cells was inhibited by107 peptide as revealed in a FACS assay. 109 peptide was used as the negative control.

Task 2. To evaluate immune parameters associated with DNA vaccination of plasmids encoding glycotope (months 1-16).

In this task we were supposed to characterize immune responses to 911 and 106 peptides encoded into DNA. We did characterize the immune response to 911 and 106 peptides working in the context of Meth A cells (manuscript #1 in press). As explained above we switched to 4T1 cells and now have other peptides that might work as better candidates for targeting carbohydrates on 4T1 cells. 107 peptide contains the YRY sequence, which mimic constituents of Lewis antigens and mannosyl residues in that peptide 107 is reactive with ConA, GS-1, WGA and BR55-2. Generated anti-serum reacts with both Leb and LeY antigens on the plate (Figure 9).

We decided to clone the 107 peptide in PSeqTag2/Hygro (Invitrogen) based on its ability to secret the expressed peptide out of cells. pSecTag2 is a mammalian expression vector designed for efficient secretion of fusion protein. This vector contains a secretion signal from V-J2-C region of Ig kappa chain under the control of cytomegalovirus. We designed and synthesized oligos (Table 3) for cloning in pSecTag2/HygroB. Cloning was confirmed by sequencing all constructs at the final step.

Table 3. *Oligonucleotide sequences used for cloning and making DNA constructs.*

Peptide	Oligonucleotide sequences
104	agcttGGCGGCATCATGATCCTGCTGATCTTCTCCCTGCTGTGGTTCGGCGGCGCCTAAgc ggccgcTTAGGCGCCGCCGAACCACAGCAGGGAGAAGATCAGCAGGATCATGATGCCGCCa
105	agcttGGCGGCATCTACTACCCCTACGACATCTACTACCCCTACGACATCTACTACCCCTACGACTAAgc ggccgcTTAGTCGTAGGGGTAGTAGATGTCGTAGGGGTAGTAGATGTCGTAGGGGTAGTAGATGCCGCC
107	agcttGGCGGCATCTACTACCGCTACGACATCTACTACCGCTACGACATCTACTACCGCTACGACTAAgc ggccgcTTAGTCGTAGCGGTAGTAGATGTCGTAGCGGTAGTAGATGTCGTAGCGGTAGTAGATGCCGCC

Based on our first year experience using 106 and 911 peptide with IL-12 and promising data obtained with co-immunization with IL-12 (manuscript #1 in press), we decided to use IL-12 as our adjuvant and directly test the immunization strategy to treat the tumor.

After preparing the above constructs their immunogenicity was evaluated. Mice were immunized and serum collected and the reactivity of the serum against cells and carbohydrate antigens was measured. We performed ELISA assays with DNA-generated serum against the 107 peptide. ELISA plates were coated with Leb and Ley antigens and reactivity of the serum was detected against them. The 107 DNA-induced serum reacts with both carbohydrates with an endpoint titer of 1:2000. The result of anti-Leb reactivity is shown as representative data (Fig 9a). As shown in figure 8a DNA immunization induces antibodies reactive with 4T1 cells of IgM origin.

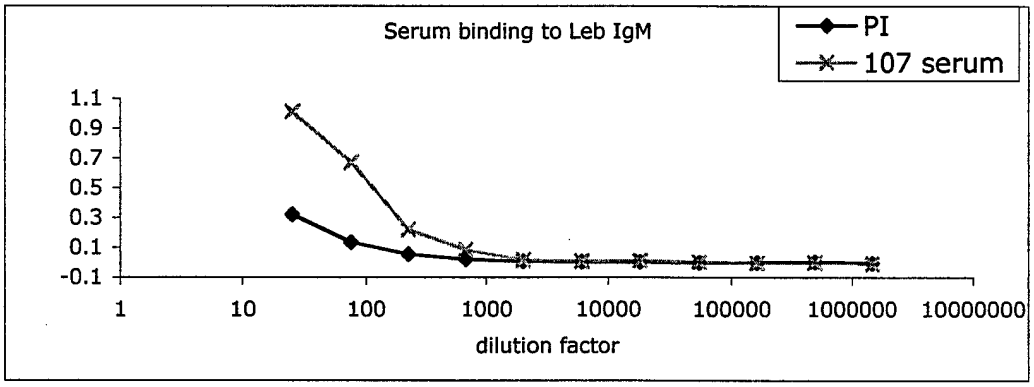


Figure 9a. *Serum response to DNA immunization. Mice (10/group) were immunized three times with 107-coding sequence and the respective vector plasmid pSec. Animals were bled 10 days after the last boost. For each group sera were pooled for 10 mice. ELISA plate was coated with Leb antigen and reactivity of IgM portion of serum antibodies was detected.*

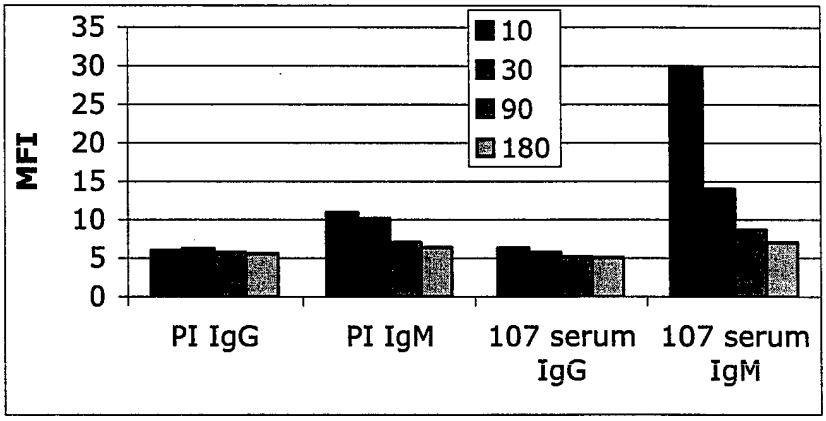


Figure 9b. *Binding of serum antibodies to 4T1 cells was detected by FACS and presented by mean fluorescence intensity (MFI). Mice were pre-bled (PI) and then immunized with the DNA construct (50 ug/mouse) of 107 in PBS. 107 serum is the serum that was collected 10 days after boost. 10, 30, 90 and 180 show dilutions of sera as in 1:10, 1:30, 1:90, and 1:180, respectively.*

GS-1 induces apoptosis in 4T1 cells.

GS-1 induces apoptosis in a number of murine cell lines. To validate if the lectin induces apoptosis and cytotoxicity in 4T1 cells, cells were incubated with GS-1 lectin and cytotoxicity measured by percentage of dead cells after 24 hours of incubation. We observed 55% of cytotoxicity in cells that were incubated with GS-1 (data not shown). Supplementary experiments proved that GS-1 induces cytotoxicity in a dose-dependent manner. Then we used the Vybrant Apoptosis Assay Kit (Molecular Probes inc) for detection of apoptosis in a 4-hour assay. The assay is designed based on phosphatidylserine translocation on the cell surface and annexin V binding. For detection, FITC-conjugated annexin V and PI were added to cells. As shown in figure 10a necrotic (FL2 channel) and apoptotic (FL1 channel), percentage of positive cells were increased after preincubation with GS-1. Likewise we observed that IgM serum antibodies raised by 107 DNA immunization also mediated apoptosis (Figure 10b) (manuscript #3 in preparation).

