Published in final edited form as:

Arch Surg. 2012 May ; 147(5): 480–482. doi:10.1001/archsurg.2011.2206.

Glycolipid Antigens for Treating Hepatic Colorectal Cancer Metastases and Their Effect on the Therapeutic Efficacy of Live Attenuated *Listeria monocytogenes*

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Abstract

Previous work demonstrated that a subset of natural killer T cells in mice decreased the antitumor efficacy of live attenuated *Listeria monocytogenes* where the actin A and internalin B genes were genetically deleted (LMD) against murine hepatic colorectal cancer metastases. Therefore, we hypothesized that the use of specific glycolipids known to selectively stimulate natural killer T-cell subsets used alone or co-administered with LMD would increase survival. We found that early or multiple administrations of glycolipids after tumor challenge had a strong impact on survival with or without LMD. Solitary administration or treatment given later was less efficacious but still showed a strong trend toward enhancing the antitumor activity of LMD. These results underscore the potential of glycolipids in the treatment of hepatic metastases and encourage further investigations into the immunomodulation of natural killer T cells to enhance the antitumor activity of LMD.

We previously reported that attenuated *Listeria monocytogenes* where the actin A and internalin B genes were genetically deleted (LMD) induces antitumor responses against hepatic metastases from colorectal cancer¹ and that the antitumor efficacy of LMD was significantly enhanced in CD1 day-deficient mice, which lack a population of natural killer T (NKT) cells, suggesting the presence of an inhibitory subset of NKT cells. Natural killer T

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Financial Disclosure: None reported.

Author Contributions: Dr Olino had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Olino, Pan, Schulick, Yoshimura, and Weber. *Acquisition of data:* Olino, Meckel, Pan, Thuluvath, Schulick, and Weber. *Analysis and interpretation of data:* Edil, Pan, Thuluvath, Pardoll, Schulick, and Weber. *Drafting of the manuscript:* Olino, Edil, Schulick, and Weber. *Critical revision of the manuscript for important intellectual content:* Olino, Meckel, Pan, Thuluvath, Pardoll, Schulick, Yoshimura, and Weber. *Statistical analysis:* Olino. *Obtained funding:* Olino, Edil, Schulick, and Weber. *Administrative, technical, and material support:* Meckel, Pan, Pardoll, Schulick, Yoshimura, and Weber. *Study supervision:* Olino, Schulick, Yoshimura, and Weber.

cells share receptor structures and functions of classic T cells and natural killer cells.^{2,3} In previous studies, NKT cells appeared to play a dual role in tumor immunity because they were shown to promote^{4–7} as well as suppress^{8–11} tumor immunosurveillance through regulatory cytokines. Natural killer T cells have the potential to produce the T-helper (Th) cell type 1 cytokine, interferon γ , Th2 cytokines, and interleukin (IL) 4 and 10. It has been shown that Th2 cytokines downregulate antitumor immunity. Interleukin 13 signals through the IL-4R-STAT6 pathway and is known to be released by NKT cells.⁹

Recently, a novel model involving subsets of NKT cells with opposing functions has been proposed.¹² These subsets can be selectively stimulated or inhibited by specific glycolipid antigens such as α -galactosylceramide (α -GalCer) and sulfatide.^{13,14} α -Galactosylceramide is a strong agonist of type I NKT cells and induces a preferential release of Th1 cytokines, whereas sulfatide has been reported to selectively stimulate non- α -GalCer-reactive type II NKT cells.^{12–14} In the present study, we hypothesize that both α -Gal-Cer and sulfatide would modulate the survival of tumor-bearing mice in combination with LMD.

