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A *Listeria*-Derived Polypeptide Promotes In Vivo Activation of NK Cells for Antitumor Therapy

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ABSTRACT

Immunotherapies have shown promise in the treatment of cancer, but more potent and targeted therapies are needed. NK cells are lymphocytes with an innate ability to recognize and lyse tumor cells. When activated, they also produce type II IFN-γ to orchestrate the activity of other immune cells. Strategies to elicit NK cell activation in vivo have potential usefulness in antitumor immunotherapies. In this study, we report on a strategy to stimulate NK cell activation and antitumor activity in mice with established B16.F10 murine melanomas. We and others previously observed that NK cells are rapidly activated during infection by pathogens such as the bacterium *Listeria monocytogenes*. A secreted *L. monocytogenes* virulence protein, p60, and a fragment of p60 termed L1S were previously shown to stimulate innate immune responses and promote NK cell activation. We purified recombinant L1S and characterized its activity in cell culture studies. Recombinant L1S protein was also observed to promote accumulation and robust NK cell activation in the lungs when given via intratracheal instillation to control and tumor-bearing mice. Importantly, therapeutic administration of a single L1S dose was found to significantly reduce the number and area of metastatic tumor nodules on the lungs of mice with established B16.F10 murine melanomas. Depletion studies showed that these antitumor effects were dependent on NK cells and IFN-γ. These data provide proof of concept that administration of a single immune-modulating microbial polypeptide can be used to therapeutically boost NK cell in vivo activation and promote antitumor responses.


INTRODUCTION

Tumor cells that metastasize and establish in distal tissues are difficult or impossible to locate and resect. Hence metastatic tumors are the primary cause of cancer-related deaths (1). Immune cells can hunt and kill individual tumor cells. Strategies to boost the antitumor activity of immune cells thus have potential use in the treatment of metastatic and hematologic cancers. Melanoma is a type of skin cancer that frequently metastasizes to the lungs, liver, bones, and brain (2, 3). Metastatic melanomas cause over 10,000 deaths annually in the United States (4). A subset of human patients with metastatic melanomas has been found to respond well to treatment with T cell–activating checkpoint inhibitor immunotherapies, and such therapies have also been effective in the commonly used murine B16.F10 melanoma model (5, 6). However, these current T cell–based therapies are not completely effective. Hence, there remains a need for additional or improved immunotherapeutic approaches to treat metastatic melanoma and other cancers.

Boosting of innate antitumor immune responses could in theory be used synergistically to supplement or improve antitumor immunotherapies based on stimulation of adaptive immunity. NK cells are an innate immune cell type that is a promising target for development of such immunotherapies. The presence of IFN-γ–producing NK cells in tumor tissue correlates with improved prognosis in both murine models and clinical studies.
of human patients (7–10). When appropriately primed and activated, NK cells recognize tumor cells and can induce cytolysis to directly kill the tumor cells in the absence of specific tumor Ags. Activated NK cells also produce cytokines such as IFN-γ that can regulate other innate and adaptive immune cells. The ability of NK cells to mediate killing and cytokine production is regulated by the presence of activating and inhibitory cell surface receptors on the tumor cell, as well as by cytokines and other priming signals provided by dendritic cells (DC) or other accessory cells. Priming of NK cells increases their cytolytic activity and ability to produce immunostimulating cytokines such as IFN-γ (11). Cytokines that are important for NK cell priming and activation include IL-18, IL-1β, IL-15, and IL-12 (7, 12–16). Previous work has shown that administration of specific NK cell–activating cytokines such as IL-2, IL-12, and IL-15, or a combination of IL-2, IFN-α, and GM-CSF can promote NK cell responses in cancer patients (17). However, alternative or additional approaches may more specifically boost NK cell activity. The availability of multiple methods to prime or boost the activity of NK cells in vivo could also facilitate the development of more effective combination immunotherapies or therapies involving sequential activation of patient NK cells.