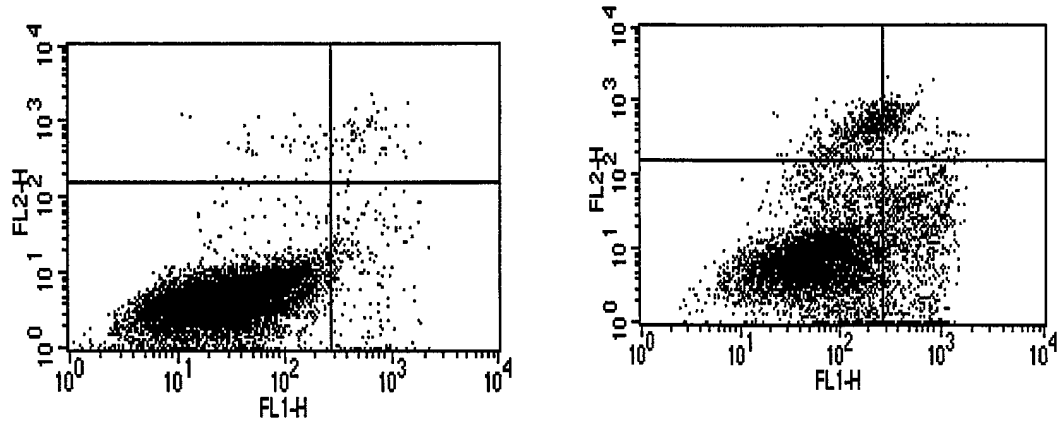


Figure 10a. *Cells were coincubated with (right) or without (left) GS-I for four hours. Cells then were harvested and stained with propidium iodide (FL2) and annexin V(FL1).*

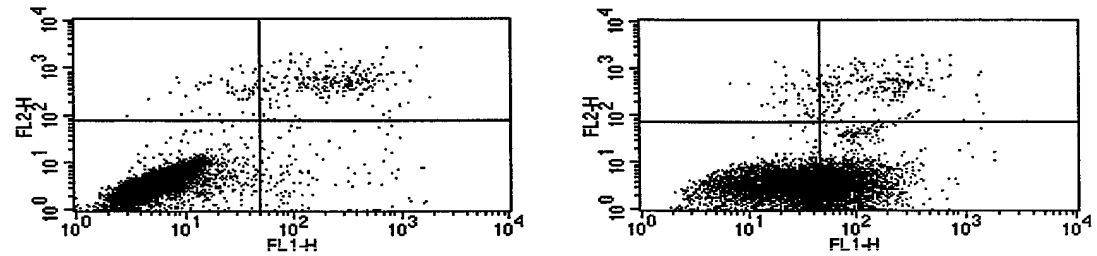


Figure 10b. *4T1 cells were incubated with 10% serum-supplemented medium from pSec (vector, left) or 107-pSec (right) immunized animals for 6 hours, then cells were harvested and stained with propidium iodide(FL-2) and annexin V (FL-1).*

Task 3. Evaluate priming and boosting effect of immunogens. (Months 10-20).

Carbohydrate conjugates have been used extensively for induction of the immune responses to carbohydrates. We decided to conjugate peptide mimotopes to carbohydrates in order to see if we are able to increase the efficacy of the serum in functional assays (Fig 11). We performed priming and boosting with peptide and BSA conjugated peptide form and looked at antibody responses by CDC as a functional assay. We performed prime/boost regimen to increase the specificity of augmented response. Conjugation works in increasing serum antibody reactivity. We also performed immunization with MCF7 cells and used the serum as control. While immunization with conjugated peptides is promising to increase the endpoint titer, it does help little in functional assays like CDC. Monoclonal antibodies or use of cell immunization is still superior.

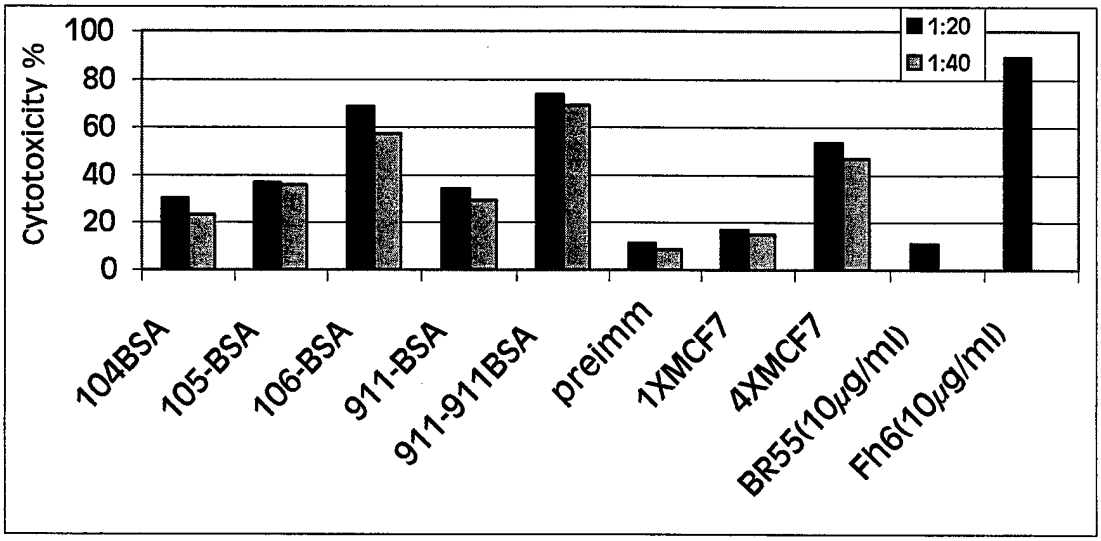


Figure 11A. *Mice were immunized with indicated antigens and serum was collected. Functionality of sera was assessed in a CDC assay against Meth a cells.*

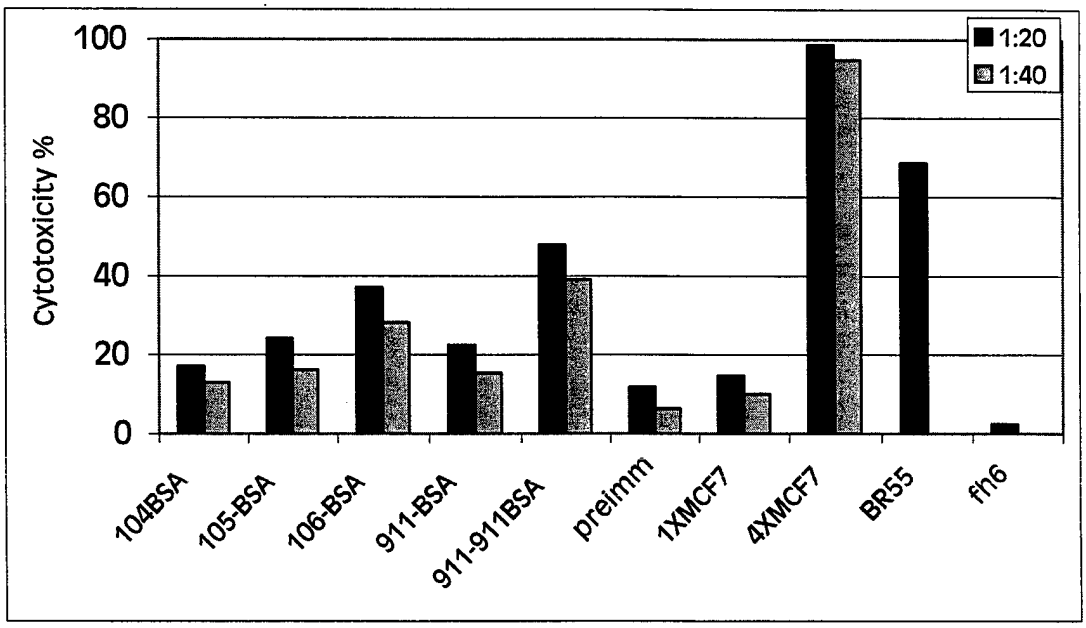


Figure 11B. *CDC assay as performed in figure 11A, only on MCF7 cells.*

We are designing prime/boost experiments using cells as booster of peptide primed animals and hope to increase the efficacy of complement-dependent cytotoxicity. We have performed priming and boosting effect with DNA, sugars and peptide and already concluded that while carbohydrate are able to boost the response induced by the DNA immunization, peptides or their DNA version are superior antigens.