METHODS

ANIMALS AND TUMOR CELL LINES

BALB/c mice (8–10 weeks old, female) were purchased from the National Cancer Institute and treated in accordance with the Johns Hopkins Animal Care and Use Committee. Colon tumor 26 cells were maintained as previously described.¹

LIVE AND ATTENUATED LISTERIA MONOCYTOGENES

Listeria monocytogenes, courtesy of Anza Therapeutics, is the actA-deleted and IntB-deleted strain. Bacteria were grown and maintained as previously described.¹ For injection, bacteria were diluted in 100 μ L of phosphate-buffered saline at $0.1 \times$ median lethal dose in BALB/c mice.¹⁵

TREATMENT OF MICE WITH LMD AND GLYCOLIPID ANTIGENS IN THE HEPATIC METASTASIS MODEL

Mice were given isolated hepatic metastases using a hemispleen injection technique.^{16,17} Mice that received LMD treatment received intraperitoneal injections of $0.1 \times$ median lethal dose (1×10^7 colony-forming units) on days 3, 6, and 9 after tumor challenge. Synthetic α -GalCer was dissolved in chloroform/methanol (2:1), dried overnight at room temperature, and stored at -80°C . For injection, α -GalCer was dissolved in 100% dimethyl sulfoxide, heated to 80°C for 1 minute, and further diluted in phosphate-buffered saline (1:200). Sulfatide was dissolved in 100% dimethyl sulfoxide, heated to 37°C for 10 minutes, and stored at -20°C . For the study, the stock solution was further diluted in phosphate-buffered saline (1:20). Finally, single or multiple injections of 1 or 4 μ g of α -GalCer or 25 μ g of sulfatide were performed intraperitoneally in a total volume of 200 μ L at different points after tumor challenge.

QUANTITATIVE POLYMERASE CHAIN REACTION

Natural killer T cells were stained with DX5 and CD3-specific monoclonal antibodies (BD Biosciences) and sorted by flow cytometry on days 7 and 10 after tumor challenge. Total cellular RNA was extracted from sorted cells using the RNeasy Mini Kit (Qiagen) and reverse transcribed. Specific sequences from β -actin housekeeping and *IL-4*, *IL-10*, and *IL-13* genes were amplified by quantitative polymerase chain reaction (Invitrogen). The cytokine gene expression was normalized by using housekeeping gene expression in the same sample as reference. Data are expressed as fluorescence units and reported as average of triplicate samples.¹⁷

STATISTICAL ANALYSES

Statistical analyses for differences in gene expression were done by Mann-Whitney *U* test and for survival experiments by log-rank test. $P < .05$ was considered statistically significant.

RESULTS

LMD ENHANCES IL-13 GENE EXPRESSION IN HEPATIC NKT CELLS

Neither tumor challenge nor LMD injection modified the gene expression of *IL-4* and *IL-10* in NKT cells isolated from the liver and spleen on the days under investigation. However, we found a trend toward enhanced *IL-13* gene expression in hepatic NKT cells early after LMD treatment and enhanced *IL-13* gene expression in hepatic NKT cells of untreated mice at later points after tumor challenge ($P = .12$, Table).

TREATMENT WITH α -GalCer IMPROVES SURVIVAL

We found that administration of 1 μ g of α -GalCer on day 1 after tumor challenge provided 87.5% survival (1 of 8 treated mice died on day 45) compared with 0% survival in a group of 9 mice that were not treated ($P < .001$). The impact of single applications of α -GalCer on survival decreased daily after tumor challenge, reaching 14.3% survival when applied on day 3 (Figure 1). This survival benefit was enhanced by multiple administrations on days 3, 6, and 9, although this difference did not reach statistical significance (57.1% vs 14.3%, Figure 1).

GLYCOLIPIDS ENHANCE THE ANTITUMOR ACTIVITY OF LMD

Given the enhanced *IL-13* gene expression in hepatic NKT cells early after LMD treatment and our survival results, we focused on early co-administration of glycolipids. We found a strong trend toward increased survival when a single dose of 1 μ g of α -GalCer or a single dose of 25 μ g of sulfatide on day 3 was added to LMD on days 3, 6, and 9 ($P = .07$ for LMD vs LMD α -GalCer, $P = .02$ for LMD vs LMD sulfatide; Figure 2).