Infections by a variety of bacterial and viral pathogens potently elicit NK cell activation and IFN-γ secretion (15). Triggering of NK cell activation by pathogens has also been associated with antitumor activity (18). Listeria monocytogenes is a Gram-positive bacterial pathogen known to potently stimulate NK cell activity in infected mice (19–21). A secreted L. monocytogenes virulence protein, p60, contributes to this effect, promoting NK cell activation and IFN-γ secretion both in the context of L. monocytogenes infection and, importantly, as a recombinant protein in absence of intact L. monocytogenes (18, 19). The ability of recombinant p60 to promote NK cell activity maps to a fragment termed LIS (18). In cell culture models, intact p60 protein or the LIS region indirectly stimulate activation of murine and human NK cells to secrete IFN-γ due to their effects on DCs (18, 20).

Given its stimulatory effects in vitro, we reasoned that recombinant LIS might also be useful to stimulate beneficial NK cell activity in disease settings. In this study, we provide proof of concept that the recombinant LIS protein can be used to promote activation of lung NK cells in mice in a therapeutic setting. Administration of LIS protein to lungs of mice with established B16.F10 murine melanoma tumors triggered significant NK cell– and IFN-γ–dependent reductions in tumor burden. Hence, LIS has potential use in promoting NK cell activity in the context of anticancer immunotherapies.

MATERIALS AND METHODS

Preparation of LIS protein

LIS protein was expressed in Escherichia coli with a pTrcHis (Invitrogen) construct and purified as previously described (18). Briefly, log phase bacteria were incubated 8 h with isopropyl β-D-thiogalactoside to induce protein expression. Following cell lysis and DNase treatment, lysates were centrifuged to pellet cellular debris. Supernatants were filtered in a 0.22 μm syringe filter and loaded on to an AKTA fast protein liquid chromatography machine for Ni column affinity purification of the 6 × His-tagged protein. Aliquots of fractions eluted from the column were evaluated using SDS-PAGE and Coomassie staining. In some experiments, polymyxin B columns were used to further purify LIS protein. Fractions containing LIS protein were concentrated using Millipore 10 kDa columns and protein concentrations were determined using bicinchoninic acid.

Mice

C57BL/6J (B6) and B6.tlr4−/− mice were obtained from the Jackson Laboratory and maintained at the National Jewish Health and the University of Colorado Anschutz Medical Campus. Mice were used at 8–12 wk of age. Mice were anesthetized for intratracheal (i.t.) instillations by i.p. inoculation with 150 μl containing 80–100 mg/kg ketamine and 5–10 mg/kg of xylazine. A heat lamp was used to maintain body temperature until this point. To sacrifice mice, 500 μl Fatal Plus (pentobarbital) was given i.p. and mice were exsanguinated via cardiac stick. For depletion experiments, purified endotoxin-free Abs were purchased from Bio X Cell. Clones used were: anti-NK1.1 clone PK136, anti-Ly6G clone 1A8, and anti–IFN-γ clone XMG1.2. All depletion Abs were given at 500 μg per mouse in a 200 μl i.p. injection.

Lung digestion and flow cytometry

Lungs were perfused with 10 ml of ice cold PBS through the heart. One lobe was taken and frozen for lysate ELISA. Remaining lobes were separated and chopped utilizing surgical scissors. Lungs were digested in 1 mg/ml of Collagenase IV (Worthington) or 1 mg/ml of Collagenase VIII (Sigma) for 30 min at 37°C. To stop the reaction, 60 μl of 5 mM EDTA was added to each organ. Digested organs were brought into single-cell suspensions by processing through an 18G needle three to five times, then passed through at 100 μm cell strainer twice. A total of 2 × 106 cells were obtained and plated in RPMI 1640 complete medium with GolgiPlug, Brefeldin A, protein transport inhibitor (eBioscience) at 1:1000 for 3 h. Following treatment with protein transport inhibitor, the cells were treated with FC block for 30 min. Surface staining used the following Abs and was performed for 30 min in FACS buffer at 4°C: Ly6G-A18 (PerCP-Cy5.5; BioLegend), NK1.1-PK136 (PerCP-Cy5.5; eBioscience), CD3-17A2 (BV510; BioLegend), and NKp46-29A1.4 (Pe-Cy7; eBioscience). For intracellular staining, cells were treated with a PFA-saponin mixture for 15 min at room temperature to fix the cells and prepare them for intracellular staining. Cells were then stained for intracellular cytokines in PBS-saponin for 45 min at room temperature using the following Abs: IFN-γ–XMG1.2 (APC; eBioscience). Samples were run on LSRII and LSR Fortessa (BD Biosciences) and FACS analysis was done through FlowJo Software.
Isolation and growth of bone marrow DCs
Bone marrow was extracted from the hind-leg bones of mice. Bone marrow was suspended and plated in 300 μl of RPMI 1640 complete medium containing GM-CSF. Cells were grown over 7 d and new media was added on days 2 and 4. On day 7, nonadherent cells were collected and plated in 300 μl of RPMI 1640 complete medium in 24-well plates at 3 × 10⁵ per well overnight. On day 8 the medium was changed and experiments were conducted.

