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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

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 that 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 auto-antigens on tumor cells and conseqeuntly they are precieved as viable targets for immunotherapy. 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). In clinical trials, carbohydrate vaccine formulations induce humoral responses that are considered beneficial, because these responses are associated with better prognosis for the patients. While pure carbohydrate antigens elicit diminished immune responses because of their T cell independent nature, conjugate vaccine technology has overcome some of the limitations of carbohydrates as vaccine antigens because of the T-dependent (TD) help conferred by the protein (1,2). Conjugation of carbohydrates to a carrier protein that elicit carrier-specific T- and B cell responses does not necessarily enhance carbohydrate immunogenicity (3). In the case of responses to conjugated carbohydrate, it can be speculated that the type of T helper cells (Th1 or Th2) can modulate the amount and isotype switch of the anti-carbohydrate IgG. Despite their promise, carbohydrate-conjugates induce responses that are often deficient in many respects, including the lack of induction of the Th1 associated murine IgG2a isotype (4). In this regard, a role for IFNγ and lymphocytes in initial tumor development has been documented and more specifically a Th1- and CTL-mediated response with production of IFNγ is thought to be necessary for the induction of antitumor immunity. Carbohydrate-conjugates are characteristic of traditional vaccines inducing Th2-biased immune responses with a high titer of antibodies and almost without a significant induction of cell-mediated immunity against the respective antigen. Consequently, new formulations or alternative ways to augment carbohydrate reactive immune responses are being evaluated. We are demonstrating that peptides mimicking breast associated neolactoseries antigens are capable of activating carbohydrate specific cellular immune responses (manuscript #1) that limit tumor growth in vivo. Thus, carbohydrate-mimicking peptides represent a new and very promising tool to increase the efficiency of the immune response to carbohydrates.

Body

During the initial funding period we have focused on characterizing the immune response parameters upon vaccination with peptide mimetics. This was specific aim 1. We have published 1 paper and preparing another manuscript for submission. The basis of the second paper was presented at the 6th International symposium on Predictive Oncology and Intervention Strategies held in Paris France and was a winner (1st place) of the Symposium Presidents' Award for Scientific Excellence (http://www.cancerprev.org/Meetings/2002/Awards). However, we deviated to some extent from our initial tasks to further validate the use of peptide mimetics to augment the response to tumor cells.

Task 1. Establish murine breast tumor model (months 1-3). The purpose of this Task was to develop a model system with two objectives. One, to use a cell type relevant to Breast cancer. The other to use a cell line that expresses relevant neolactoseries carbohydrate structures. We had established by FACS analysis that murine 4T1 cells do not express LeY nor sLex or sLea. This cell line (H2-d) is highly metastatic and is weakly immunogenic. In Task 1 we planned to transfect murine 4T1 cells with fucosyltransferases. However, in our present studies we used the murine cell line Meth A (H2-d) as a model. Meth A is a Methylcholanthrene-induced sarcoma of BALB/c origin. We identified a clone by FACS sorting with the anti-extended sLex structure

reactive FH6 antibody. Sorted cells were passed as an ascitic tumor. It was thought that many-proof of principle experiments could be conducted with this cell line early on. In our first year we established that immunization with a peptide mimetic of core structures of the neolactoseries structure that reacts with an anti-LeY monoclonal antibody and reacts with an anti-extended sLex reactive monoclonal elicits a cellular response to Meth A cells. Peptide presentation can be of the form of a multiple peptide or encoded into a DNA plasmid. We are now beginning the transfection experiments with 4T1.

Peptide mimetics activate cellular responses to Model carbohydrate expressing cell line (manuscript #1).

The metastatic potential of some tumor cells is associated with the expression of the sLex sLea as they are ligands for selectins. We have shown that peptide mimetics of these antigens can potentiate IgG2a antibodies, which is associated with a Th1 type cellular response. As L-selectin is preferentially expressed on CD4+ Th1 and CD8+ T cell populations, specific induction of these phenotypes could augment a response to L-selectin ligand expressing tumor cells. We demonstrated that immunization with a multiple antigen peptide (MAP) mimetic of sugar constituents of neolactoseries antigens induces a MHC dependent peptide specific cellular response that triggers IFNγ production upon peptide stimulation, correlating IgG2a induction upon mimetic immunization. Surprisingly, T lymphocytes from peptide immunized animals were activated in vitro by sLex, also triggering IFNγ production in a MHC dependent manner. Stimulation by peptide or carbohydrate resulted in loss of L-selectin on CD4+ T cells confirming a Th1 phenotype. We also observed an enhancement in CTL activity in vitro against sLex expressing Meth A cells using effector cells from Meth A primed/peptide boosted animals. CTL activity was inhibited by both anti-MHC Class I and anti-L-selectin antibodies. These results further support a role for L-selectin in tumor rejection along with the engagement by T cell receptor for most likely processed tumor-associated glycopeptides. Peptide mimetics therefore appear promising to mediate carbohydrate reactive cellular responses.

BALB/c female mice were immunized with a multivalent antigen peptide (MAP) called 106 MAP with the 106 sequence GGIYWRYDIYWRYDIYWRYD and splenocytes were collected seven days after the last immunization. Cell proliferation was assessed in the presence of the peptide **(Fig 1A)**, with purified T lymphocytes alone and after addition of MMC inactivated antigen presenting cells (APCs) **(Fig. 1B)**. The results indicate that proliferation is APC dependent. Proliferation of the peptide specific T lymphocytes was not observed in the absence of APCs. Proliferation was inhibited by the addition of anti-Class II, and to a lesser extent with anti-Class I antibodies **(Fig. 1C)**. The addition of anti-CD1 antibody inhibited proliferation to almost the same extent as addition of anti-Class I antibody (manuscript #1). Therefore, the 106 peptide might be presented in multiple ways to T cells. IFN-γ production was found to be peptide specific **(Fig. 1D)**, with no detectable presence of IL-4 or IL-10.

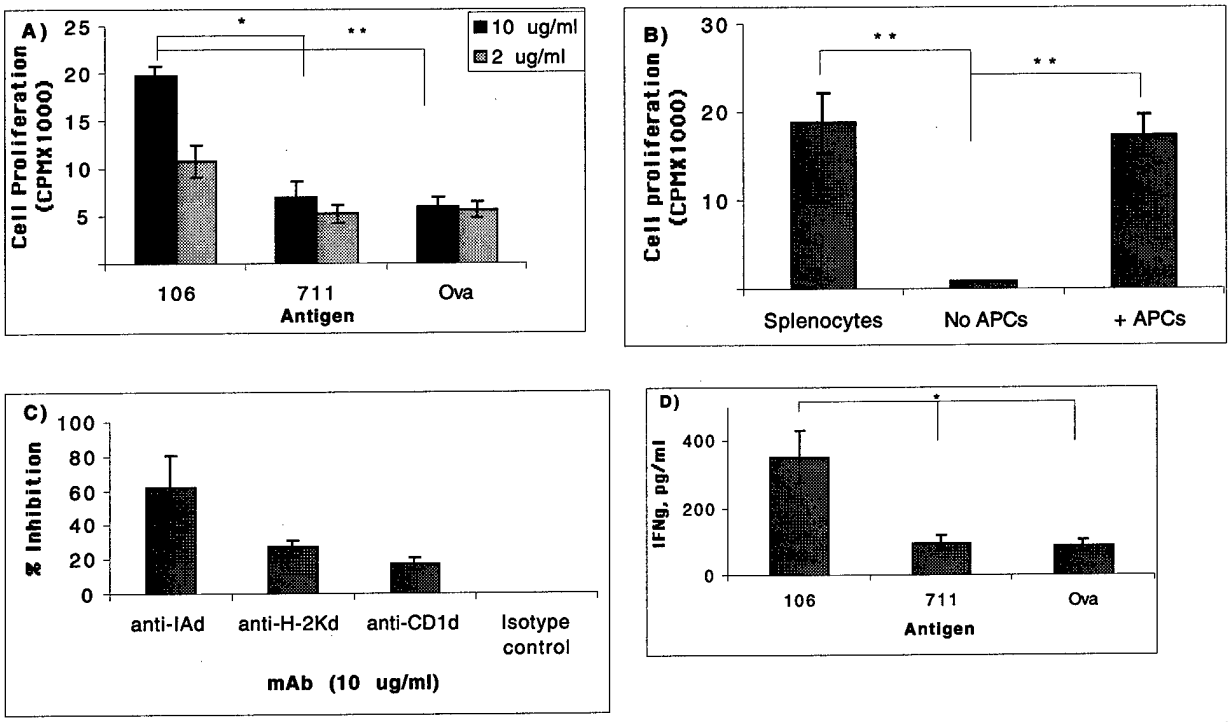
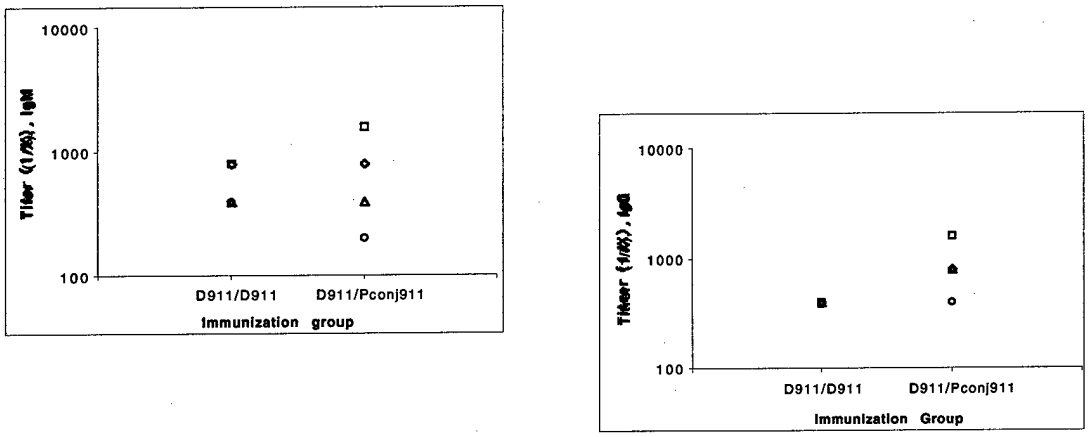


Figure 1. *Immunization of BALB/c mice with 106 MAP induces an APC-dependent cell proliferation which, is blocked by anti-MHC class II and class I antibodies (10 ug/ml). Stimulation of splenocytes with 106 peptide (10 ug/ml) induces IFNγ production. All results are expressed as the mean ± SEM of four independent experiments with triplicate samplings *P < 0.05. **P < 0.01. mAb; Monoclonal antibody, Ova; Ovalbumin*

Task 2. To evaluate immune parameters associated with DNA vaccination of plasmids encoding glycotope along with molecular adjuvants (months 1-16)**.** This task was associated with our first objective. We were interested in inducing both cross-reactive antibody and cellular responses. We intended to manipulate the immune response to peptide 106 and peptide 911, having the sequence YRYRYGRYRSGSYRYRYGRYRSGS, in different directions by co-immunization with genes encoding IL-12 and GM-CSF. We made the peptide encoded constructs and tested them in a vaccination study. Addition of recombinant IL-12 to the 911 plasmid did not affect the outcome. We established that recombinant IL-12 could enhance the efficacy of immunization with the 106 peptide. We further established that peptide/plasmid immunization mediates a Th1 response, as does immunization with 106 peptide. While we have not finished characterizing the humoral response parameters (isotype distribution, CDC, ADCC) to Meth A, clearly vaccination mediates tumor growth inhibition. This is attributed to T cells and by the in vitro cell proliferation and CTL studies.

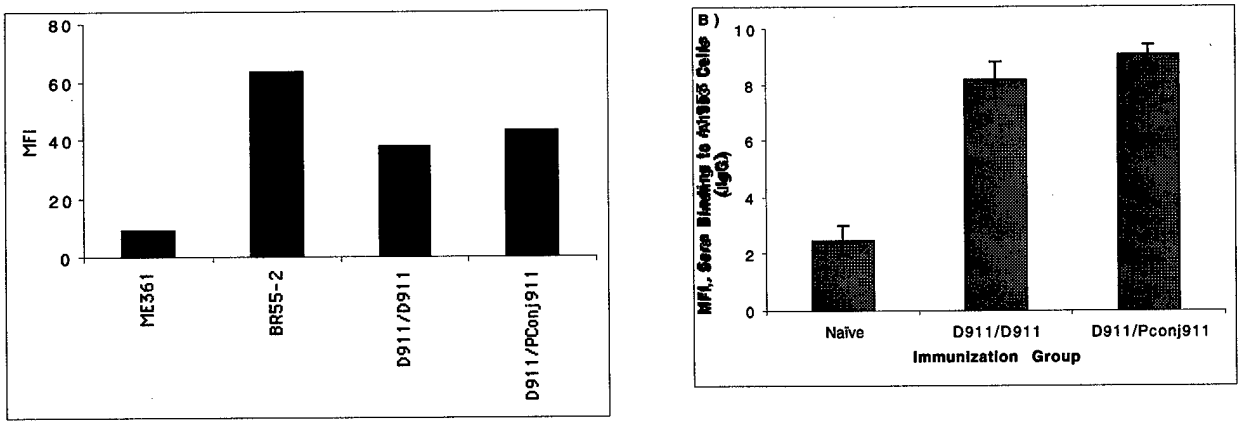
Using Meth A cells have jumped ahead to elements of Task 3 (months 10-20) and Task 4 (months 18-36) in that we have started to look at priming and boosting and challenge experiments. We will continue to assess a role played by GM-CSF and will follow through with a complete assessment of the subtasks as described in the proposal using 4T1 cells expressing neolactoseries antigens as they become available.