Task 4. Perform tumor challenge experiments (months 18-36).
We have shown previously that immunization with a peptide mimotope of tumor-cell surface carbohydrate antigens induces anti-carbohydrate antibody responses leading to Meth A tumor eradication (manuscript#1 in press). In order to evaluate our mimotope based vaccination strategy in immunotherapy of breast cancer, we employed the 4T1 tumor model. We established the 4T1 tumor in mammary fat pads and then started immunization with the plasmids containing the sequences of 104, 105 and 107 mimotopes. As shown in (Fig.12A), immunization with plasmid encoding 107peptide induces slight tumor shrinkage. Tumor shrinkage is temporary and the growth gets back to its previous paste very soon. However, analyzing survival time (Fig. 12B) indicates that immunization with 107 DNA significantly ($p = 0.021$) increased survival time of the tumor-bearing animals as compared with immunization with vector DNA only. While we see a marginal effect on the size of the tumor in the primary site of inoculation, a significant increase in survival rate is observed. We know that 4T1 cell line is a highly metastatic line and animal death happens because of the complications after metastasis to multiple organs. We hypothesize that immunization may interrupt cell metastasis

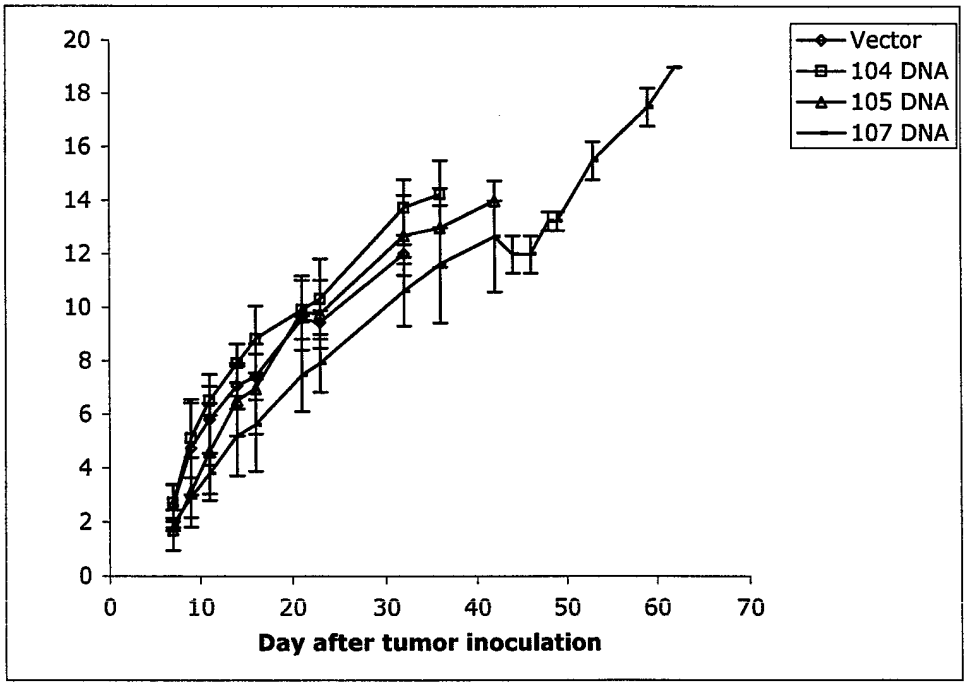


Figure 12 A. *Immunization of tumor-bearing animals with DNA construct of the 107 peptide induces slight tumor regression and an increase in survival rate. Mice were inoculated with 10^5 cells intraductal and immunization started 4 days later with 4-5 days intervals. Il-12 was administered at 0.2 ug/mouse/daily for 5 days, starting the day*

after the last immunization Average of mean tumor diameter in groups of mice immunized with indicated peptide construct.

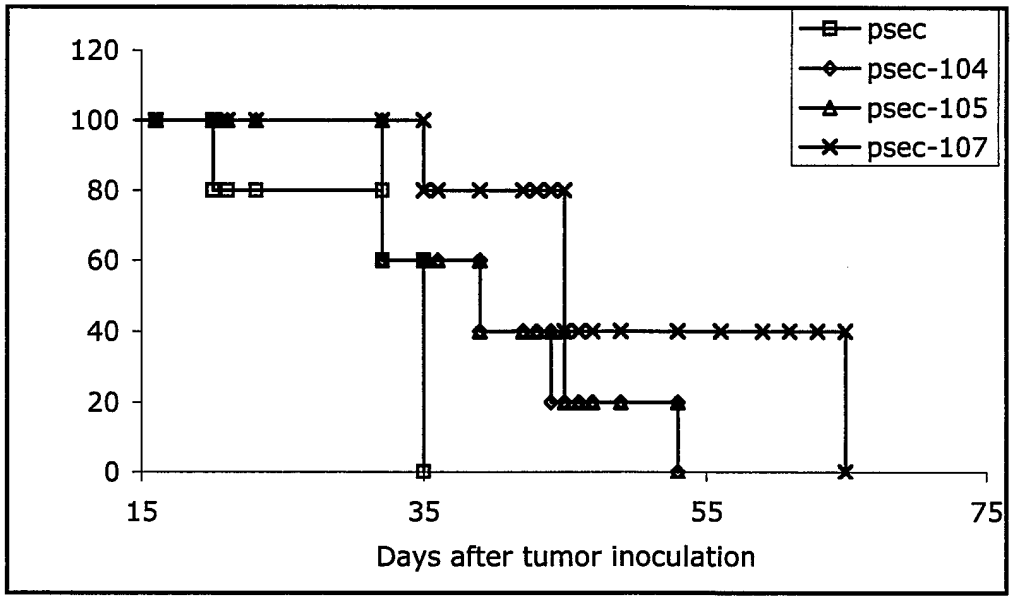


Figure 12 B. *The percentage of survived animals in experimental groups from above.*

by blocking the process or establishment or by killing the cells. To further characterize the effects of DNA administration on metastasis to distant organs, we repeated the challenge experiment as above and 30 days post tumor transplant, lung, liver and bone samples were harvested and metastatic lesions were quantified (Table 4). Immunization had no effect on bone and lung metastasis but significant positive effects on reducing liver metastasis (Fisher exact test, $p = 0.018$). Out of 12 mice used for 107 therapeutic DNA immunization only 2 found positive for tumor in liver compare with 8 out of 12 positive livers in vector immunized animals.

Table 4. Number of mice detected positive for distant organ metastasis.

Immunization	Organs		
	Lung	Liver	Bone marrow
psec (vector)	12	8	4
Psec-107	12	2*	3

*$p = 0.018$ as compared with vector immunized by Fisher exact test.

Is the antigen shared with human breast cancer cell lines?

We know that LeY is present on the breast tumors. We looked at possible competition between Leb and LeY for binding to the anti-107 serum. ELISA plate was coated with Leb antigen and the anti-serum binding to Leb was abrogated by serial pre-adsorption with both LeY and Leb (Fig. 13). This result implies that the antigen is a common constituent of both antigens meaning that whatever happens in the mouse model might happen in human.

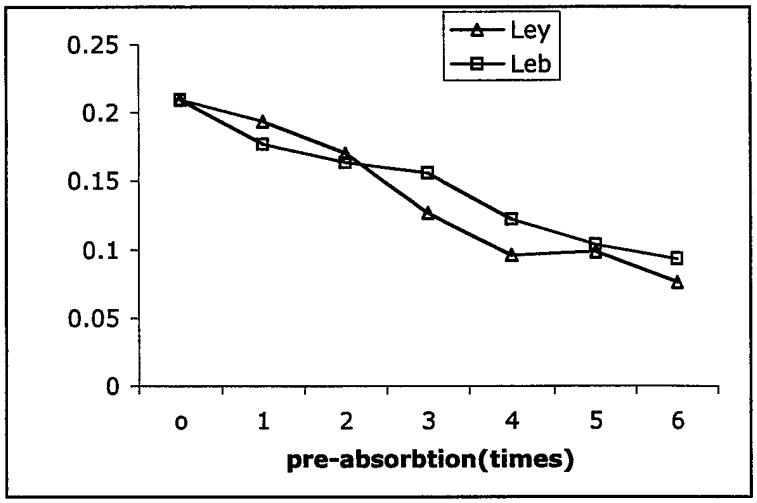


Figure 13. *Mice were immunized with 107 peptide/QS21 and serum was collected. Reactivity of the serum against Leb antigen was detected by ELISA. Reactivity was wipped out by preabsobtion of the serum with both leY and leb antigens.*

Key research accomplishment

1. **We characterized the carbohydrate profile of 4T1 cell surface** and observed that KM-93, GS-1 and CON A bind to 4T1 cells.
2. **We defined potential peptide mimotopes for targeting 4T1 cells in vivo.** Based on the screening of our mimotopes against the 4T1-reactive sugars, we chose peptides that most likely mimic carbohydrate epitopes on the surface of the cells.
3. **We observed that immunization with DNA induced IgM antibodies reactive with 4T1 cells.** The sequences of peptide mimotopes were converted to DNA and cloned in a vector that secrets coded peptides. Immunization with constructed plasmids induced IgM antibodies against Leb and leY antigen, which are reactive with 4T1 cells.
4. **We observed that DNA administration of 4T1-tumor bearing animal temporarily reduces the burden of tumor.** Mice were inoculated with tumor cells and 3-4 days after transplant therapeutic immunization was started with DNA constructs. A temporary shrinkage of tumor mass was observed in group immunized with the construct encoding 107 peptide.
5. **DNA administration of the 107 peptide significantly increases survival rate of animals.** Survival experiments showed that immunization of tumor-bearing animals significantly increases survival rates.
6. **We observed that administration of 107 DNA inhibits liver metastases.** Two weeks after the completing of the immunization, mice were sacrificed, organs were harvested and distant-site metastases was measured by clonogenic assay.