Cell culture and coculture experiments
Bone marrow–derived DCs (BMDCs) were treated with 10 μg per well (33.3 μg/ml) of protein following priming with PBS (none), IL-12 (2 ng/ml), polynosinic:polycytidylic acid (20 μg/ml), or LPS (10 ng/ml). Supernatants were collected 24 h after treatment and tested for IL-1β or IL-18 via ELISA. Nylon-wool nonadherent cells (NWNA) or purified NK cells were isolated for coculture experiments and added at 3 × 10⁴ cells per well. Then 24 h later supernatants were collected and tested for IFN-γ via ELISA. BD-OptEIA kits were used to quantify IL-18. Numbers were calculated by clicker-counter with the edges being counted on only one side. To estimate the total tumor area, images were taken of the lung surface and analyzed using the FIJI package of ImageJ. To accommodate differences in the size of various lung images, tumor area measurements for each lung were normalized to the total lung surface area within each image.

Isolation of NWNA
Sterile nylon wool (0.5 g) was inserted into 20 ml syringe columns. A three-way stopcock was attached to the column and a 20 ml syringe containing RPMI 1640 complete medium. The nylon wool was primed with 20 ml of RPMI 1640 medium and passed at 37°C. Whole spleens were isolated and digested into a single-cell suspension using 70-μm nylon sheets. RBCs were lysed using ACK RBC lysis buffer and resuspended in RPMI medium. Splenic cells were placed on nylon wool and incubated for 1 h at 37°C to remove adherent myeloid and B cells.

B16.F10 culture
B16.F10 murine melanoma cells were obtained from American Type Culture Collection (CRL-6475). B16.F10/RFP murine melanoma cells were a gift from D. Matthew and R. Torres (University of Colorado). Cells were maintained in culture with DMEM as described (22, 23). Cells were sequentially passaged at 80–90% confluency. Cells thawed from frozen aliquots were passaged five to eight times prior to harvest at <50% confluence for use in vivo. Cells were reconstituted into cold PBS prior to i.v. injection.

Lung immune activation model
Mice were given i.t. inoculations of 125 μg of protein, 125 ng of LPS, or PBS in 50 μl vol. The mice were sacrificed 24, 48, or 96 h to analyze immune parameters. Lungs were prepared for single-cell suspension and flow cytometric analysis as above.

Assay for tumor cell lysis
To isolate effector NK cells, mice were given i.t. inoculations of LIS protein or PBS as above and sacrificed 24 h later. Lungs were perfused with 10 ml of ice cold PBS through the heart. The lobes were separated and chopped utilizing surgical scissors. Lungs were digested in 1 ml of collagenase/hyaluronidase mixture (catalog number 07912; STEMCELL), DNase at 10 μg/ml, and 1 ml of RPMI 1640 complete media for 45 min at 37°C, one milligram per milliliter of Collagenase IV (Worthington), or 1 mg/ml of Collagenase VIII (Sigma) for 30 min at 37°C. To stop the reaction 60 μl of 5 mM EDTA was added. Digested lungs were brought into a single-cell suspension by processing through an 18G needle three to five times and then passed through a 100 μm cell strainer twice. NK cells were then isolated utilizing STEMCELL EasySep Mouse NK cell isolation kit (catalog number 19855). Purified lung NK cells were added at the indicated effector:target ratios to B16.F10/RFP target cells that had been plated the day before. After a 4 h coculture, cells were lifted, stained for NK1.1, and analyzed by flow cytometry. A known quantity of fluorescent beads was spiked into the samples to facilitate accurate counting of total cell numbers (CountBright absolute counting beads number C36950). Live single cells were gated and the number of RFP+ NK1.1 cells per 1000 beads was determined. Specific lysis was quantified by calculating the reduction in number of tumor cells following the culture with NK cells: % specific lysis = 100 × [(number of target cells in control) – (number of target cells with NK cells)/(number of target cells in control)].