COMMENT

Our study suggests that an inhibitory effect of NKT cells in the LMD-treated hepatic metastasis model is at least in part mediated by *IL-13* signaling as evident by a trend toward enhanced expression at early points after LMD treatment. However, NKT cells are known to constitutively express cytokine genes and maintain preformed messenger RNAs to rapidly

respond to antigenic stimulation.¹⁸ Hence, measuring the cytokine gene expression may not fully represent the level of active proteins. Our findings are in line with previous reports indicating that IL-13 plays a critical role in the downregulation of tumor immunosurveillance by NKT cells^{8,9} and further suggest that future studies on the clinical effects of IL-13 antagonists should focus on early points after LMD treatment.

The correlation between the expression of the invariant V α 14J α 18 T-cell receptor α -chain (type I vs type II NKT cells) and the Th1 vs Th2 cytokine pattern of NKT cells is controversial. Little is known about type II NKT cell physiologic function and its role in tumor immunity. We found that α -GalCer, a strong agonist of type I NKT cells that induces a preferential release of Th1 cytokines,¹⁴ improved survival and showed a strong trend toward enhancing the antitumor activity of LMD. However, the use of sulfatide, a selective stimulant of type II NKT cells,¹³ also showed a synergistic trend in combination with LMD. Further studies are necessary to characterize the role of functional NKT-cell subsets in our model, including other glycolipid antigens, as well as to better characterize the down-signaling effects of IL-13.

Acknowledgments

Funding/Support: Dr Olino's work was supported by grant T32 DK007713-14 from the National Institutes of Health. Dr Weber's work was supported by grant PBB5B-118840 from the Swiss National Science Foundation and a grant from the Novartis Foundation.

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Figure 1.

α-Galactosylceramide (α-GalCer) treatment improves survival. Survival of mice after tumor challenge on day 0 (1×10^5 colon tumor 26 cells) followed by either no treatment (NT), treatment with a single dose of 1 ug of α-GalCer on day 3 (α-GalCer d3), or 3 treatments of 1 ug of α-GalCer on days 3, 6, and 9 (α-GalCer d3, 6, 9; n = 5–7 per group). $P = .006$ for α-GalCer on day 3 vs no treatment. $P = .002$ for α-GalCer on days 3, 6, and 9 vs no treatment. P value is not significant for α-GalCer on days 3, 6, and 9 vs α-GalCer on day 3.

Figure 2.

α -Galactosylceramide (α -GalCer) and sulfatide enhance the impact of *Listeria monocytogenes* (LMD) on survival. Survival of mice after tumor challenge on day 0 (1×10^5 colon tumor 26 cells) followed by either no treatment (NT), 3 treatments of LMD on days 3, 6, and 9 (LMD), both LMD on days 3, 6, 9 and 1 μ g of α -GalCer on day 3 (LMD α -GalCer), or both LMD on days 3, 6, and 9 and 25 μ g of sulfatide on day 3 (LMD sulfatide; $n = 6-7$ per group). $P = .04$ for LMD vs no treatment. $P = .004$ for LMD α -GalCer vs no treatment. $P = .005$ for LMD sulfatide vs no treatment. $P = .07$ for LMD α -GalCer vs LMD. $P = .02$ for LMD sulfatide vs LMD. P value is not significant for LMD α -GalCer vs LMD sulfatide.

Table

Doubly Attenuated LMD Enhances IL-13 Gene Expression in Murine NKT Cells of the Liver Early After Treatment^{a,b}

	Day 7	Day 10
Naïve	1.28×10^{-8}	7.71×10^{-10}
LMD	7.96×10^{-4}	6.23×10^{-10}
No treatment (CT26 alone)	7.16×10^{-9}	1.59×10^{-3}
CT26 and LMD	1.60×10^{-3}	6.19×10^{-9}

Abbreviations: CT, colon tumor; IL, interleukin; LMD, *Listeria monocytogenes*; NKT, natural killer T.

^a Meanwhile, gene expression in NKT cells of untreated mice is increased at later points after tumor challenge.

^b Data are expressed as fluorescence units by using as reference β -actin expression and reported as average of triplicate samples.