Reportable outcome. One manuscript is in press, and two more in preparation.

Manuscript #1 in press. A mimic of tumor rejection antigen associated carbohydrates mediates an anti-tumor cellular response. Behjatolah Monzavi-Karbassi, Ping Luo, Fariba Jousheghany, Marta Torres-Quiñones, Gina Cunto-Amesty, Cecile Artaud, Thomas Kieber-Emmons. Cancer Research 2004.

Conclusions

Defining new targets for designing novel immunotherapeutic approaches is crucial for the therapy of solid tumors. Aberrant expression of carbohydrate structures on malignant cells can be used to develop immune-based approaches for treatment. Overall in the second year of our research, we have established that:

1. Therapeutic administration of DNA format of a peptide mimotope stimulates tumor mass regression and diminishes metastasis to some organs.
2. Immune responses to mimotopes increase survival rate of tumor bearing animals.
3. In metastatic disease, carbohydrate profile of tumor surface changes, so, targeting a single antigen in an invasive metastatic cell line is not enough.

Appendix Cover Sheet

In press Cancer Research

A mimic of tumor rejection antigen associated carbohydrates mediates an anti-tumor cellular response

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Keywords: Cancer vaccine, tumor immunotherapy, peptide mimotope, carbohydrate antigens

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Abstract

Tumor associated carbohydrate antigens are typically perceived as inadequate targets for generating tumor specific cellular responses. Lectin profile reactivity and crystallographic studies demonstrate that MHC class I molecules can present to the immune system post-translationally modified cytosolic peptides carrying O-beta-linked GlcNAc. Here we report that a peptide surrogate of GlcNAc can facilitate an in vivo tumor specific cellular response to established Meth A tumors that display native O-GlcNAc glycoproteins on the tumor cell surface. Peptide immunization of tumor bearing mice had a moderate effect on tumor regression. Inclusion of IL-12 in the immunization regimen stimulated complete elimination of tumor cells in all mice tested, while IL-12 administration alone afforded no tumor growth inhibition. Adoptive transfer of immune T cells into tumor-bearing nude mice indicates a role for CD8+ T cells in tumor regression. This work postulates that peptide mimetics of glycosylated tumor rejection antigens maybe further developed for immune therapy of cancer.

Introduction

The presence of carbohydrate antigens on the surface of common human malignant tumor cells has led to studies directed toward development of synthetic carbohydrate-based anticancer vaccines (1). While these vaccines elicit antibody responses, it would also be advantageous if T cells could be directed to tumor-associated carbohydrate antigens (2-6). Post-translationally modified cytosolic peptides carrying O-beta-linked GlcNAc can be presented by class I MHC molecules to the immune system that activate Cytotoxic T Lymphocytes (CTLs), as resolved by wheat germ agglutinin (WGA) binding profiles reacting with GlcNAc containing glycopeptides in the MHC Class I binding site (7, 8). Crystal structure analysis of T-cell receptor (TCR) binding to model glycopeptides has indeed shown that T cells can recognize GlcNAc linked glycopeptides bound by the MHC molecule (9, 10). T cells therefore have the potential to react with the GlcNAc moiety of glycopeptide antigens, suggesting that T cells can target to presented carbohydrate antigens on tumor cells.

In an effort to further define strategies to augment immune responses to tumor cells we have been developing peptide mimics of tumor associated carbohydrate antigens and have demonstrated that peptides synthesized as multivalent peptides can emulate or mimic the native clustering or presentation of tumor cell displayed carbohydrate antigens (11). We have shown that prophylactic vaccination with a peptide surrogate, having the sequence GGIYWRYDIYWRYDIYWRYD (and referred to as peptide 106), induces a tumor specific humoral response inhibiting tumor growth of a Methylcholanthrene-induced sarcoma cell line (Meth A) in vivo (11). This peptide also activates an in vitro Meth A specific cellular response with IFN-γ production upon activation of lymphocytes with peptide (12). Characterization of the

cellular response indicated that peptide specific CD8+ T cells played an important role in mediating the tumor specific CTL response which was inhibited by anti-Class I antibody (12).

Here, we demonstrate the ability of this peptide to stimulate the regression of established Meth A tumor in a murine model via the activation of specific anti-tumor cellular responses. We demonstrate that peptide 106 is a mimic of O-GlcNAc, an antigen presented on Meth A surface expressed glycoproteins as resolved by reactivity with WGA to which the peptide also binds. Immunohistochemistry demonstrates infiltrates of lymphocytes targeting Meth A tumor cells in peptide immunized mice and adoptive transfer of peptide specific T cells into tumor bearing nude mice verifies a role for CD8+ T cells in mediating tumor regression. These studies highlight a new function for peptide mimotopes of carbohydrate associated antigens by demonstrating that they possess in vivo antitumor activity with CD8+ T cells as the primary effector cells.

Materials and Methods

Mice and Tumor Inoculation. 6-8 week-old BALB/c female mice were purchased from The Jackson Laboratory (Bar Harbour, ME). BALB/c nude mice (BALB/cAnNTac-*Foxn1nu* N9, *nu/nu*) were purchased from Taconic Farm Inc. (Germantown, NY). To establish tumor each mouse was inoculated subcutaneously into the right flank with 5 x 10^5 Meth A cells (Methylcholanthrene-induced sarcoma of BALB/c origin) (11). Tumor growth was measured using a caliper and was recorded as the mean of two orthogonal diameters ((a+b)/2).

Immunization. As in our previous studies (11, 12), peptide 106 having the sequence GGIYWRYDIYWRYDIYWRYD was synthesized as a multiple antigen peptide (MAP; Research Genetics, Huntsville, AL). Each mouse received 100 ug of 106 MAP and 20 ug of QS-21 (Antigenics Inc., Framingham, MA) intraperitoneally (i.p.), both re-suspended in 100ul of

PBS three times at 5 day intervals. Recombinant murine IL-12 (Sigma, St Louis, MO) was administered i.p. once daily for 5 days, starting on the day of the last peptide immunization.

Flow Cytometry. Acquisition and analysis performed as described earlier (12). Cells were re-suspended in a buffer containing, Dulbecco's Phosphate Buffered Saline, 1% BSA and 0.1% Sodium Azide (FACS buffer), and incubated with biotinylated peanut agglutinin (PNA) or WGA (10 ug/ml, Vector laboratories, Burlingame, CA) for 30 min on ice. Cells were then stained with FITC-conjugated streptavidin at 1:500 dilution for another 30 minutes on ice.

ELISA and inhibition assays. ELISA was performed as described (11). Briefly, plates were coated with 106 MAP. Biotinylated WGA was added, and binding was visualized with streptavidin-HRP (Sigma, St Louis, MO). Absorbance was read, using a Bio-Tek ELISA reader (Bio-Tek instruments, Inc, Highland Park, Vermont). For inhibition assay, GlcNAc and GalNAc, attached to a polyacrylamide polymer (PAA) (GlycoTech Corporation, Rockville, MA), were used as carbohydrate competitor. After coating the plate with the peptide as above, biotinylated WGA (2.5μg/ml) combined with serial concentrations of carbohydrates and incubated overnight at $+4^0$C. Lectin/carbohydrate mix was added to the plate and lectin binding was visualized by streptavidin-HRP as above. Mean absorbance was calculated from duplicates for each carbohydrate concentration and percentage of inhibition calculated as: {1-(mean of test wells/mean of control wells)} x 100.