Lung metastasis model
Mice were injected with 2–6 × 10⁵ B16.F10 cells via the tail vein. After 8 d, tumor-inoculated mice were randomized into groups and given i.t. inoculations with 50 μl of PBS with or without 125 μg LPS protein or 125 ng LPS. The mice were sacrificed after 24, 48, or 96 h to analyze tumor burden and immune parameters. Lungs were inflated with PBS and removed from the animal. Lungs were separated and imaged on both sides. Each black lung nodule was counted by clicker-counter with the edges being counted on only one side. To estimate the total tumor area, images were taken of the lung surface and analyzed using the FIJI package of ImageJ. To accommodate differences in the size of various lung images, tumor area measurements for each lung were normalized to the total lung surface area within each image.

Peritoneal tumor model
Mice were injected i.p. with 1 × 10⁵ B16.F10 cells on day 0. After 5 d, mice were given a single i.p. injection of 200 μl PBS ± 0.5 mg LIS protein. Mice were sacrificed for analysis on day 10 (5 d after treatment). Visible tumor masses from each mouse were dissected from the peritoneum and weighed to determine overall tumor burden.

Data analysis and statistics
Data was processed using GraphPad Prism Version 5.0b. One-way ANOVA analysis was conducted utilizing Newman–Keuls posttest to calculate the p value. Significant differences are indicated with asterisks (*) when p < 0.05.

Ethics statement
The Animal Care and Use Committees for National Jewish Health (Protocol AS2682-08-16) and the University of Colorado School of
RESULTS

**Purified recombinant LIS protein can be used to promote NK cell activation**

Our previous results using cell coculture assays support the model that p60 or its LIS fragment indirectly promote NK cell activation (Fig. 1A). LIS and p60 stimulate inflammasome-dependent production of IL-18, which is required for the NK cell activation in these cultures, as is cell-contact between BMDCs and NK cells (18, 24). As for other NLRP3-inflammasome activators, the ability of LIS to induce IL-1β and IL-18 processing and secretion requires priming of cultured BMDCs using TLR agonists or cytokines.

To study the potential for therapeutic use of LIS, we first expressed His-tagged recombinant LIS protein in E. coli and purified the protein using Ni-affinity chromatography. The protein was free of contaminants as judged by SDS-PAGE (Fig. 1B). However, in some preparations limulus assays detected trace amounts of LPS (<10 ng/µg). Consistent with the presence of this priming agent, LIS purified using single-step Ni-affinity chromatography–stimulated secretion of IL-1β and IL-18 by unprimed BMDCs (Supplemental Fig. 1). LIS purified in this way also promoted secretion of IFN-γ in cocultures comprised of NWN1A splenic lymphocytes and unprimed BMDCs (Fig. 1C). Activated NK cells are responsible for the IFN-γ production in these cocultures (18, 25). When detoxified LIS (dLIS) was prepared by a second purification step on polymyxin B columns, the protein failed to stimulate NK cell IFN-γ secretion in the cell cocultures unless the BMDCs were first primed with IL-12 or LPS (Fig. 1C). These priming agents did not induce IFN-γ production in the absence of LIS protein (Fig. 1C), nor was heat-inactivated LIS capable of inducing IFN-γ secretion in these cocultures (Fig. 1D). These data are consistent with our previous results and confirm that preparations of purified LIS exert protein-dependent immune stimulatory activity.

We next sought to evaluate the ability of purified LIS to stimulate NK cell activity in vivo. To this end, Ni-affinity purified protein was instilled directly into the lungs of naive B6 mice using an established i.t. inoculation method (26). Lungs were harvested 24 h later and digested to prepare single-cell populations for flow cytometric analysis. Compared to mice given an equal volume of PBS ± 125 ng LPS, the proportion and number of lung NKL1.1 CD3− NK cells staining positive for IFN-γ− was significantly increased in mice treated with 125 µg of purified LIS protein (Fig. 1E). The frequency of lung NK cells activated to produce IFN-γ was proportional to the dose of LIS given (Supplemental Fig. 1), and we primarily observed responding NK cells in the site inoculated with LIS (lung or peritoneum) at the doses used. Interestingly, lung NK cell activation was also seen to occur in B6.1tlr4−/− mice (Fig. 1F), which lack the LPS receptor TLR4. Furthermore, dLIS protein induced NK cell activation and IFN-γ production when instilled into the lungs (Fig. 1G). These data demonstrate that LIS protein itself is responsible for the NK cell activation. The absence of a priming agent requirement to induce NK cell activation in the lungs may be due to in situ priming of DCs by commensal lung bacteria. Taken together, these results demonstrate that purified LIS protein can be administered to naive mice to elicit activation of lung NK cells in vivo.