As we have characterized peptide 106 encoded DNA (5) we immunized groups of mice with the DNA version of 911 peptide (D911 with plasmid design described in our original proposal) and then boosted them, after three weeks, with DNA (D911/D911), or BSA-conjugated peptide (D911/Pconj911). Analysis of humoral response by ELISA against LeY antigen (Fig. 2A,B) showed that DNA immunization primes for both IgM and IgG reactive antibodies. Either DNA or BSA-conjugated peptide boost similar levels of IgM and IgG. We examined binding of IgG portion of generated sera to MCF-7 and A1953 cell lines. As shown in figure 3 (A,B) we did not detect significant differences in binding between sera. A CDC assay was also performed, with an overall cytotoxicity of about 30%, and did not show any preference for DNA or peptide conjugate immunized serum over MAP (data not shown).



A. **B.**

Figure 2. *DNA or peptide conjugate immunization of DNA primed animals induce similar serum reactivity. Mice (groups of four) were primed with DNA and boosted with either the same DNA construct or conjugated form of MAP at three-week interval and bled two weeks after boost. Sera were separated for each individual mouse. Anti-LeY end-point titers of IgM (A) and IgG (B) were measured for each individual.*



A. **B.**

Figure 3. *Collected serum was pooled and it's binding toMCF-7 (A) and A1953 (B) cells was detected by FACS. For FACS assay serum was diluted 1-100 and the binding is presented by Mean Fluorescence Intensity values (MFI – SD). Each assay was repeated twice with similar results. Positive control monoclonal for MCF7 cells was the anti- LeY BR55-2. Negative control was the anti-ganglisoside antibody ME361.*

Mimotopes are superior immunogens than carbohydrates

A closer look at the above data revealed to us that mimotope immunization might induce a type of memory for generation of serum IgM, which is long lasting. To examine this issue we primed mice with 911 MAP and then boosted them with sLex (sialyl Lewis ^{x}-PAA). Serum reactivity was then checked against sLex. As shown in figure 4, 911 peptide primes for IgM-sLex reactive Abs. Immunization with sLex boosts the response and it lasts longer than it does in immunization with sugar only. Sugar boosted the response and stabilized it for a longer time period. The data suggest that there is a priming effect, with peptide immunization, for generation of cross-reactive IgM and the generated IgM is longer lasting. We also analyzed the cross-reactive IgG response during the course of experiments, as we reported before we also detected a prime and boost effect for IgG of low titer (data not shown). The results suggest that despite the induction of a state of tolerance in carbohydrate immunization, mimotope immunization induce a type of memory for the IgM portion of the serum, priming for later sugar immunization.

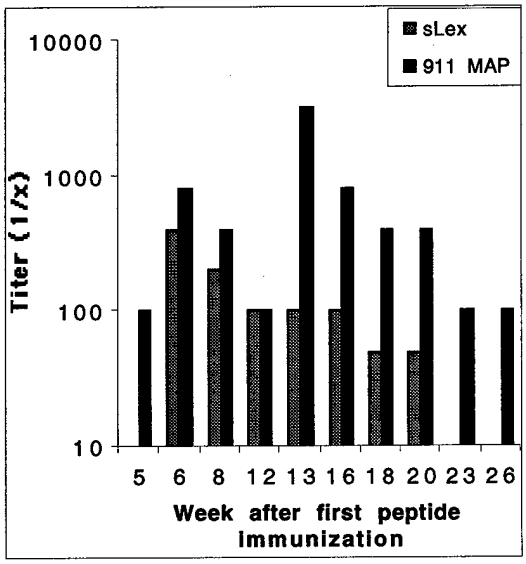


Figure 4. Peptide immunization primes for an anti-carbohydrate cross-reactive memory IgM response. Mice were pre-bled and groups of mice were immunized with 911 MAP twice at weeks one and four. Other groups were immunized with sLex (sialyl Lewis ^{x}-PAA) once at week four. Serum was collected at week 0 (pre-bled), after peptide boost or after first sugar immunization for every two-three succeeding weeks. All groups received a sugar immunization at week 12. Mice were bled individually, collected sera pooled and the reactivity against indicated carbohydrates was detected by standard ELISA. The highest serum dilution with a 2SD higher OD than pre-immune serum was determined as end-point titer. Data are representative of three independent experiments.

Therapeutic peptide immunization induces tumor regression

In Task 2 we had described studies using IL-12 encoded DNA to augment the potential Th1 and possible CTL response. We had decided to try a series of pilot experiments using recombinant IL-12 first. To study the outcome of peptide immunization on the growth of solid tumors *in vivo*, we evaluated the antitumor cellular response on established Meth A tumors. BALB/c females were inoculated sub-cutaneously with 5 X 10^5 Meth A cells and seven days later treatment was started with the peptide injected intraperitionealy (3 times at 4-5-day intervals). As shown in Figure 5A, immunization moderately affected Meth A sarcoma growth, as 6 mice out of 11 immunized were cured. Treatment of animals with IL-12 following peptide immunization, intending to enhance the immune response, was successful as it mediated the complete eradication of established tumors (Fig. 5B). Treatment of tumor-bearing mice with only IL-12 did not affect tumor growth (Fig. 5C). We further determined that Peptide/IL-12

combination therapy is highly effective even in lower doses of IL-12, as 100 ug of daily IL-12 treatment in combining therapy, but not alone, eradicated tumors in 5 mice of five challenged (Fig. 5D,E). The time of the beginning of immunization and the size of tumor at this time apparently affect the efficacy of immunization. With immunizations started at day 14 or later or treating tumors with mean diameter of larger than 7 mm the efficacy of immunization dropped significantly (Fig. 5F).

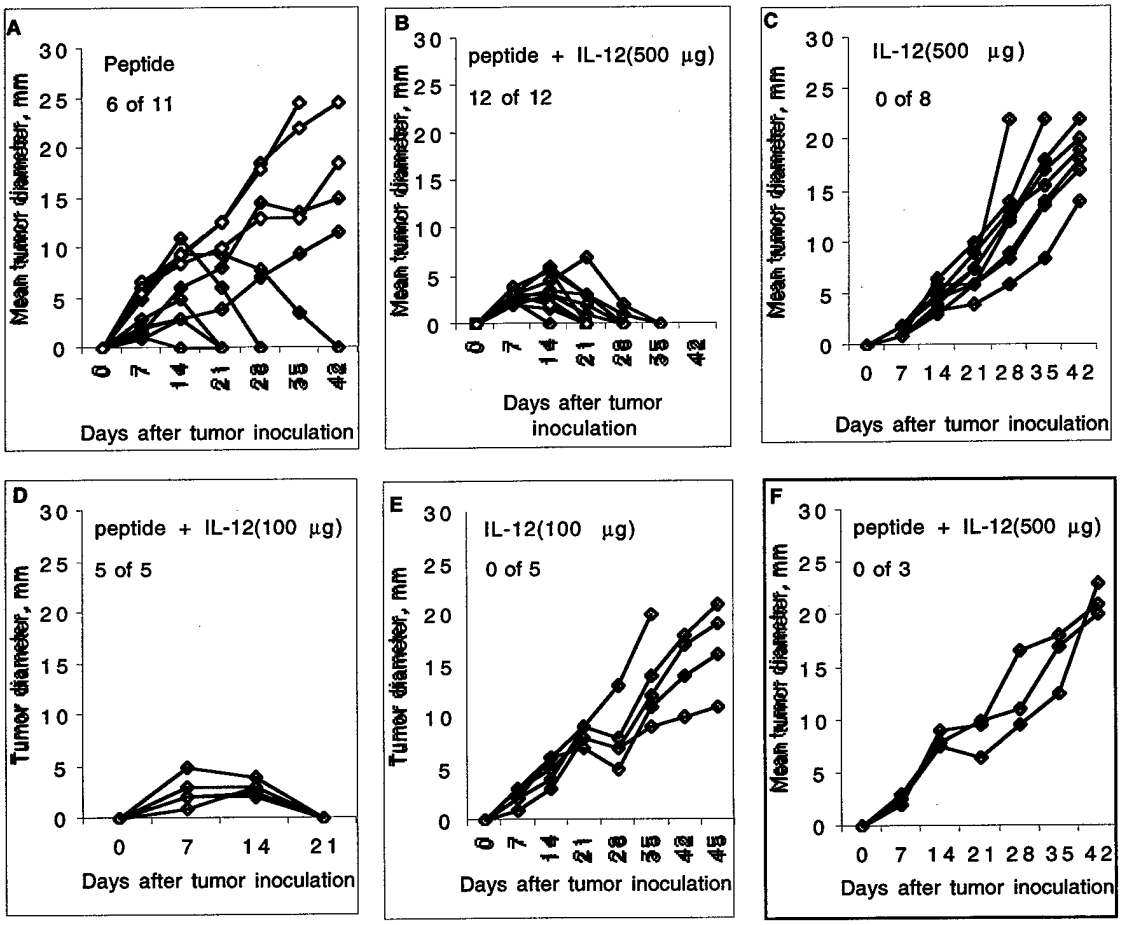


Figure 5. *Effect of peptide immunization on Meth A tumor growth and regression. BALB/cfemale mice were inoculated s.c. with 5 X 10^5 Meth A cells on day 0. 7* ***(A,B,D)*** *or 14* ***(F)*** *days after tumor inoculation peptide immunization started, mice were immunize i.p. with 106 MAP/QS21 for three times at 4-5 day intervals. IL-12 was administered alone* ***(C,E)*** *or following peptide immunization* ***(B,D,F)*** *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. The results are representative of two experiments.*

Peptide immunization leads to an increase in T-cell population and IL-12 responsiveness of T cells

Next we asked if the T cell population was increased after immunization with peptide. We used flow cytometry to examine the expression of CD3, CD4, CD8 and NK marker (DX5) on the surface of freshly isolated splenocytes. As shown in Figure 6A, the proportion of CD4+ and CD8+ T cells increased in immunized/cured mice compared with non-immunized/tumor-bearing animals. We did not detect any difference between groups of mice regarding NK cell population using the DX5 antibody.

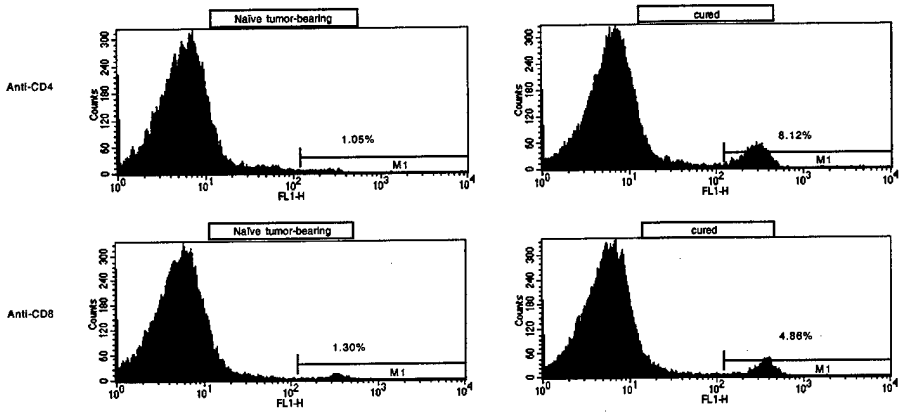


Figure 6A. *Peptide immunization induces higher proportion of CD4+ and CD8+ cells among splenocytes and IL-12 responsiveness among T cells. Naïve (non-immunized), tumor bearing, and peptide immunized/IL12 treated, cured, mice were sacrificed, splenocytes collected and deleted from erythrocytes. Freshly prepared splenocytes were stained with anti-CD4 and -CD8 FITC-conjugated mAbs. The results are representative of two experiments.*

To determine whether this population could be stimulated by IL-12, an IL-12 responsiveness test was performed on the T-cell population. Individual mice from non-immunized/tumor-bearing, and immunized/cured groups were sacked and T-cells purified using nylon wool followed by depletion of NK cells and a subsequent positive selection for Thy1.2 using MACS. Purity of T cells was more than 97% as assessed by expression of CD3 molecule by FACS. Purified T cells were seeded and stimulated with various concentrations of IL-12 and induction of IL-12 responsiveness was determined by measuring IFN-γ production. Figure 6B shows that T cells from peptide-immunized animals produced IFN-γ upon stimulation with IL-12.