T-cell purification. Splenocytes were harvested from spleens and prepared by lysis of erythrocytes and consequent washing several times with fresh media (12). Splenocytes were first passed through nylon wool and then, using MiniMACS (Miltenyi Biotec, Auburn, CA), NK cells were depleted using anti-NK cell (DX5) Microbeads. Finally, T cells were positively purified by Thy1.2 coated beads. Purified T cells were tested for purity as > 97% positive for anti-CD3

antibody. For cell transfer experiments, after nylon wool passage, cells were enriched in CD4+ or CD8+ population using MiniMACS and depletion of unwanted cell populations.

IFN-γ production by purified T cells. Purified T cells (1 x 10^6/ml) were cultured in 96-well or 24-well plates with various doses of rIL-12. After 48 hours of stimulation supernatant was harvested and stored at –20 until use. Concentration of IFN-γ was measured using a quantitative ELISA kit (BioSource International Inc., Camarillo, CA) according to the manufacturers instructions.

Adoptive transfer of cells. Splenocytes were collected from cured mice after tumor eradication and were used in transfer experiments. 1.5 x 10^7 immune splenocytes were transferred i.p. to syngeneic nude tumor-bearing mice 7 to 10 days after inoculation of 0.5 x 10^6 Meth A cells into the right flank. In order to in vitro deplete CD4+ and CD8+ cells, splenocytes (1.5 x 10^7/each sample) were first passed through nylon wool column and then using MACS, CD4+ and CD8+ cells were depleted.

Histology. Tumors with surrounding tissues were excised and fixed in 10% formalin. Fixed samples were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Sections were analyzed histologically for lymphocyte infiltration.

Statistical analysis. Statistical analyses were performed using Student's *t* test and the chi-square test; Ps < 0.05 were regarded as statistically significant. Excell® and Statistica® software's were used for analyses. All experiments were performed at least three times.

Results

Peptide mimic of GlcNAc moiety

It has become evident that both CD4+ and CD8+ T cells can recognize glycopeptides carrying mono- and disaccharides in a MHC-restricted manner provided the glycan group is

attached to the peptide at suitable positions (13). Reactivity patterns of lectins with Meth A cells indicate that GlcNAc glycosyl epitopes are more highly expressed on Meth A tumor cells than the T antigen Galβ1-3GalNAc epitope as WGA displays greater reactivity with Meth A cells than Peanut agglutinin (PNA) (Figure 1A, B). WGA binds to the peptide 106 mimotope in a concentration dependent manner as assessed by ELISA (Fig 1C). This binding is selectively and significantly inhibitable by WGA reactive GlcNAc in a concentration dependent manner (Figure 1D), further indicating that the peptide mimotope is reactive with the GlcNAc binding site of WGA and therefore peptide 106 is an effective antigenic mimic of GlcNAc.

Therapeutic peptide immunization induces tumor regression

To study the outcome of peptide immunization on the growth of solid tumors *in vivo*, we evaluated the anti-tumor effect of the peptide 106 on established Meth A tumors. BALB/c females were inoculated sub-cutaneously with Meth A cells and seven days later treatment was started with the peptide. As shown in Figure 2A, immunization moderately affected the growth of Meth A sarcoma, as 6 mice out of 11 immunized were cured (Chi-square test, $P = 0.01$ as compared with animals administered with IL-12 only). Figure 2B demonstrates that treatment of animals with IL-12 following peptide immunization tended to enhance the immune response and was successful in mediating complete eradication of established tumors (Chi-square test, $P = 0.008$ as compared with peptide immunized only). Treatment of tumor-bearing mice with only IL-12 did not affect tumor growth (Fig. 2C) in keeping with other such studies (14).

We further determined that Peptide/IL-12 combination therapy is highly effective even at lower doses of IL-12, as 100 ng of daily IL-12 treatment in the combined therapy, but not alone (Chi-square test, $P = 001$), eradicated tumors in 5 mice of five challenged (Fig. 3A,B). The time of the beginning of immunization and the size of tumor at the time off immunization affect the

efficacy of immunization. When immunizations were started at day 14 or later or when treating tumors with a mean diameter larger than 7 mm, the efficacy of immunization dropped (Fig. 3C) ruling out the possible effect of hyper-immunization per se on the outcome of the challenge experiments. To further rule out non-specific effects of hyperimmunization, we observed that cell-based vaccination using 10^6 mitomycin-C-inactivated Meth A cells followed by IL-12 administration also failed to induce tumor regression (Fig. 3D). This latter result confirmed a previous study in which Meth A immunization along with IL-12 failed to induce tumor regression (14). Our results are in agreement with other therapeutic vaccine studies on Meth A cells, where enhancement of anti-tumor T-cell responses led to quick eradication of established tumors (15, 16).

Adoptive transfer of splenocytes stimulates eradication of tumors in nude mice

To further assess whether the anti-tumor activity mediated by peptide/IL-12 therapy is T-cell dependent, we evaluated our therapeutic strategy in nude mice. BALB/c-nu/nu mice bearing Meth A tumors were immunized with the peptide followed by IL-12 treatment. Combined peptide/IL-12 therapy had no effect on tumor growth of nude mice, indicating the dependence of mediated tumor regression on T cells (data not shown). Next, nude mice were transplanted with Meth A cells and injected ip with fresh splenocytes, isolated from cured mice, 10 days later (Fig 4). Immune cells transferred had a dramatic effect on tumor size as by day 15 after transfer, tumor was eradicated completely in all four mice tested (Chi-square test $P = 0.005$).

In a follow-up study splenocytes were depleted of B cells and enriched for CD4+ or CD8+ cells, in vitro, and then transferred to tumor-bearing nude mice. Our data indicate that CD8+ cells are required for efficient eradication of tumor, however the process seems dependent on both CD4+ and CD8+ cell (Fig. 5). Histologic sections of tumor sites and surrounding tissues

were prepared (Fig. 6). Contrary to non-immunized tumor-bearing mice, we detected lymphocytes around the periphery and infiltrating into tumor mass of immunized mice (Fig. 6A,B). Staining of sections obtained from the tumor site of a cured mouse shows the presence of lymphocytes, whereas no tumor is detectable microscopically (Fig. 6C).

Discussion

Carbohydrates are abundantly expressed on the surface of malignant cells and induction and enhancement of a cell-mediated immune response toward these antigens has outstanding implications in vaccination and treatment of cancer. T cell recognition of nonpeptidic and modified peptide antigens is however still poorly understood. Peptide mimetics of carbohydrate antigens can activate peptide-specific cellular responses but they have also been shown to activate cellular responses that might be cross-reactive with carbohydrate moieties (12, 17). The induction of carbohydrate reactive T–lymphocytes with peptide mimics is based upon a functional definition of T-cell mimotopes. One possible explanation is that the peptide mimotope activates cross-reactive CTLs that recognize a processed O-linked glycopeptide associated with MHC class I. It is also possible to generate carbohydrate-specific unrestricted CTL responses with MHC class I-binding carrier peptides (18). However, we previously showed that anti-MHC Class I antibody blocks CTL killing of Meth A cells in vitro by T cells derived from peptide 106 immunized mice (12).

Immunization with cells in combination with IL-12 had no obvious enhancement of anti-tumor immune effects. Our data propose that replacing cell immunization with peptide 106 enhanced a potential immune responses resulting in a significant but moderate tumor eradication. Further treatment of peptide immunized mice with IL-12 helped significantly in stimulating eradication of established tumor in all animals tested. Lack of tumor shrinkage upon cell-based

immunization rules out the possibility that hyperimmunization had a bearing on tumor regression. Taken together, these results indicate that peptide immunization enables an effective anti-tumor immune response, the potential of which can be significantly enhanced with IL-12 administration. Other groups have performed therapeutic immunization on Meth A cells by immunization with p53 mutant epitope, starting the immunizations 7 days after tumor inoculations and demonstrated an efficient enhancement of anti-tumor cellular immune responses leading to eradication of tumor mass in the majority of animals within two weeks after the first immunization (15, 16). Our findings are in concert with the results of these studies.