**LIS treatment induces a limited and transient inflammatory response in the lungs**

The kinetics and extent of the inflammatory response elicited by LIS treatment was further evaluated. A modest and transient, but significant, increase in the total number of NK cells recovered from the lungs was observed at 24 h after LIS treatment (Fig. 2A) It was not determined if this increase resulted from the recruitment of new NK cells or proliferation of lung-resident NK cells. The proportion of lung NK cells producing IFN-γ increased substantially after LIS treatment, peaking at 24 h after treatment and returning to basal levels by 96 h (Fig. 2B). LIS instillation was also associated with a significant and transient increase in the number of neutrophils in the lungs (Fig. 2C). The kinetics of neutrophil accumulation correlated with those of NK cell IFN-γ production, suggesting a generalized inflammatory response following LIS treatment. Consistent with this interpretation, B and T cell numbers were also modestly increased at 24 h after LIS installation (Fig. 2D, 2E). These data demonstrated the ability of LIS treatment to stimulate a rapid and transient accumulation and activation of NK cells in the lungs, a response that was further associated with the accumulation of other immune cells.

To evaluate the efficacy of LIS in stimulating NK cell activation in tumor-bearing mice, we instilled the protein i.t. into animals with established metastatic melanomas. Mice received a single dosing of LIS 8 d after they received an i.v. inoculation with B16.F10 melanoma cells. As in the naive mice above, the total number of NK cells was significantly increased in the lungs of tumor-bearing mice 24 h after LIS treatment (Fig. 3A). The number of total lung NK cells returned to baseline by 96 h after LIS treatment (Fig. 3A). Intracellular staining for IFN-γ showed that the number of NK cells actively secreting IFN-γ was also transiently increased after LIS treatment in the tumor-bearing mice (Fig. 3B). Neutrophils also transiently accumulated in the lungs of tumor-bearing mice following the LIS stimulation (Fig. 3C). These data showed that the presence of established melanoma tumor nodules did not interfere with the ability of i.t. LIS treatment to trigger transient inflammation and NK cell activation in the lungs.

**LIS instillation is therapeutic against metastatic disease**

To evaluate if tumor burdens were reduced by the elicited inflammatory and NK cell response, mice were treated with LIS on day 8 and harvested for analysis 4 d later (Fig. 4A). Upon harvest, the lungs from mice treated with LIS were clearly distinguished by

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a reduced abundance of dark melanoma nodules (Fig. 4B). Blinded quantification of these visible nodules confirmed there was a significant reduction in their numbers in the L1S-treated mice (Fig. 4C). The average size of the tumor nodules also appeared to be reduced in the L1S-treated group (see Fig. 4B). To quantify this parameter, we used image analysis methods to estimate
FIGURE 2. Kinetics of inflammatory cell accumulation and activation in lungs of naive mice treated with L1S.

As above, mice received a single i.t. instillation with PBS ± L1S protein or LPS. At the indicated times, lungs were harvested and single-cell suspensions prepared for flow cytometry analysis. (A) NK cell (NK1.1+CD3−) number per lung. (B) Proportion of activated NK cells (NK1.1+CD3−) as measured by intracellular staining for IFN-γ/NK1.1+CD3−. (C) Neutrophil (Ly6G+CD11b+) numbers per lung. (D) B cell numbers per lung (B220+CD3−). (E) T cell (CD3+CD20−) numbers per lung. (F) Proportion of T cells (CD3+CD20−) staining positive for intracellular IFN-γ at indicated time. Each data point represents data pooled from at least three experiments with at least three mice per experiment. Cells are gated on live → singlets → respective group(s) of interest. Error bars indicate SEM. *p < 0.05 using ANOVA with Newman–Keuls comparison.