As it is clear from our data regarding tumor challenge and peptide boost we had a prime and boost effect, we thought to magnify our priming by immunization with inactivated cells. In this case we replaced our first peptide immunization with inactivated Meth A-cell immunization. Tumor-bearing mice seven days after tumor inoculation first were immunized with attenuated cells (10^7/mouse, i.p.), followed by a combination of peptide/IL-12 as explained before.

Unexpectedly, this immunization regimen was less efficient as we recorded 3-4 escaped mice out of 12 in each separate experiment. The data again suggested that peptide immunization is efficacious but it should be done early on, as large tumors are hard to treat. Taking advantage of other effector cells, we further tried to stimulate eradication of tumors in immunized/tumor-bearing animals by treating them with Cyclophosphamide (Cy). Cy injected littermates were immunized with the peptide mimetic after 7 days followed by 5 doses of IL-12. We speculated that using this regimen, we might activate effector cells, which may cooperate with T cells in eradicating tumors of larger size. Following Cy and peptide/IL-12 therapy a clear tumor regression was marked in two thirds of the cases (data not shown). To further understand what cell population was in charge of such distinct tumor regression, we isolated splenocytes and stained cell surface markers by flow cytometry. As shown in Figure 7, it appears T cells may be the major effector population.

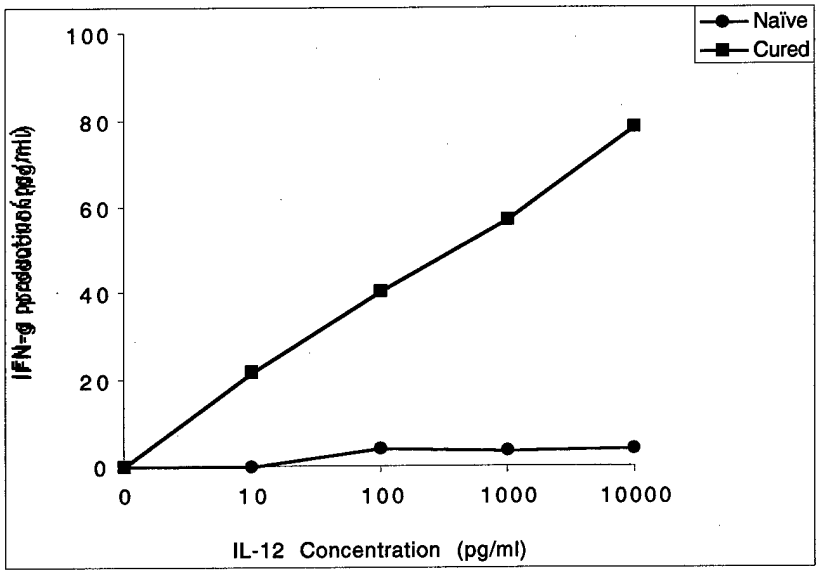


Figure 6B. *Induction of IL-12 responsiveness in T cells of transplanted mice after peptide immunization. Non-immunized/tumor-bearing and peptide-immunized/cured mice were saced, splenocytes collected and T cells purified. Purified T cells were incubated with serial dilutions of rIL-12 for 48 hours. Then supernatants were collected and concentration of IFNγ was determined. The results are representative of three independent experiments.*

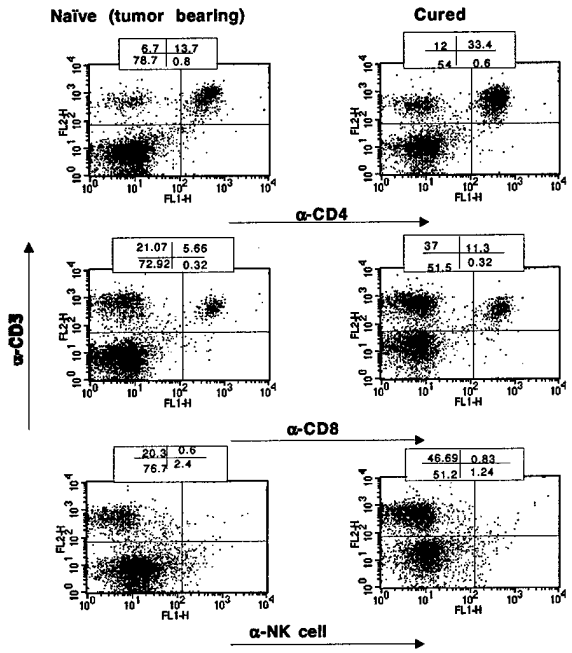


Figure 7. Treatment with Cy and Il-12 mediate an increase in CD4+ and CD8+ cells. Immunized tumor-bearing (uncured) and a mouse that underwent Cy and IL-12 treatment after or before eradication of tumor were saced and freshly prepared CD3+ splenic lymphocytes were stained with anti-CD4 -CD8 and -NK cell marker. The results are representative of two experiments.

Peptide-mediated tumor-growth suppression is dependent on cellular responses

To determine whether the anti-tumor activity mediated by peptide/IL-12 therapy is dependent on a cellular adaptive immune response 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 (Fig. 8). Combined peptide/IL-12 therapy had no effect on tumor growth of nude mice, indicating the dependence of mediated tumor

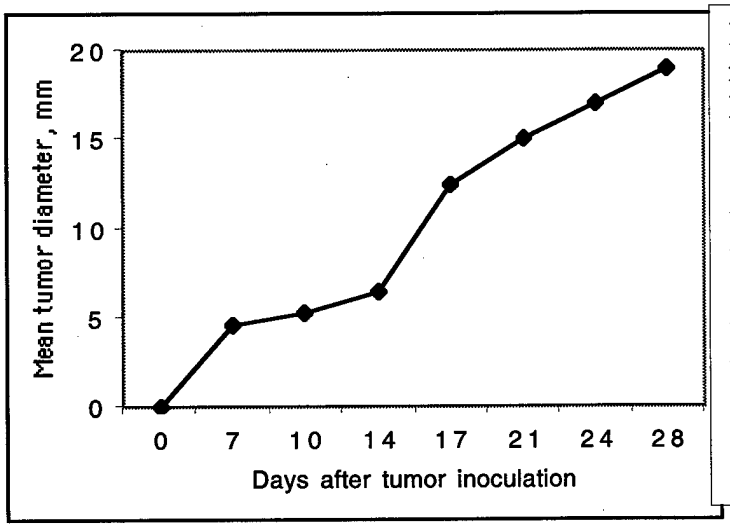
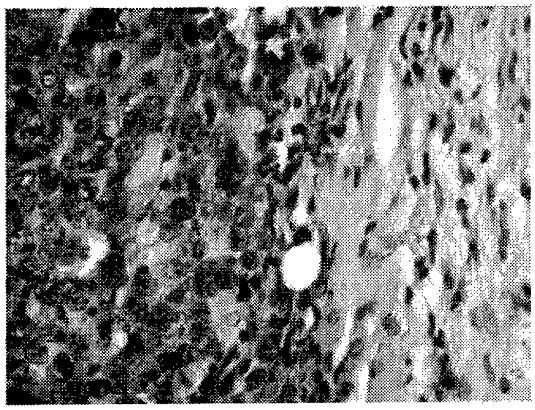


Figure 8. Peptide mediated antitumor responses depend on T cells. Nude BALB/c mice were injected with 5X105 Meth A cells into right flank. Seven days after treatment was started with peptide immunization (three times with 4-5 day intervals) followed by 5 doses of Il-12 (o.5 ug/mouse/injection/daily). Data represent the average of tumor mean diameter based on three individual mice.

regression on T cells. We prepared histologic sections of the tumor site and surrounding areas from non-immunized and immunized littermates. As shown in Figure 9, in contrast to non-immunized tumor-bearing mice, we detected lymphocytes in periphery infiltrating into tumor mass of immunized mice (Fig. 9B). Staining of sections obtained from the tumor site of an

already cured mice showed the presence of lymphocytes, with no tumor detectable microscopically (Fig. 9C).

A)



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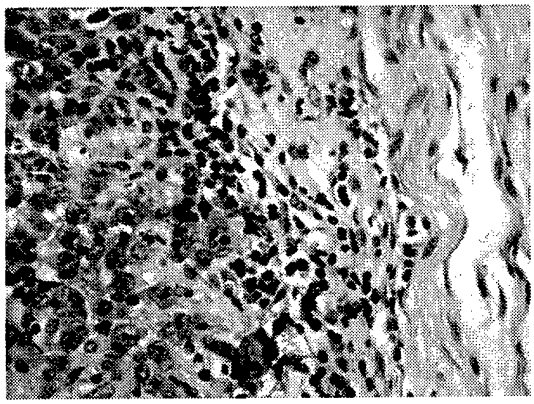

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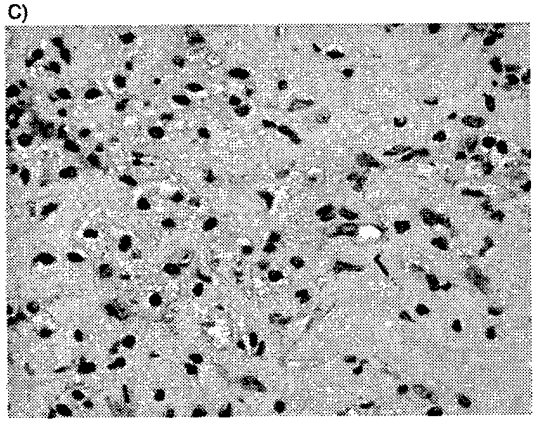

Figure 9. Lymphocytes infiltrate the Meth A challenge site in immunized mice. Fixed sections from tumor site of non-immunized tumor-beraing (A), immunized tumor-shrinking (B) and immunized after tumor elimination (C) stained with hematoxylin and eosin. Mice were transplanted and immunization started 7 days later. Samples were obtained when tumor was 16 mm (A), 2mm(B) and 5 days after tumor eradication.

Adoptive transfer of splenocytes stimulates eradication of tumors in nude mice

To further confirm a role played by T cells activated by the peptide mimotope, nude mice were transplanted with Meth A cells and injected ip with fresh splenocytes, isolated from cured mice, 10 days later (Fig 10). 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. In a follow-up study splenocytes were depleted from B cells and enriched for CD4+ and CD8+ cells, in vitro, and then transferred to tumor-bearing nude mice. Our data indicated a role for CD8+ cells in eradication of tumors (Fig. 11).

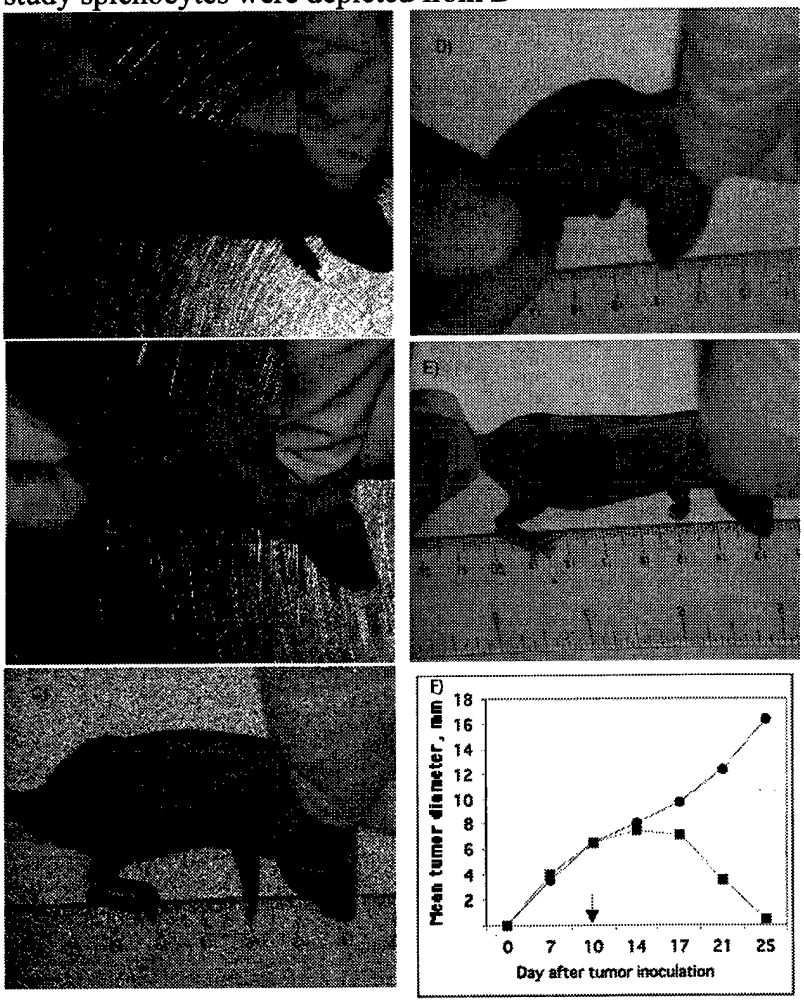

Figure 10. Adoptive transfer of fresh immune splenocytes eradicated established tumors in nude mice. Two groups (4 per group) of nude mice were inoculated s.c. with 5 x 10^5 Meth A cells into the right flank. 10 days after inoculation when average of tumors diameter was about 7 mm, one group was injected i.p. with 1.5 x 10^7 of fresh splenocytes collected from already immunized and cured BALB/c animals. Splenocytes were harvested from spleens and 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.