As resting T cells do not express the IL-12 receptor (19) and IL-12 responsiveness is only activated after TCR stimulation (20), we observed that purified peptide specific T cells were stimulated with IL-12 in vitro (data not shown). IL-12 treatment is ineffective in the Meth A tumor model (14) as further observed in our studies. Because IL-12 responsiveness of T cells is induced after T-cell receptor stimulation, the lack of IL-12 responsiveness suggests that T cells in Meth A-bearing mice are not sensitized to Meth A tumor antigen upon immunization with Meth A cells. In contrast our data suggest that peptide immunization can sensitize tumor reactive T cells that are responsive to IL-12. It is possible that peptide immunization further expands B and T cells that have been primed via shed glycoprotein(s)processing. We propose that peptide 106 immunization activated a population of Th1 and CTLs with production of IFNγ (12), and in vivo IL-12 treatment further helps to expand the T cell population and IFNγ production. In previous studies the failure of IL-12 treatment to induce tumor regression was also considered to be associated with the lack of T-cell migration to tumor sites (14). It was argued that sensitization of T cells to tumor antigens and generation of IL-12 responsiveness are insufficient

to induce tumor regression when sensitized T cells are not allowed to migrate to tumor sites. In our studies we observe lymphocyte migration to tumor sites.

In summary, this work further postulates the occurrence of saccharide epitopes for T cells linked to peptides with anchoring motifs for MHC Class I (6, 13). While analogous to the haptens trinitrophenyl and O-beta-linked acetyl-glucosamine, the potential implications of natural carbohydrates as antigenic epitopes for CTL in biology are considerable and under-studied. Consequently, it might be possible for peptide mimetics to activate T cells that recognize carbohydrate moieties on native glycopeptides (21). Peptides that mimic carbohydrate structures attached to class I or class II anchoring peptides would extend our notion of vaccine design for cancer immunotherapy in the adjuvant setting.

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Legend to figures

Figure 1. Binding of WGA to the peptide and Meth a cells and inhibition of WGA binding to the peptide. Meth A cells were incubated with biotinylated peanut agglutinin (PNA) (**A**) or wheat germ agglutinin (WGA) (**B**), washed and stained with Streptavidin-FITC for FACS assay. Continuous and broken lines show Streptavidin-FITC and lectins plus streptavidin-FITC, respectively. **C**) 96-well ELISA plates were coated with the peptide (50 ug/ml) and incubated with biotinylated WGA, after washing the plate binding was visualized by adding streptavidin-HRP. **D**) Inhibition of binding of WGA to the peptide was performed by competitive carbohydrate GlcNAc. GalNac was employed as negative control. WGA was pre-incubated with serial dilutions of carbohydrates overnight at $+4^0$C and then added to peptide-coated plates as above. Results present the mean value ± SD. $*P < 0.05$; $**P < 0.01$. $***P < 0.0005$ as compared with inhibition of GalNac at the same concentration.

Figure 2. Effect of peptide immunization on Meth A tumor growth and regression. BALB/c female mice were inoculated s.c. with 5 x 10^5 Meth A cells on day 0. **A, B**) 7 days after tumor inoculation peptide immunization started, mice were immunized i.p. with 106 MAP/QS21 three times. IL-12 was administered alone (**C**) or following peptide immunization (**B**) at indicated doses daily for 5 days, starting on the day of last peptide immunization. Tumor growth is expressed as the mean diameter for each individual mouse. *P*ac and *P*ba are *P* values of Chi-square tests comparing A with C and B with A, respectively.

Figure 3. Effect of late peptide or cell immunizations on the growth of the tumor. Tumor was established as explained in legend to figure 2. (**A, B**) The same immunization as performed in

Fig. 2 but with Lower doses of IL-12. **(C)** Peptide immunization was started 14 days after tumor inoculation. **(D)** 7 days after tumor inoculation, mice were immunized ip with 10^6 mitomycin-C-inactivated Meth A tumor cells for three times at 4-5 day intervals. IL-12 was administered alone **(B)**, following peptide immunization **(A, C)** or following cell immunization **(D)** at indicated doses daily for 5 days, starting on the day of last peptide or cell immunization. *P*ab(d) is the *P* value of Chi-square test comparing A with B or D.

Figure 4. Adoptive transfer of fresh immune splenocytes eradicated established tumors in nude mice. Two groups (4 per group) of nude mice were inoculated s.c. with 5 x 10^5 Meth A cells into the right flank. 10 days after inoculation when average of tumors diameter was about 7 mm, one group was injected i.p. with 1.5 x 10^7 of fresh splenocytes collected from already immunized and cured BALB/c animals. Splenocytes were prepared by lysis of erythrocytes and consequent washing several times with fresh media. Pictures shown are taken from a representative individual on the day of cell transfer **(A)**, 7 **(B)**, 12 **(C)** and 17 **(D)** days later. **E)** Tumor size in control group 25 days after transplant as one representative individual out of four is shown. **F)** Average of tumor diameter for 4 mice per group in naïve control (circle) and splenocyte-transferred groups (square). Arrow shows the date of injection of splenocytes.

Figure 5. CD8+ T cells are required for successful adoptive therapy of established tumors. Solid tumors were established in nude mice and at day 7 enriched splenocytes were transferred ip. For enrichment, splenocytes were passed through nylon wool after which the percentage of CD19+ cells remained were less than 7%. The percentage of CD8+ and CD4+ cells in CD4 and CD8 enriched population was less than 10%. Results present the mean value ± SD. $*P < 0.05$; $**P < 0.01$. $***P < 0.0005$ as compared with mean tumor diameter of CD4+ transferred animals.

Figure 6. Lymphocytes infiltrate the Meth A challenge site in immunized mice. Fixed sections from tumor site of non-immunized tumor-bearing (**A**), immunized tumor-shrinking (**B**) and immunized after tumor elimination (**C**) stained with hematoxylin and eosin. Mice were transplanted and immunization started 7 days later. Samples were obtained when tumor was 16 mm (**A**), 2mm(**B**) and 5 days after tumor eradication.