The proportion of the lung surface area covered by tumors (Supplemental Fig. 2). Results confirmed the ability of L1S treatment to significantly reduce lung tumor burden (Fig. 4D). NK cells isolated from lungs 24 h after L1S treatment were also found to have an increased ability to lyse B16.F10 tumor cells using a clone expressing RFP (Fig. 4E). A separate series of experiments showed that i.p. treatment with L1S could also reduce the overall mass of melanoma recovered from mice with established i.p. B16.F10 tumors (Supplemental Fig. 3). These results together argue that the inflammatory response and/or NK cell activation triggered by a single L1S treatment was sufficient to significantly reduce the masses of metastatic and local B16.F10 melanomas in the lungs and peritoneum of mice with previously established tumors.

Therapeutic effects of L1S treatment require NK cells and IFN-γ

To further investigate the mechanism responsible for the therapeutic effects of L1S treatment, mice with established tumors were depleted of various immune effectors. Tumors were established by i.v. inoculation as above. On day 7 after the tumor inoculation, mice were given single i.p. inoculations of PBS ± control IgG, α-NK1.1 (clone PK136), or α-Ly6G (clone 1A8) to deplete NK cells or neutrophils, respectively. Flow cytometric analysis confirmed that lung NK cells and neutrophils were depleted effectively in the respective mice (Fig. 5A, 5B). The day following depletion (day 8), mice received a single L1S treatment, then tumor burdens in the lungs were quantified on day 12. Results showed that L1S significantly reduced tumor burdens in mice treated with L1S + IgG control Ab, relative to control mice given IgG but not L1S (Fig. 5C, 5D). However, L1S failed to reduce tumor nodules when given to mice depleted of NK cells prior to treatment (Fig. 5C, 5D, α-NK1.1). Subsequent experiments further showed that L1S treatment was ineffective at reducing tumor burdens in mice pretreated with α-IFN-γ (Fig. 5E, 5F). By contrast, tumor burdens were similar in mice treated with L1S and IgG or α-Ly6G (to deplete neutrophils) (Fig. 5E, 5F). These results together indicate that NK cells and IFN-γ are required for the antitumor effects of L1S, whereas neutrophils are not. Thus, the inflammatory response elicited by L1S was not sufficient to mediate antitumor effects. These therapeutic antitumor effects instead appear to be due to the accumulation of NK cells and their production of IFN-γ.

DISCUSSION

As basic understanding of the immune system has increased, so has the ability of researchers and clinicians to identify and apply therapeutic approaches that harness the body’s immune response. Such efforts have the potential to improve the treatment of infectious, neoplastic diseases and others. In this study, we investigated a novel approach to promote antitumor immune responses. This approach exploits a pathogen-derived immune modulating factor to boost NK cell responses in animals with established metastatic tumors. As shown in this study, highly purified preparations of this factor (a recombinant polypeptide containing the L1S region from the L. monocytogenes p60 virulence protein) stimulate secretion of IL-18 by primed BMDC and IFN-γ production by NK cell/BMDC cocultures. We further showed that local instillation of the L1S protein into the lungs promoted activation of lung NK cells to secrete IFN-γ in both naive mice and mice with established metastatic tumors. The model tumor used in these experiments was the NK cell–sensitive B16.F10 melanoma (27, 28). Consistent with the interpretation that NK cell activation exerts antitumor effects in treated mice, we...
observed significant reductions in the number and size of tumors in L1S-treated mice. NK cells isolated from lungs of L1S-treated mice also showed an increased ability to lyse RFP-expressing B16.F10 tumor cells. Therapeutic effects were absent in mice given L1S protein after depletion of NK cells or IFN-γ, but not Ly6G⁺ neutrophils. Hence, our data suggest L1S can be used in vivo to boost the activation of tumor-reactive NK cells.

This study was founded on prior work showing expression of the p60 virulence protein by L. monocytogenes is associated with increased NK cell activation (19). Expression of p60 by L. monocytogenes was also shown to boost activation and IFN-γ secretion by NK cells cocultured with infected BMDC (18, 20, 25). The ability to promote NK cell activation appears to benefit L. monocytogenes because IFN-γ secreting NK cells responding to p60/LIS rapidly switch to secretion of IL-10 (20). IL-10 has been argued both to promote antitumor resistance in the B16.F10 model (29, 30), and to promote growth of B16.F10 melanoma cells (31, 32). However, given our finding that IFN-γ is important for the observed antitumor effects, we did not further explore NK cell IL-10 production in the present work.