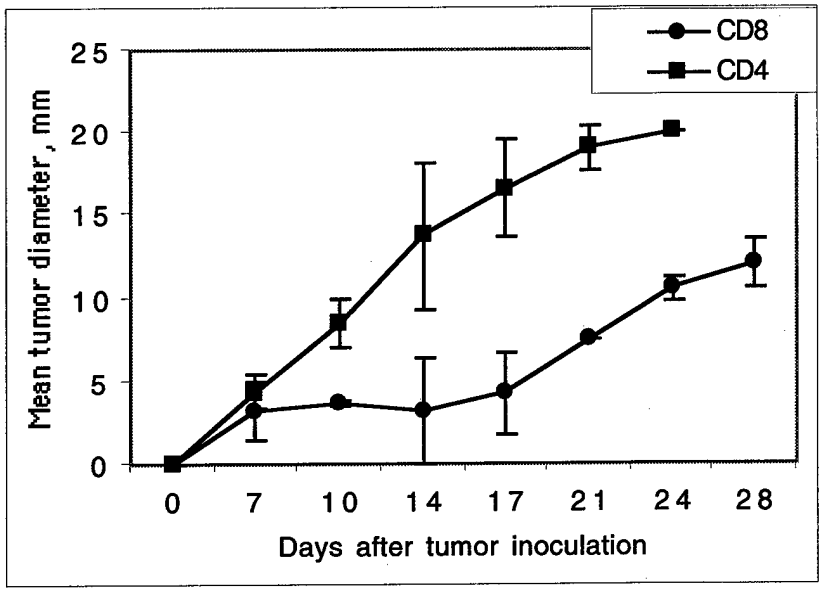

Figure 11. *Adoptive therapy of established tumors is dependent on CD8+ cells but not CD4+ T cells. Solid tumors were established in nude mice and at day 7 enriched splenocytes were transferred ip. For enrichment, splenocytes were passed through nylon wool after which the percentage of CD19+ cells remained were less than 7%. The percentage of CD8+ and CD4+ cells in CD4+ and CD8+ enriched population was less than 10%.*

Expression of FH-6-reactive antigen on escaped tumor variants

To examine a possible change in phenotype of tumors in mice that escaped our therapy, tumor masses were isolated from uncured mice and stained with FH-6 antibody. As illustrated in figure 9, tumor cells that did not respond to therapy lost the expression of the extended sLex on the surface. However, some individuals do not respond well to peptide or no strong immune response initiates after tumor transplant. IL-12 or Cy treatment later enhances T-cell responses and clears the tumors, but in those mice that tumor continued to grow the tumor phenotype might be changed to escape immunity.

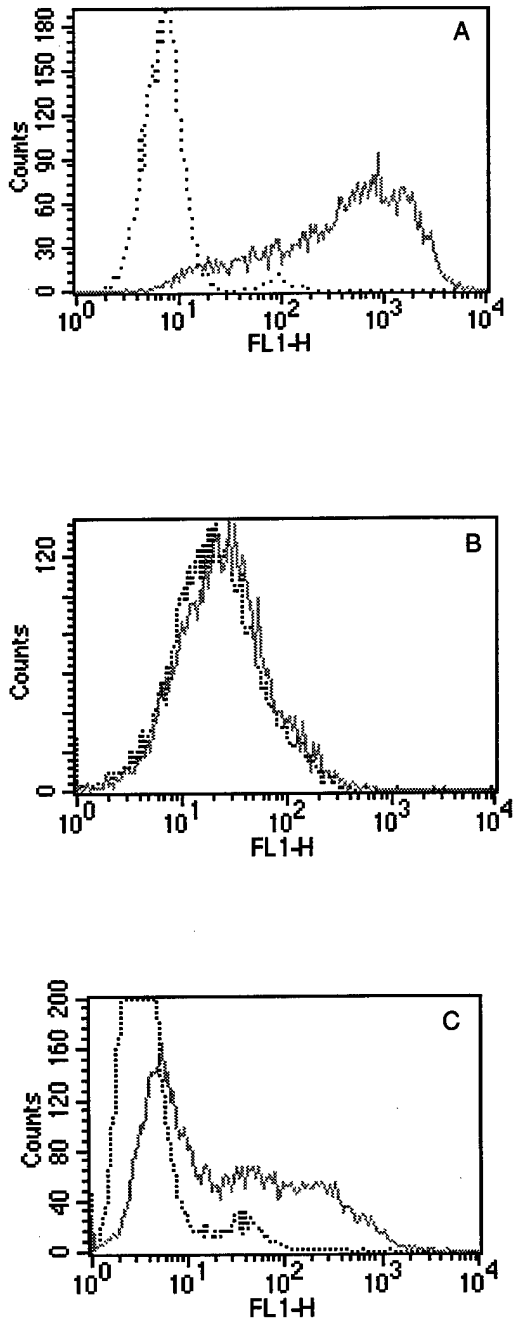

Figure 12. *In non-responding mice tumor cells undergo a change in phenotype. Mice that did not respond to treatments were sacrificed and tumor mass was isolated and cultured back in vitro for a day or two and then binding of FH-6 to normal ascitic Meth A* ***(A)*** *and these cells* ***(B,C)*** *was examined. Cells in B and C are from two individual mice, which represented non-responding animals.*

Vaccination with peptide 106 encoded DNA inhibits tumor growth. Using the DNA encoded sequences of peptides 106 and 109, groups of mice were immunized with respective DNA

constructs. After two immunizations of the respective peptide encoded DNAs, mice (respective groups) were rested for 110 days and challenged with Meth A tumor cells **(Fig. 13)**. Tumor growth inhibition was observed in mice immunized with the 106 DNA **(13A)** but not in mice immunized with the construct of peptide 911 **(13B)**. Addition of IL-12 did not affect the outcome of the 911 immunized mice.

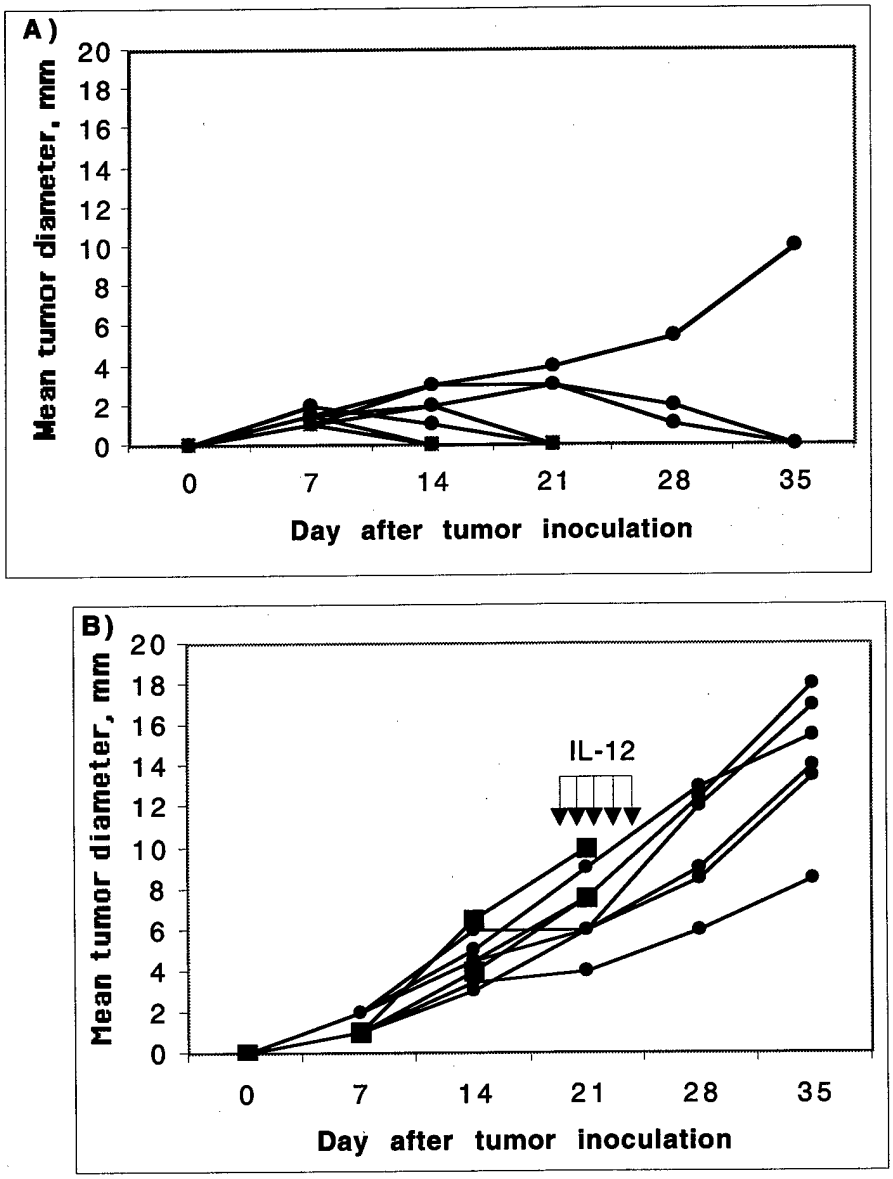

Figure 13. *Vaccination with DNA format of the peptide suppressed tumor growth in further challenging the immunized mice with Meth A cells. Mice were immunized with DNA constructs of 106 **(A)** and 911 **(B)** peptides twice at a three-week interval. 4 months after the boost mice were challenged with $5X10^5$ Meth A cells s.c.. Tumor growth is expressed for each individual mouse.*

Key research accomplishments

1. **We observed that peptide 106 can stimulate Th1 response.** We established that cell proliferation, stimulated by peptide 106, was primarily MHC Class II dependent as determined by inhibition with respective anti-MHC antibody. Cell stimulation with peptide 106 triggered IFN-γ release suggesting that peptide immunization with QS-21 as expected polarized the Th1 subset.
2. **We observed that Th1 cells activated by peptide mimetic express L-selectin.** Consistent with previous studies we observed L-selectin loss on CD4+ T lymphocytes upon peptide stimulation but also saw this loss upon stimulation with sLex. The proliferative response was peptide, sLex and Lex specific since LeY did not exhibit any cellular responses nor did splenocytes from sLex and LeY immunized animals responded to peptide 106, sLex or Lex antigens.

3. **We observed that the designed peptide 106 contained a CTL epitope.** In vivo stimulated effector cells from peptide 106 immunization displayed cytotoxicity directed toward peptide 106-pulsed MHC Class I^+ Class II^- P815 target cells, further verifying a role for $CD8^+$ T cell reactivity with peptide 106.

4. **Peptide 106 boosting can affect Meth A directed CTL.** While Meth A cell priming and boosting can lead to CTL activity against Meth A cells, peptide boosting increased the level of cytotoxicity against Meth A cells to a statistically significant level as compared with cytotoxicity against P815 cells as control target indicating a cross-reactive nature between peptide and tumor specific CTL responses. CTL activity was inhibited by the addition of either anti-Class I or anti-L-selectin antibody. However, we could not block CTL activity by the sLex reactive antibody FH6. It is possible that FH6 binds to a subtype of sLeX carbohydrate epitopes that do not always function as ligands for L-selectin just like it defines a subset that does not bind to E-selectin (6). This is also similar to that found for the antibody MECA 79 which binds to a subset of sulfated sLex different than that of L-selectin (7). L-selectin is known to bind to a variety of carbohydrates expressed on glycoproteins (8,9) It is possible that L-selectin functions as an auxiliary molecule (10) and by itself is not sufficient to mediate CTL killing, but requires engagement of antigen specific TCR (11). NK cells on the other hand also express L-selectin and NK cells appear to mediate cytolysis of tumor cells that express high levels of sLex (12). However, direct evidence that fuscosylated selectin ligands play a role in tumor rejection is still lacking.