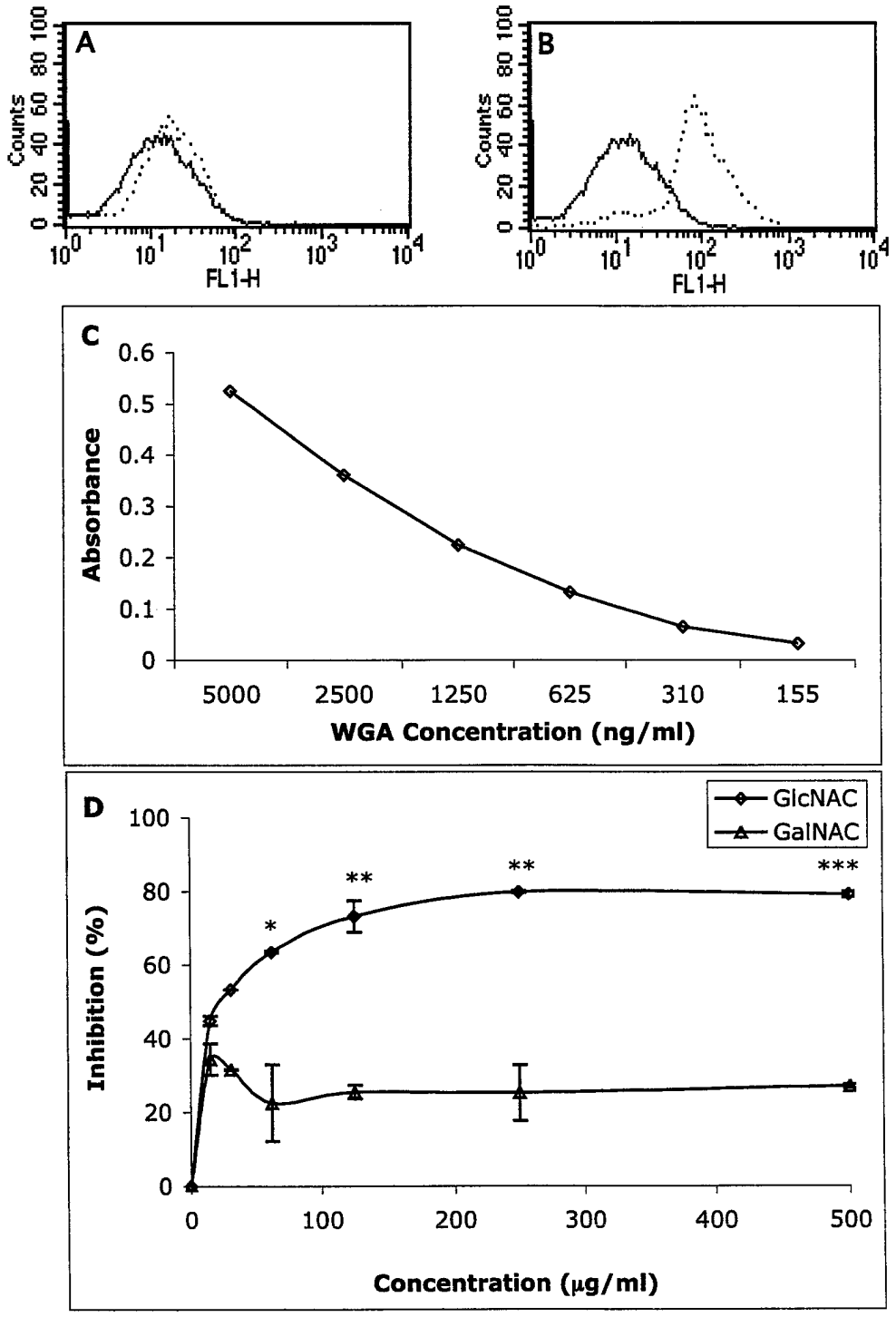

A
B
Counts
FL1-H
C
Absorbance
WGA Concentration (ng/ml)
5000
2500
1250
625
310
155
D
GlcNAC
GalNAC
Inhibition (%)
Concentration (µg/ml)
*
**
**
***

Figure 1

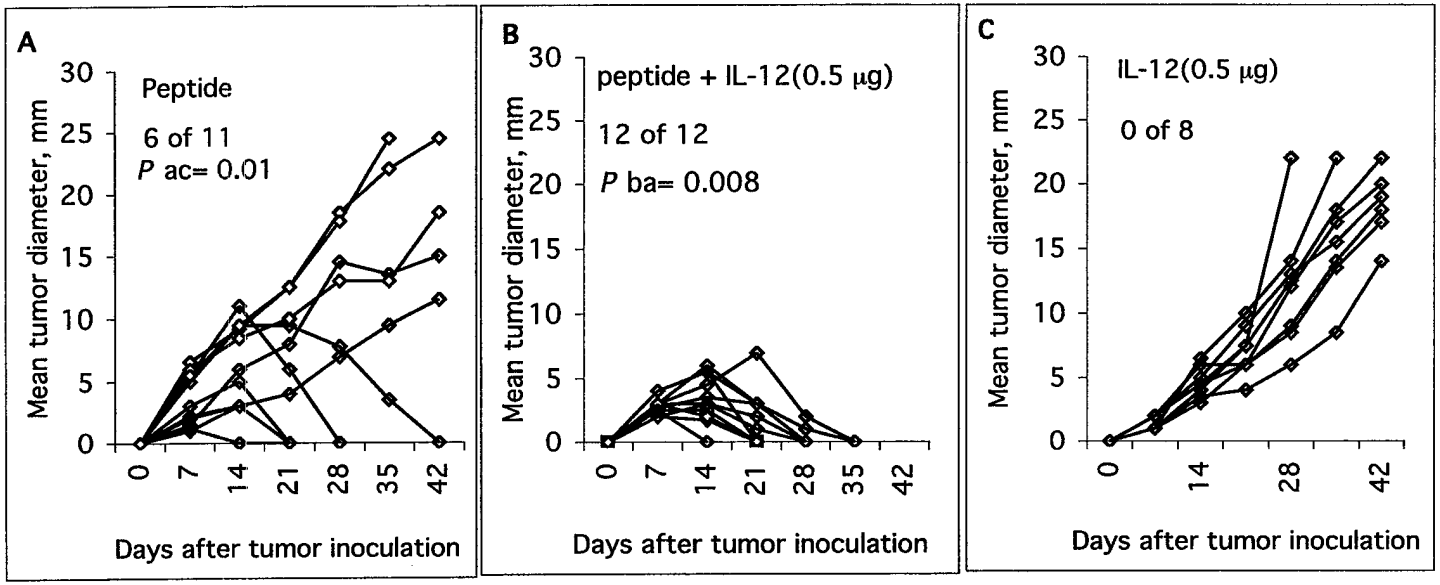

A
Peptide
6 of 11
P ac= 0.01
Mean tumor diameter, mm
Days after tumor inoculation
B
peptide + IL-12(0.5 μg)
12 of 12
P ba= 0.008
Mean tumor diameter, mm
Days after tumor inoculation
C
IL-12(0.5 μg)
0 of 8
Mean tumor diameter, mm
Days after tumor inoculation

Figure 2

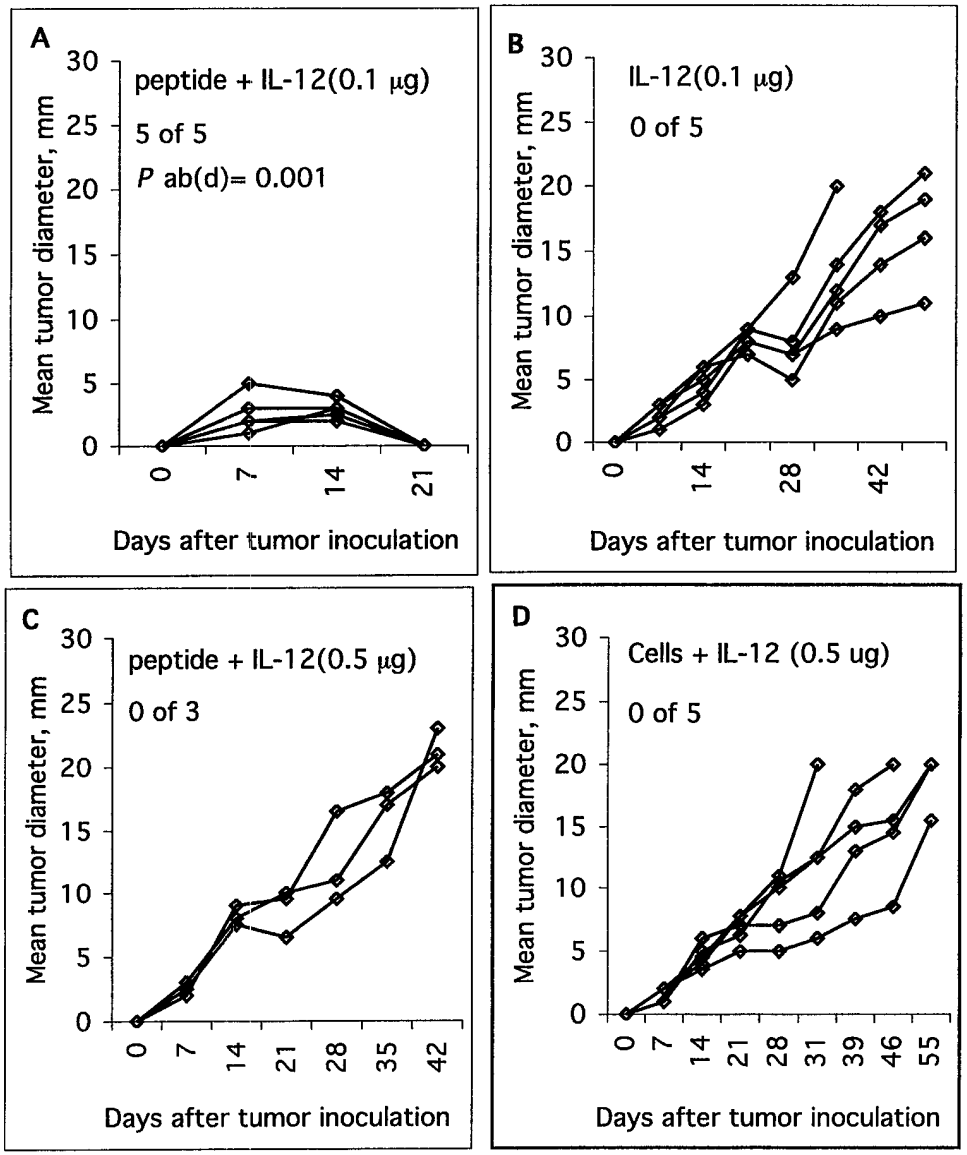
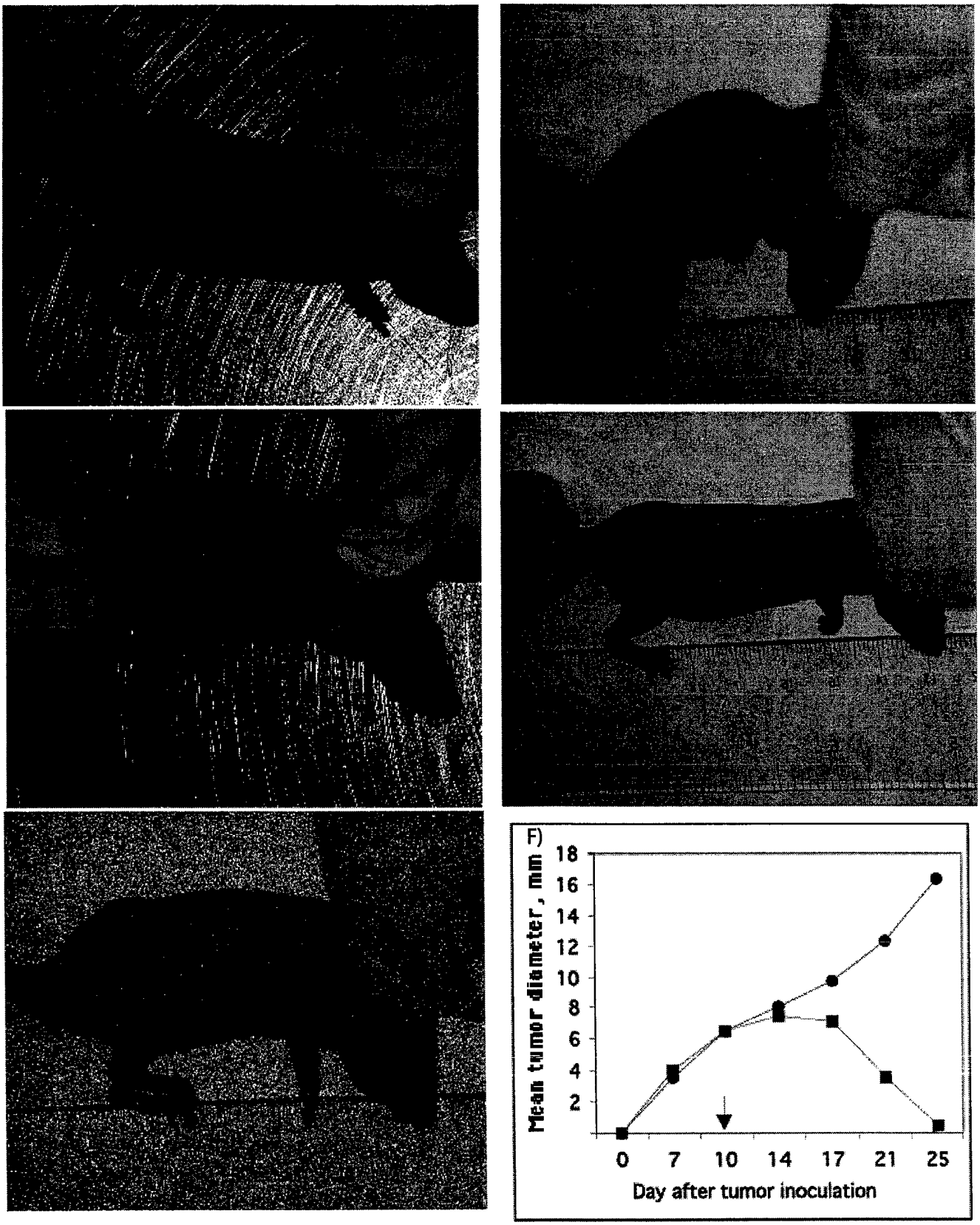

A
peptide + IL-12(0.1 μg)
5 of 5
P ab(d)= 0.001
Mean tumor diameter, mm
Days after tumor inoculation
B
IL-12(0.1 μg)
0 of 5
Mean tumor diameter, mm
Days after tumor inoculation
C
peptide + IL-12(0.5 μg)
0 of 3
Mean tumor diameter, mm
Days after tumor inoculation
D
Cells + IL-12 (0.5 ug)
0 of 5
Mean tumor diameter, mm
Days after tumor inoculation

Figure 3

Figure 4


F)
Mean tumor diameter, mm
18
16
14
12
10
8
6
4
2
0
7
10
14
17
21
25
Day after tumor inoculation

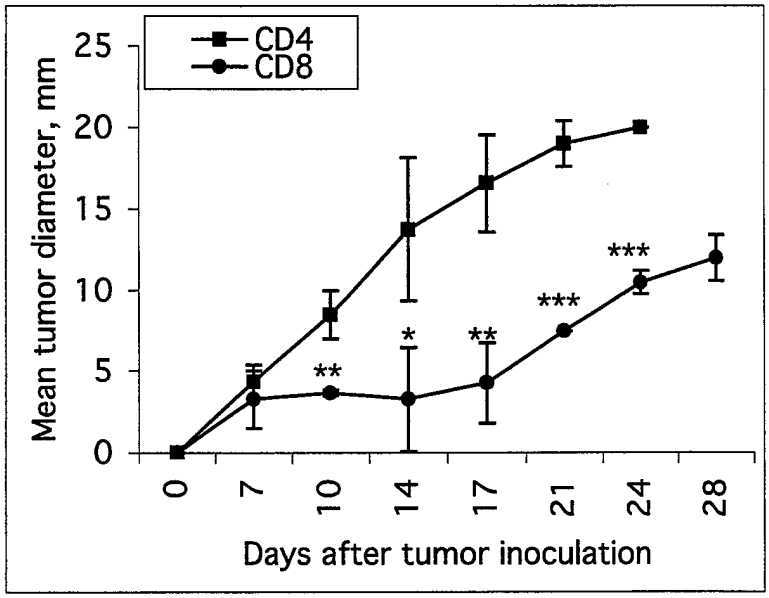
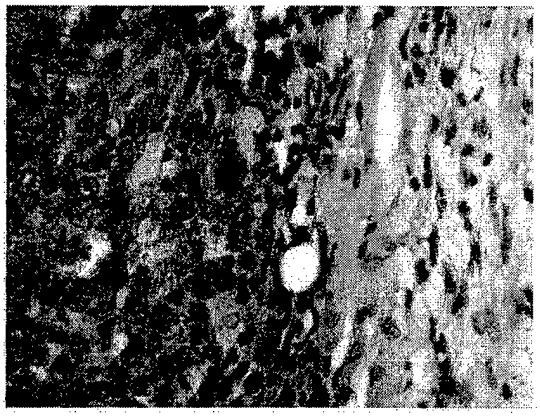

CD4
CD8
Mean tumor diameter, mm
25
20
15
10
5
0
**
*
**
***
***
0
7
10
14
17
21
24
28
Days after tumor inoculation

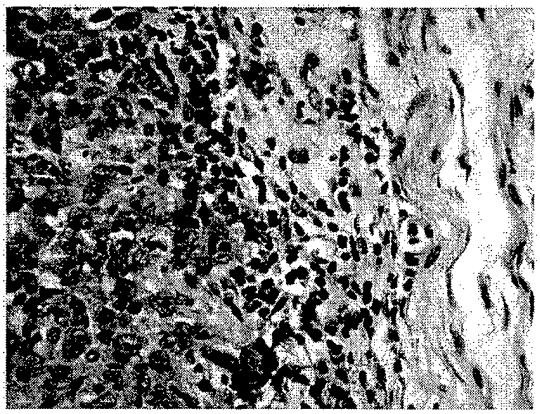
Figure 5

Figure 6

A)



B)



C)