5. **Immunization with Peptide 106 along with IL-12 can eradicate established tumors.** As it was expected, immunization with 106 MAP moderately inhibited tumor growth. It was shown that vaccination with inactivated Meth A cells had no effect on tumor growth, suggesting a superiority of 106 peptide vaccine to a cell-based one in this model. Our data proposes that immunization with only 106 peptide is not sufficient for induction of a state of reliable immunity to eradicate tumor. IL-12 is known to exhibit potent anti-tumor activity in a number of murine tumor models. Further treatment of peptide immunized

mice with IL-12 or a combination of Cy and IL-12 considerably helped in stimulating eradication of tumor.

6. **Both types of T cells seem to play a role in tumor cell erradication.** Our data shows that there is a proportional increase in the CD4+ and CD8+ T cells among splenocytes of treated mice compared to tumor-bearing mice. We did not observe any differences between splenocytes of these two groups of mice regarding the expression of NK cell marker DX5. Therefore, we propose that 106 immunization induces a population of Th1 and CTLs with production of IFNγ, and that IL-12 helps out with expanding the T cell population and activating IFNγ production. As resting T cells do not express IL-12 receptor and IL-12 responsiveness is induced after TCR stimulation, purified T cells were stimulated with IL-12 in vitro. The data indicate that in tumor-bearing animals T cells were not sensitized with tumor antigens, while peptide immunization induced T-cell responsiveness to IL-12.

7. **The designed 106 sequence is superior to the 911 sequence as a means to inhibit tumor growth.** DNA immunization with the 911 sequence did not mediate tumor growth inhibit even with the addition of IL-12.

Reportable Outcomes

One manuscript was published and another is in preparation. We have presented the results to be included in the manuscript in preparation at a conference in which it was judged meritoriously. We have shown that immunization with peptide 106 induces cellular responses that are not achievable by immunization with carbohydrate alone. Although cellular responses generated by the peptide mimotope may enhance CTL induction, vaccination with peptide alone appears not to be completely sufficient in the effector phase when challenged with a very high tumor burden (13). Our CTL data using effectors from peptide only immunized mice on Meth A cells as targets confirm this fact with marginal CTL activity observed.

Our results are very narrow with regards to the breadth of carbohydrate directed cellular responses. However, constituents of sLex and sLea are proposed to be influential to the metastatic properties of a variety of human tumor cells. Consequently, further efforts to optimize and isolate the carbohydrate moieties associated with presented glycopeptides may facilitate vaccine applications for eradication of metastatic lesions by both antibody dependent lysis and cellular responses. This possibility has yet to be proved with the appropriate models but suggests that for certain carbohydrate antigens like sLex and sLea, peptide mimetics might augment cellular responses other than delayed-type hypersensitivity like responses in future vaccine applications. The generated data support the idea of successful treatment of established tumors. As a challenge model we used BALB/c Meth A sarcoma, as we know that respective carbohydrates are expressed on the surface of this cell line.

Conclusions:

Defining new targets for designing effective and broad-based vaccines is considered crucial for the immunotherapy of solid tumors. 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. We have established that:

1. Immunization with 106 MAP induces a Peptide specific cellular response.
2. Vaccination of mice with peptide 106 eradicates established tumor.
3. Immunization with DNA format of the peptide suppresses tumor growth in further challenge.
4. Induced immunity has a cellular nature as it is transferred to nude mice by transferring splenocytes from cured mice.

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Immunization with a carbohydrate mimicking peptide augments tumor-specific cellular responses

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Keywords: cancer vaccine, carbohydrate, L-selectin, Meth A cells, peptide mimeotope

Abstract

The metastatic potential of some tumor cells is associated with the expression of the neolactoseries antigens sialyl-Lewis x (sLex) and sialyl-Lewis a (sLea) as they are ligands for selectins. We have recently shown that peptide mimetics of these antigens can potentiate IgG2a antibodies, which are associated with a T_h1-type cellular response. As L-selectin is preferentially expressed on $CD4^+$ T_h1 and $CD8^+$ T cell populations, specific induction of these phenotypes could augment a response to L-selectin ligand-expressing tumor cells. Here we demonstrate that immunization with a multiple antigen peptide (MAP) mimetic of sugar constituents of neolactoseries antigens induces a MHC-dependent peptide-specific cellular response that triggers IFN-γ production upon peptide stimulation, correlating with IgG2a induction. Surprisingly, T lymphocytes from peptide-immunized animals were activated *in vitro* by sLex, also triggering IFN-γ production in a MHC-dependent manner. Stimulation by peptide or carbohydrate resulted in loss of L-selectin on $CD4^+$ T cells confirming a T_h1 phenotype. We also observed an enhancement in cytotoxic T lymphocyte (CTL) activity *in vitro* against sLex-expressing Meth A cells using effector cells from Meth A-primed/peptide-boosted animals. CTL activity was inhibited by both anti-MHC class I and anti-L-selectin antibodies. These results further support a role for L-selectin in tumor rejection along with the engagement by the TCR for most likely processed tumor-associated glycopeptides, focusing on peptide mimetics as a means to induce carbohydrate reactive cellular responses.

Introduction

Tumor-associated carbohydrate antigens are correlated with metastatic phenotype and poor survival in epithelial malignancies of different origins. The expression of the neolactoseries antigens represented by sialyl-Lewis x (sLex), Lewis x (Lex) and Lewis Y (LeY) are increased significantly on a variety of carcinomas (1–6), with sLex and sialyl-Lewis a (sLea) also reported to be expressed on melanoma cells (7). Early studies indicated a possible relationship between metastatic properties of tumor cells and the expression of these antigens leading to tumor cell dissemination (8–11). An inhibitory effect on the establishment and growth of metastatic colonies of tumor cells expressing these antigens has been noted when anti-sLea or anti-sLex antibody was administered to mice in a pancreatic tumor murine model (12).

A cellular role played by L-selectin expressed on lymphocytes has also been noted in that anti-L-selectin antibody (Mel14) can influence cytotoxic T Lymphocyte (CTL) sensitization and metastatic colony formation (13), and Mel 14 can inhibit CTL activity of effector cells *in vitro* derived from immunization with selectin-ligand expressing cells (14). These results strongly suggest that blood-borne tumor cells may utilize these antigens with selectin molecules when tumor cells adhere to the endothelia at metastatic sites (1,12,15–17). Over-expression of selectin ligands on tumor cells are also targeted by NK cells (18). Consequently, targeting these antigens may thwart tumor cell dissemination.

We have recently shown that peptide mimetics of selectin ligands can induce a humoral response that targeted sLex in a murine tumor model facilitating tumor growth inhibition (19). We have gone on to show that immunization with peptide

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mimetics can elicit carbohydrate-reactive IgG2a antibodies, associated with T_h1 (20,21). As QS-21 potentiates a T_h1 response (22) and L-selectin is preferentially expressed on $CD4^+$ T_h1 cells (23) and on $CD8^+$ T cells (14,24), we were in the first instance interested in determining if peptide mimetic immunization activated lymphocytes that expressed L-selectin. As glycopeptides are also presented by MHC class I that express carbohydrate constituents (25,26) and T cells can recognize carbohydrate moieties on MHC-associated glycopeptides (27), we were also interested in whether mimeotope immunization could augment a CTL response to fibrosarcoma Meth A cells.

We observed that peptide immunization promotes a specific cellular response with IFN-γ production upon activation of splenocytes and T lymphocytes with immunizing peptide or with sLex and its homologue Lex. More importantly, we observed that immunization with peptide mimetic facilitated a specific cellular response to Meth A cells. Peptide boosting of mice primed with sLex-expressing Meth A cells enhanced the Meth A directed *in vitro* CTL activity. This activity was blocked not only by anti-MHC class I but also by anti-L-selectin antibody. This latter observation parallels other studies describing a role for L-selectin in T cell-mediated rejection of cells that express its ligands, such as sLex (14). The possibility of boosting lymphocyte subsets that cross-react with tumor cells that express L-selectin ligands opens new perspectives in designing cancer vaccines for further reducing micrometastases.

Methods

Immunization of animals

Peptides were synthesized as multiple antigen peptides (MAP; Research Genetics, Huntsville, AL) made by Fmoc synthesis on polylysine groups, resulting in the presentation of eight peptide clusters. Multivalent sLex, Lex and LeY were obtained from GlycoTech (Rockville, MD). Each BALB/c mouse received i.p. injections with 100 μg of MAP, or 50 μg of carbohydrates, and 20 μg of the adjuvant QS-21 (Aquila Biopharmaceuticals, Framingham, MA), both re-suspended in 100 μl of PBS as we described earlier (19). QS-21 was used in all peptide and carbohydrate immunization as adjuvant. As controls we used naive mice, animals injected with QS-21 (20 μg per mouse) or with multivalent sLex and LeY (50 μg of carbohydrates and 20 μg of the adjuvant QS-21 per mouse) antigens.

sLex-expressing Meth A cells were selected by sorting of positive cells with anti-sLex FH-6 antibody from an original Meth A cell line and were 100% $sLex^+$ upon prolonged culture. Meth A cells were repeatedly passed *in vitro* to decrease their tumorgenicity. Mice were immunized i.p. with 3×10^5 of these cells. Non-tumor bearing mice were used for analysis or for further experiments.

Antibodies

All antibodies were purchased from PharMingen (San Diego, CA). Anti mouse I-A^d (AMS 32.1), H-$2K^d$ (SF1-1.1) and CD1d (1B1), and their IgG2a and IgG2b isotype controls were first dialyzed in PBS buffer and then used in the proliferation cultures. For fluorescent staining of the splenocytes we used biotinylated rat anti-mouse anti-CD62L (anti-L-selectin Mel-14) and its isotype control IgG2a; anti-CD4–FITC (L3T4) and its isotype control IgG2b–FITC.

Flow cytometry

Fresh or *in vitro* stimulated splenocytes were blocked before staining with PBS containing 1% BSA and 1% rat serum for 10 min at 4°C. Cells were subsequently stained with rat anti-mouse mAb labeled with biotin, FITC or phycoerythrin. Appropriate rat isotype controls were used to set up background fluorescence. Streptavidin–FITC or avidin–phycoerythrin (Sigma, St Louis, MO) were used after biotin-labeled antibodies, as required. Acquisition of data was performed immediately after staining by using the FACScan analyzer and analysis performed by CellQuest software (both from Becton Dickinson Immunocytometry Systems, Mansfield, MA).

In vitro proliferation assays

Spleens were aseptically removed from each group. Splenocytes were harvested from spleens, and isolated as the responder cells by lysis of erythrocytes and consequent washing several times with fresh media. Prepared responder cells were used for detection of cell proliferation, as described (28,29). Briefly, cells (2.5×10^5/well) were cultured in flat-bottom 96-well plates and incubated with MAP, carbohydrates, ovalbumin (OVA) or media only. After 3 days of incubation, 1 μCi of [^{3}H]thymidine was added to each well and cells were incubated for an additional 16–18 h. Cultures were then harvested and radioactive emission counted on a Betaplate liquid scintillation counter (EG & G Wallac, Turku, Finland).

Proliferation assay was also performed using CellTiter 96 Aqueous One Solution (Promega, Madison, WI) based on the manufacturer's instructions. Cell culture was exactly performed as above and at the third day of incubation the provided solution was added to each well. Plates were incubated for an additional 1–2 h in a humidified, 5% CO_2 incubator at 37°C. Absorbency was measured immediately at 490 nm using a 96-well plate reader (Spectra Fluor; Tecan, Triangle Park, NC) as a measure for cell proliferation.

For detection of cell proliferation in T lymphocyte-enriched populations, a single-cell suspension of isolated splenocytes was depleted of B cells by positive selection after incubation with pan-B (B220) magnetic beads (Dynal, Oslo, Norway), at 4°C, for 30 min. To separate adherent from non-adherent cells, the B cell-depleted population was resuspended in RPMI media with 20% FBS and incubated for two consecutive adherence periods of 1 h each, at 37°C in humidified atmosphere with 5% CO_2. T lymphocytes were recovered and washed 3 times with RPMI/10% FBS and used in cell proliferation assays with or without mitomycin C (MMC)-treated antigen-presenting cells (APC). Adherent cells were recovered separately, treated with MMC (100 μg/ml in RPMI supplemented with 10% FBS, for 40 min at 37°C) and used as APC in proliferation of purified T lymphocytes at 10% ratio. Purified T cell populations, checked by FACS analysis, were assessed as >95% $CD3^+$, with no $CD19^+$ cells, and used in cell proliferation assays as explained above. Medium used for the proliferation assays was RPMI 1640 (Life Technologies,

Table 1. Peptides used in this study

Peptide	Sequence	Source	Predicted H-2K binding motif
106	GGIYWRYDIYWRYDIYWRYD	designed	RYDIYWRYDI (2000.0)
109	GGARVSFWRYSSFAPTY	phage display	RYSSFAPT (60.00)
711	GGPGQPGQPGQPGQ	designed	QPGQPGQPGQ (0.144)

Values in parenthesis are estimated half-times of disassociation of a molecule containing this sequence using a subsequence of 10 for scoring (33). The score for 109 ranges from 20 to 60 for different subsequences. The score for 106 changes from 50.0 for a subsequence of 8 to 80.0 for a subsequence of 9. The score for 711 does not go above 1.0 for subsequences of 8 or 9.

Rockville, MD) supplemented with 5% heat-inactivated FCS, L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml).

Cytokine production

Supernatants were collected from the co-cultures of splenocytes or purified T lymphocytes 72 h after *in vitro* stimulation with carbohydrate, peptides and media. The concentrations of IL-4, IL-10 and IFN-γ were measured by quantitative capture ELISA (PharMingen), according to the manufacturer's instructions.

CTL assay

Cytotoxic activity was measured by a 5 (P815 cells)- or 16 (Meth A cells)-h ^{51}Cr-release assay, as described elsewhere (30,31). Briefly, effector cells were stimulated for 5 days in the presence of stimulator cells and 10% RAT-T-STIM without concanavalin A (Becton Dickinson Labware, Bedford, MA). Peptide-pulsed murine mastocytoma P815 cells were used as stimulators (10:1 effector:stimulator ratio) and targets in 5-h assay, as described before (30,32). Meth A cells were treated with MMC and used as stimulator in 300:1 effector:stimulator ratio. For MMC treatment, 1×10^7 cells were treated in 1 ml of RPMI media supplemented with 10% FBS, with 100 µg MMC at 37°C for 40 min. Cells were washed 3 times after treatment.

Untreated Meth A cells were used as target cells. All target cells were labeled with 100 µCi/ml $Na_2{}^{51}CrO_4$ and mixed with effector cells at E:T ratios ranging from 50:1 to 3:1. Maximum and minimum (spontaneous) releases were determined by lysis of target cells in 5% Triton X-100 and medium respectively. Lysis was calculated as [(experimental – spontaneous)/(maximum – spontaneous)]×100. An assay was not considered valid if the values for the 'spontaneous release' count were >20% (for 5-h assay) or 30% (for 16-h assay) of the 'maximum release'. To calculate specific lysis of targets, the percent lysis of non-specific targets (P815 cells) was subtracted from the percent lysis of specific targets (peptide-pulsed P815 cells).

Statistical analysis

Data were analyzed by using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant. All experiments were performed at least 3 times.

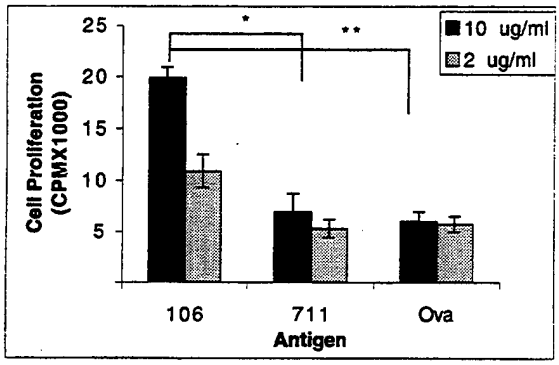

Fig. 1. Proliferative response of splenocytes from peptide 106-immunized mice. (A) Mice (four per group) were immunized 3 times with peptide 106 formulated in QS-21 every other week. Seven days after the third injection, spleens were collected and splenocytes were incubated with two concentrations (10 and 2 µg/ml) of the indicated MAP or OVA for 72 h. A ^{3}H-incorporation assay was performed as described in Methods. Results represent the mean value ± SEM based on four independent experiments with triplicate samplings. $^*P < 0.05$; $^{**}P < 0.025$.

Results

Peptide immunization primes for a specific cellular response

To facilitate a cellular response, we had designed peptide 106 (Table 1) with the potential to bind to H2-K^d and I-A^d. MHC binding prediction calculations identifies an H2-K^d and I-A^d binding motif centered on the RYDIYWRYDI sequence of the 106 peptide (Table 1) (33). Furthermore, the WRYDI sequence tract of the 106 peptide also displays similarity to a peptide sequence tract (DIYRW) identified in a peptide that binds to CD1 (34), which may play a role in activating NK T cells.

To analyze the cellular response upon peptide 106 immunization, BALB/c mice were immunized with the mimeotope and the proliferative response of isolated splenocytes to the peptides 106, 711 (Table 1) as MAP forms, as well as OVA (as an additional control) was determined. As expected, *in vivo* primed splenocytes from 106 peptide-immunized animals were specifically activated by peptide 106, and not control peptides, in a statistically significant concentration-dependent manner (Fig. 1). Peptide 106 did not stimulate splenocytes from naive animals or from animals immunized with QS-21 alone (data not shown). These results indicate

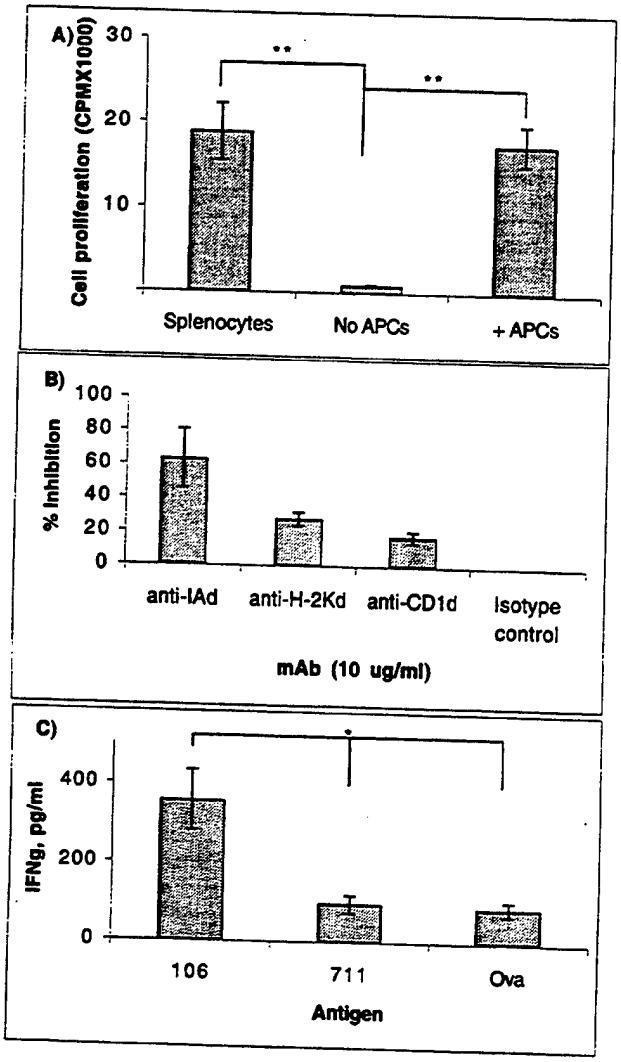

Fig. 2. Cell proliferation is APC dependent, and is blocked by anti-MHC class II and I antibodies. Mice were immunized with 106 peptide as explained in the legend to Fig. 1 and splenocytes were collected 7 days after the last immunization. (A) Proliferation assay was performed on splenocytes and purified T lymphocytes (with or without addition of inactivated APC) with a peptide concentration of 10 μg/ml. (B) Anti-I-A^d, -H-$2K^d$ and -CD1d, and IgG2a and IgG2b isotypes were used for blocking of proliferation of splenocytes. Isotype controls acted very similarly, so just one of those is presented. (C) Splenocytes were stimulated using peptides 106, 711 and OVA (each 10 μg/ml), and IFN- γ levels were quantified. All results are expressed as the mean ± SEM of four independent experiments with triplicate samplings. $^*P < 0.05$; $^{**}P < 0.01$.

that immunization with peptide 106 mediates a peptide-specific cellular response.

Anti-MHC class II and I inhibit proliferative response

A standard cell proliferation was performed with purified T lymphocytes alone and after addition of MMC-inactivated APC (Fig. 2A). The results indicate that proliferation is APC dependent. Proliferation of the T lymphocytes was not observed in the absence of APC. Proliferation was inhibited by the addition of specific anti-I-A^d anti-class II and to a lesser extent with anti-class I antibodies (Fig. 2B). The addition of anti-CD1 antibody inhibited proliferation to almost the same extent as addition of anti-class I antibody. Therefore, the 106 peptide might be presented in multiple ways to T cells; however, MHC class II-dependent presentation appears to be the major pathway (60% inhibition by anti-class II antibody).

For analysis of cytokine profiles, plates for proliferation were doubled and the supernatant of duplicated plates were harvested after 72 h of *in vitro* activation. IFN-γ production was found to be peptide specific (Fig. 2C), with no detectable presence of IL-4 or IL-10 (data not shown). The sensitivity for IL-4 and IL-10 detection was 7.8 and 31.3 pg/ml respectively. Taken together, these results demonstrate that 106-MAP-peptide immunization along with QS-21 potentially activates lymphocyte populations with a predominant T_h1 phenotype.

Activation down-modulates L-selectin

We examined L-selectin expression on $CD4^+$ T cells. L-selectin is preferentially expressed on T_h1 over T_h2 cells (23). The activation of T cells through the TCR results in the differential regulation of L-selectin (24). Using FACS analysis, we studied the expression of L-selectin on $CD4^+$ T cells from naive and peptide-immunized animals before and after *in vitro* stimulation with peptide 106 to assess activation (Fig. 3). In keeping with other studies, a population of cells from peptide 106-immunized mice remained clearly L-$selectin^+$ after *in vitro* stimulation (24). The demonstrated presence and loss of L-selectin on the surface of $CD4^+$ T cells occurring upon stimulation to the 106 peptide further confirms a T_h1 nature for these cells. It was observed that sLex could activate these cells as effectively as peptide 106 as assessed by L-selectin loss (Fig. 3).

Carbohydrate stimulates proliferation of 106 peptide-primed splenocytes

In separate experiments, isolated splenocytes were incubated with sLex, Lex and LeY (Fig 4A). sLex and Lex, but not LeY stimulated a proliferative response with splenocytes from 106-immunized animals. Splenocytes from mice immunized with the carbohydrate antigens sLex or LeY did not proliferate upon incubation with peptide 106, LeY, sLex or Lex (Fig. 4B and C). This result suggests that priming with the peptide mimeotope leads to a specific carbohydrate cross-reactive cellular response.

MHC class II molecule is involved in proliferation of peptide-primed T lymphocytes by carbohydrate

sLex-stimulated proliferation of peptide-primed T lymphocytes was inhibited by the I-A^d-specific anti-MHC class II antibody, suggesting a role played by the class II molecule in the cross-reactivity between the carbohydrate antigen and peptide-primed responder cells and APC (Fig. 5A). The specificity of the antibody for I-A^d has been assessed by others, indicating specific MHC class II inhibition of proliferation (35–37). IFN-γ production for sLex activation was detected (Fig. 5B). As in the peptide case, we could not detect any IL-4 and IL-10 production after activation. This production was specific for peptide-immunized mice compared with T lymphocytes derived from mice immunized with QS-21 alone or from naive animals.

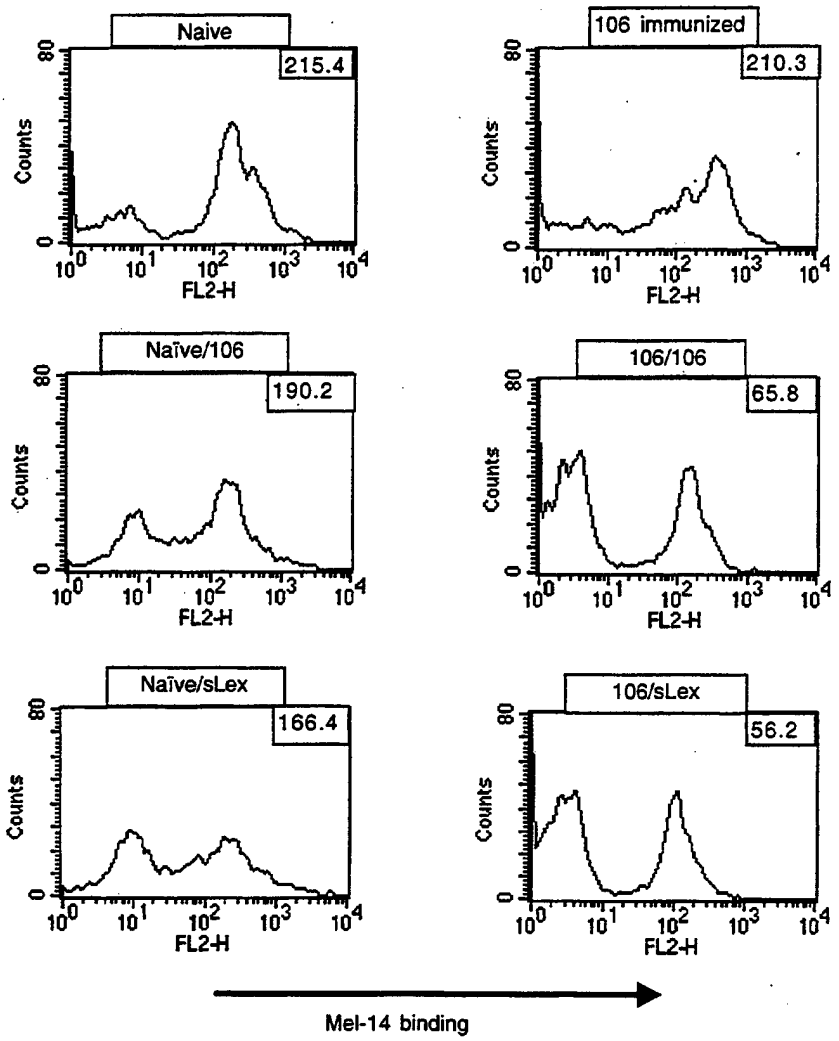

Fig. 3. Analysis of L-selectin expression on the surface of $CD4^+$ lymphocytes obtained from naive and 106/QS21-immunized animals. Staining was performed with phycoerythrin-conjugated anti-mouse L-selectin (Mel-14) and anti-CD4–FITC (L3T4) mAb before and after 3 days of *in vitro* stimulation. For stimulation either 106 or sLex (10 μg/ml) were used. Analysis of Mel-14 binding was performed on gated $CD4^+$ lymphocytes. Mean fluorescence values for L-selectin expression on $CD4^+$ cells are shown in the top right corner of the histograms. Naive, cells from naive mice before proliferation. 106 immunized, cells from 106-peptide immunized mice before proliferation. Naive/106 and 106/106, cells from naive and 106 immunized mice stimulated *in vitro* for 3 days with 106 peptide. Naïve/sLex and 106/sLex, cells from naive and 106 immunized mice stimulated *in vitro* for 3 days with carbohydrate sLex.

Peptide boost of Meth A cell-primed animals enhances CTL activity against Meth A cells

In vitro stimulated effector cells from peptide 106-injected animals showed high specific lysis against peptide-pulsed P815 cells, confirming the peptide's ability to function as a MHC class I target (Fig. 6A). As expected, cytotoxicity was reduced in experiments in which $CD8^+$ cells were depleted. When Meth A cells were used as stimulators and targets, we observed moderate cell killing (Fig. 6B). In contrast, no significant cytotoxicity was detected on the P815 cells, used as a negative control. P815 cells did not express sLex, as assessed by FACS using the mAb FH-6 (data not shown) nor did serum to peptide 106 bind to P815 cells, also assessed by FACS (data not shown). We did not detect any significant increase in lysis of peptide pulsed Meth A cells (not shown). *In vitro* stimulated splenocytes from naive or QS-21 immunized mice did not show significant cytotoxicity on Meth A cells.

To examine the hypothesis that peptide immunization could augment cellular responses to Meth A-cells, we studied the proliferative effects of peptides and Meth A cells in groups of mice immunized with Meth A cells only or primed with Meth A cells and boosted with peptide. The results are summarized in Fig. 7(A). Immunization with Meth A cells stimulated a proliferative response to the cells, as expected. Boosting of Meth A cell-immunized mice with peptide 106 increased the proliferative response to peptide 106 and to Meth A cells. As the control peptide for proliferation we used a peptide referred to as peptide 109 (Table 1). We did not detect cross-reactivity between

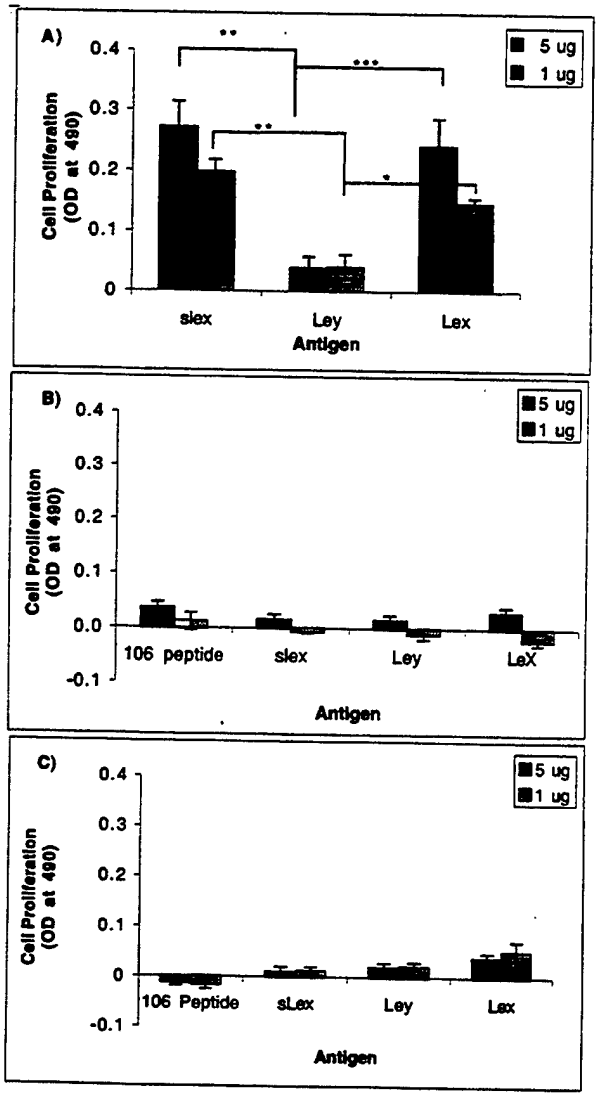

Fig. 4. Cell proliferative response to carbohydrates in splenocytes from 106 peptide (A)-, sLex (B)- and LeY (C)-immunized mice. Mice (four per group) were immunized 3 times at a 2-week interval. Seven days after the last immunization spleens were removed, and splenocytes collected and prepared as explained in Methods. Cells were incubated with peptide and carbohydrates for 3 days. Proliferation was measured at the third day after incubation. For recording the proliferation response the number of viable cells was detected using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega). The absorbance in the presence of culture medium is subtracted as background proliferation. All results present the mean value ± SD of triplicate samplings. Data are representative of three independent experiments with pooled cells from four mice. $^{*}P < 0.05$; $^{**}P < 0.025$; $^{***}P < 0.01$.

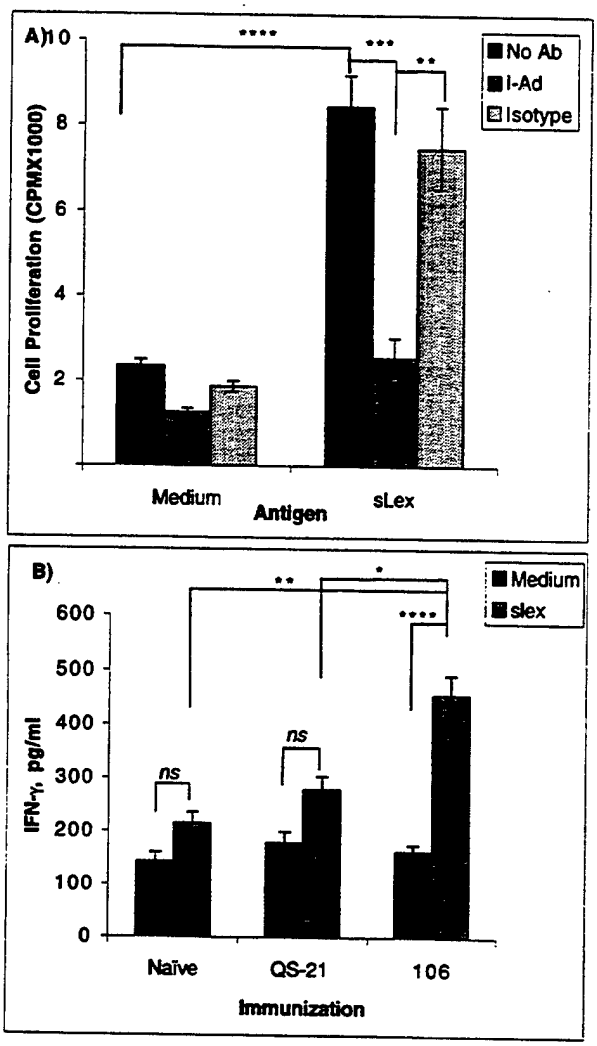

Fig. 5. (A) Anti-MHC class II antibody blocks activation of T cells by sLex. Purified T lymphocytes from 106 peptide/QS21-immunized (performed as explained above) animals were used in a proliferation assay with or without sLex (10 μg/ml) in the presence of MMC-treated APC. Incubation was done in the presence or absence (No Ab) of anti-MHC class II antibody (10 μg/ml). Isotype control was used at 10 μg/ml. (B) IFN-γ production in the supernatant of purified T lymphocytes stimulated with sLex (10 μg/ml) for 3 days in the presence of MMC-treated APC. Supernatant from stimulated T lymphocytes was collected and IFN-γ was measured. All results are presented as the mean value ± SEM based on four independent experiments with duplicate samplings. Medium, proliferation in culture medium used as background. $^{*}P < 0.05$; $^{**}P < 0.025$; $^{***}P < 0.01$; $^{****}P < 0.005$. ns, not significant.

peptide 109 and Meth A cells, but this peptide showed cross-reactivity in cellular response with 106 peptide (not shown; attributed to the sequence homology between the peptides). We investigated the role of Meth A and P815 cells in stimulation of splenocytes from mice immunized with peptide 106, sLex and OVA as control. Only splenocytes from 106 peptide immunization showed a proliferative response in co-culture with MMC-treated Meth A cells (data not shown), we did not detect significant proliferation using P815 cells as stimulator (data not shown).

We observed a cytotoxic enhancement effect upon immunization with peptide 106 using effector cells from Meth A-immunized mice compared with those from Meth A-primed/peptide-boosted mice (Fig. 7B). The level of CTL was consistent with those observed in similar studies (31,32). A clear increase in lysis was observed in the cell-primed/peptide-boosted animals compared to mice immunized with Meth A cells only. We did not detect any cytotoxicity using re-

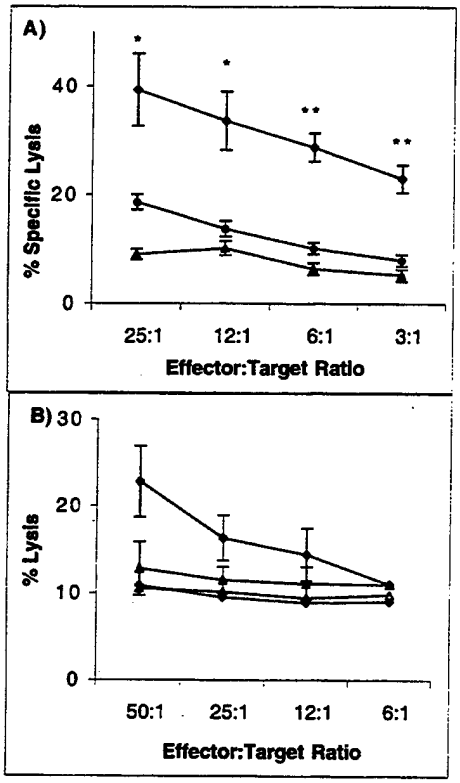

Fig. 6. Induction of cross-reactive CTL response to Meth A cells following immunization with peptide 106. Mice (four per group) were immunized with peptide 106 at 3 times at a 2-week interval and 7 days after the last immunization, spleens were collected and isolated effector cells were re-stimulated with either peptide pulsed P815 cells or Meth A cells *in vitro* for 5 days. CTL was performed using 106 peptide-pulsed P815 (A) or Meth A (non-pulsed) (B) cells as stimulators and targets. ◆, Bulk effector cells from 106-immunized mice; ●, $CD8^+$ T cell depleted effector cells from 106-immunized mice; ▲, Bulk effector cells from naive mice. To calculate specific lysis of targets (as shown in A), the percent lysis of non-specific targets (P815 cells) was subtracted from the percent lysis of specific targets (peptide-pulsed P815 cells). Cytotoxicity against P815 cells as control target (open symbols) is shown in (B). All error bars (SD) were calculated based on triplicates. All data are representative of three independent experiments using pooled cells from four mice. Statistically significant compare with cytotoxicity levels of naive effector cells at $^*P < 0.05$ and $^{**}P < 0.025$ respectively.

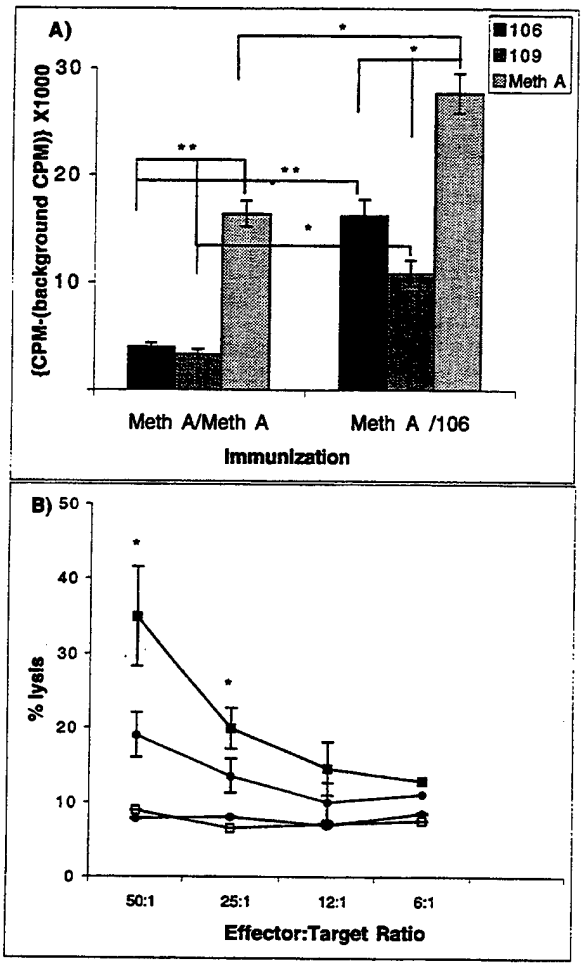

Fig. 7. (A) 106 peptide boosts cellular responses in the Meth A cell-primed animals. Mice were immunized with Meth A cells, rested for 1 month, and then were boosted with the 106 peptide and compared with Meth A cell primed/boosted (at the same interval) only. In each immunization regimen, splenocytes were collected 7 days after boost, and proliferative response was measured using peptides 106, 109 and Meth A cells as antigens. Meth A cells were treated with MMC and then used in the assay. Background c.p.m. is [^{3}H]thymidine incorporation with used medium only. (B) Anti-Meth A cells CTL. Mice were primed and boosted with Meth A cells (circle) or primed with Meth A cells and boosted with 106 peptide (square), as explained above. Cytotoxicity was measured against Meth A (closed symbols) or P815 cells (open symbols). Splenocytes were stimulated *in vitro* with Meth A cells as described in Methods. All bars show SD based on three replications. All data are representative of three independent experiments using pooled cells from four mice. $^*P < 0.05$; $^{**}P < 0.025$. In (B) asterisks compare the levels of cytotoxicity of effector cells from Meth A/106-immunized animals with the cytotoxicity levels of the same cells detected against P815 cells as targets.

stimulated effectors from either immunization groups targeting P815 cells. Levels of cyotoxicity against Meth A cells were compared with those levels against P815 cells, statistically significant cytotoxicity against Meth A cells was detected only with effectors from peptide boosted animals. Representative results from 50:1 E:T ratio of a separate experiment are summarized in Table 2. Cytotoxicity was dependent on both $CD4^+$ and $CD8^+$ cells as assessed by respective depletion of the $CD4^+$ and $CD8^+$ subsets. Meth A cell-mediated lysis was found to be inhibited by both anti-MHC class I and L-selectin antibodies, but not by anti-class II antibody as Meth A cells do not express MHC class II molecules.

These results further suggest that immunization with peptide or Meth A cells activates $CD4^+$ and $CD8^+$ T cells that affect cytotoxicity. Meth A cells are known to activate both T cell subsets that are involved in cytotoxicity targeting Meth A cells (31,32). However, in the case of peptide mimetic boost of Meth A cell-immunized animals, MHC class I-restricted/$CD8^+$-dependent T cells showed a predominant role in the CTL response. Our results further indicate that L-selectin may participate in the lysis process as described previously (14).

Table 2. Percentage of specific lysis (± SD)[a] on Meth A[b] cells as targets at 50:1 E:T ratio

Immunization[c]	Cell treatment					
	None	Anti-I-A[d]	Anti-H-2k[d]	Mel-14	$CD4^+$ depleted	$CD8^+$ depleted
Naive	5.7 (0.9)	ND[d]	ND	ND	ND	ND
Meth A/Meth A	20.98 (6)	ND	ND	3.4 (4.2)	15.8 (8)	14 (7)
Meth A/106	30.06[e] (4.9)	27.8[e] (4.1)	13.3 (6.7)	13.09 (7.7)	21.8 (8.5)	14.2 (6)
Meth A/QS-21	11.2 (4.2)	15 (6.36)	13.63 (2.9)	11.8 (5.8)	ND	ND

[a]Values are calculated means ± SD from three replications and are representative of three independent experiments using pooled cells from four mice in each.
[b]P815 cells were also used as targets and no significant lysis (6%) was detected.
[c]Groups of mice were primed with Meth A cells, rested for 1 month and then boosted with Meth A, 106 petide/QS-21 or QS-21 alone. Splenocytes were collected 7 days after the boost.
[d]ND, not determined.
[e]Statistically significant ($P < 0.05$) as compared with cytotoxicity level of the same effectors against P815 cells as targets or of naive effectors against Meth A cells.

With naive mice and QS-21-immunized mice, as before, we did not detect any significant lysis.

Discussion

We had previously demonstrated that immunization with a multivalent, repetitive peptide mimetic of sLex (peptide 106) induced an anti-sLex cross-reactive antibody response to Meth A tumor cells (19). Antibody responses to sLex can mediate complement-dependent killing of sLex-expressing tumor cells inhibiting the establishment and growth of metastatic colonies as observed in several animal models (12,19,38).

Meth A tumor growth inhibition upon immunization with attenuated Meth A cells is reported to be dependent on $CD4^+$ and $CD8^+$ T cell responses to undefined glycoproteins (31,32,39–41). Commitment of lymphocytes to the T_h1 and to $CD8^+$ T cell phenotypes as characterized by the expression of IFN-γ, may be critically involved in tumor rejection (42,43). However, the expression of L-selectin on these cell types may also lend to CTL activity (14). IFN-γ and lymphocytes work together to find and eliminate tumor cells (44). As the adjuvant QS-21 promotes the T_h1 phenotype, which expresses L-selectin, and peptide 106 can induce a T_h1-associated humoral response, we examined if immunization with peptide 106 along with QS-21 could augment cellular responses to sLex-expressing Meth A tumor cells.

Immunization with the mimeotope led to peptide-specific cell proliferation that was concentration dependent (Fig. 1). As expected, cell proliferation was primarily MHC class II dependent as determined by inhibition with respective anti-MHC antibody (Fig. 2). Cell stimulation with peptide 106 triggered IFN-γ release, suggesting that peptide immunization with QS-21 as expected polarized the T_h1 subset (Fig. 2C). Consistent with previous studies we observed L-selectin loss on $CD4^+$ T lymphocytes upon peptide stimulation, but also saw this loss upon stimulation with sLex (Fig. 3). The proliferative response was peptide, sLex and Lex specific since LeY did not exhibit any cellular responses nor did splenocytes from sLex- and LeY-immunized animals responded to peptide 106, sLex or Lex antigens (Fig. 4).

We observed that sLex activated T lymphocytes from 106 immunized animals to proliferate and secrete IFN-γ (Fig. 5). Cell activation by sLex was inhibited by anti-MHC class II antibody addition, suggesting a possible role for this molecule in the presentation process (Fig. 5B). *In vivo* stimulated effector cells from peptide 106 immunization displayed cytotoxicity directed toward peptide 106-pulsed MHC class I^+ class II^- P815 target cells, further verifying a role for $CD8^+$ T cell reactivity with peptide 106 (Fig. 6). While Meth A cell priming and boosting can lead to CTL activity against Meth A cells, peptide boosting increased the level of cytotoxicity against Meth A cells to a statistically significant level as compared with cytotoxicity against P815 cells as control target (Fig. 7 and Table 2), indicating a cross-reactive nature between peptide and tumor specific CTL responses.

CTL activity was inhibited by the addition of either anti-class I or anti-L-selectin antibody (Table 2). However, we could not block CTL activity by the sLex-reactive antibody FH-6 (data not shown). It is possible that FH-6 binds to a subtype of sLex carbohydrate epitopes that do not always function as ligands for L-selectin just like it defines a subset that does not bind to E-selectin (2). This is also similar to that found for the antibody MECA 79 which binds to a subset of sulfated sLex different than that of L-selectin (45). L-selectin is known to bind to a variety of carbohydrates expressed on glycoproteins (46,47). It is possible that L-selectin functions as an auxiliary molecule (48) and by itself is not sufficient to mediate CTL killing, but requires engagement of antigen-specific TCR (14). NK cells, on the other hand, also express L-selectin and other lectin-type molecules, and NK cells appear to mediate cytolysis of tumor cells that express high levels of sLex (18). However, direct evidence that fuscosylated selectin ligands play a role in tumor rejection is still lacking.

The activation of cross-reactive T cells has been described in many systems. What is the specificity of the $CD8^+$ T cells targeting Meth A? Meth A cell-primed T cells maybe glycopeptide/glycoprotein specific (32), which are cross-reactive with peptide 106, as glycopeptides are known to activate T cells that recognize the carbohydrate moiety on MHC-associated glycopeptides (49–55). Direct interaction of the TCR with the carbohydrate (27,56) is dependent on the chemical structure

of the glycan as well as its position within a peptide. Glycopeptides with GlcNAc residues known to associate with class I (25) also induce CTL responses (26). T cells primed to glycopeptides carrying more complex saccharide antigens sometimes show a complicated pattern of cross-responses to glycopeptides with simpler glycan moieties (51). It argues that the presentation of carbohydrate antigens on Meth A should be more thoroughly characterized in terms of the structure of glycopeptides, glycoshingolipids, etc., which is beyond the scope of the present paper.

Proliferation by sLex and Lex is tougher to reconcile. Carbohydrate antigens have been proposed to associate with MHC directly (57); however, this type of association has not yet been confirmed in our system. It is possible that proliferation is associated with some yet undefined superantigen-like association as we observed up-regulation of CD15s on sLex activated T cells (data not shown). Superantigens but not mitogens are capable of inducing up-regulation of E-selectin ligands on human T lymphocytes (58). Likewise, bacterial polysaccharides and mimicking peptides with a distinct charge motif involving Lys and Asp residues have been shown to activate T lymphocytes and that this activity confers immunity to a distinct pathologic response to bacterial infection (59). Peptide 106 has a Arg residue homologous to Lys, with the Asp residues identical to peptides that induce cross-reactive cellular responses in bacterial studies (59). The molecular details of how this occurs are not as yet known. Further analysis of binding affinities of peptide and carbohydrate with I-A^d will be illuminating. However, immunization with sLex did not induce T lymphocytes that reacted with peptide 106 nor itself, an effect of sLex being a T cell-independent antigen.

Immunization with peptide 106 induces cellular responses that are not achievable by immunization with carbohydrate alone. Nevertheless, the induction of optimal systemic anti-tumor immunity involves the priming of both $CD4^+$ and $CD8^+$ T cells specific for tumor-associated antigens. Although cellular responses generated by the peptide mimeotope may enhance CTL induction, vaccination with peptide alone appears not to be completely sufficient in the effector phase when challenged with a very high tumor burden (19). Our CTL data using effectors from peptide-only-immunized mice on Meth A cells as targets (Fig. 6B) confirm this fact with marginal CTL activity observed. Consequently, we did not observe complete tumor protection in our previous study (19).

Our results are very narrow with regard to the breadth of carbohydrate directed cellular responses. However, constituents of sLex and sLea are proposed to be influential to the metastatic properties of a variety of human tumor cells. Consequently, further efforts to optimize and isolate the carbohydrate moieties associated with presented glycopeptides may facilitate vaccine applications for eradication of metastatic lesions by both antibody-dependent lysis and cellular responses. This possibility has yet to be proved with the appropriate models but suggests that for certain carbohydrate antigens, peptide mimetics might augment cellular responses other than delayed-type hypersensitivity like responses (60) in future vaccine applications.

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Abbreviations

APC	antigen-presenting cell
CTL	cytotoxic T lymphocyte
LeY	Lewis Y
MAP	multiple antigen peptide
Meth A cells	methylcholanthrene-induced fibrosarcoma
MMC	mitomycin C
OVA	ovalbumin
sLex	sialylated Lewis x
sLea	sialylated Lewis a

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