Our depletion experiments indicated that NK1.1⁺ cells and IFN-γ are important for the antitumor effects of L1S therapy. Accumulation of neutrophils (Ly6G⁺CD11b⁺) was also seen after LIS instillation, and neutrophils have previously been observed to associate with tumors (33–35). However, depletion of Ly6G⁺ cells did not impact LIS therapeutic efficacy. Like NK cells, NKT cells express the NK1.1 marker. Because we did not observe increased IFN-γ production or numbers of NKT cells in LIS-treated lungs (data not shown), the failure of mice treated with αNK1.1 to reduce tumor burdens in the presence of LIS is likely due to the depletion of NK, not NKT, cells. NK cells were first described as cytolytic innate effector cells in 1975 (36). The ability of NK cells to recognize and lyse THP-1 and YAC-1 tumor cells is also known to be induced or enhanced by exposure to cytokines such as TNF and IFN-γ, which NK cells themselves produce (37–39). These cytokines increase target-cell expression of NK cell activating or adhesion ligands, such as ICAM1 (38). IFN-γ can also act on NK cells to increase their cytolytic antitumor activity by promoting expression of granule proteins and death receptor ligands such as TRAIL (40). Increased expression of the IFN-γ-inducible activation marker CD69 has also been associated with increased NK cell cytolysis (41, 42), and we observed increased CD69 expression on NK cells from LIS-treated lungs (data not shown). IFN-γ can also directly promote apoptosis of tumor cells (43), and can enhance M1 differentiation and thus tumoricidal activity of tumor associated monocytes and macrophages. Future studies will be necessary to better define which of these mechanisms is most important for the LIS-induced antitumor effects.

In addition to mouse DC and NK cells, recombinant LIS protein stimulates human DC and NK cells in cell culture models (20). Activation in these cell cultures requires the presence of contaminating or exogenous priming agents in addition to purified recombinant LIS protein itself. The requirement for a priming agent could in theory limit the efficacy or safety of LIS in human anticancer therapies. However, we previously found that Food and Drug Administration–approved agents such as monophosphoryl lipid A suffice for priming in cell culture (18). We also show in this study that i.t. treatment with dLIS stimulated NK cell activation and secretion of IFN-γ. This likely reflects the presence of endogenous priming agents within the lung microenvironment. Priming could be due to microbes or microbial products present in the air or commensal microbes in the lung or other mucosal tissues (44, 45). Evidence that lung DC are constitutively primed comes from a study by Ichinohe et al. (46), who found that depletion of microbiota via the use of an antibiotic mixture lead to reductions in lung responses to inflammatory stimuli. Providing exogenous LPS to the antibiotic-treated mice was found to restore their responsiveness to inflammatory stimuli. Further studies will be needed to directly test if the observed responses to dLIS are

FIGURE 3. Kinetics of inflammatory cell accumulation and activation in lungs of tumor-bearing mice treated with L1S.

Mice were inoculated i.v. with B16.F10 melanoma cells. After 8 d, they received a single i.t. instillation with PBS ± L1S protein or LPS as above. Mice were sacrificed and lungs digested for flow cytometry analysis of total NK cells (NK1.1⁺CD3⁻) at (A) 24 and 96 h after L1S treatment. Intracellular staining for IFN-γ was also used to determine proportion of activated NK cells (NK1.1⁺CD3⁻) (B) 24 and 96 h after the L1S treatment. (C) Total neutrophils (Ly6G⁺CD11b⁺) in the lung were analyzed at 24 and 96 h after L1S treatment. Each graphed bar represents data pooled from at least three experiments with at least three mice per experiment. Cells are gated on live → singlets → respective group(s) of interest. Error bars indicate SEM. *p < 0.05 using ANOVA with Newman–Keuls comparison.
dependent on constitutive priming of lung DCs and suffice to induce antitumor responses.

Efforts to better understand immune regulation have led to the development and application of several novel immune-based therapies that show great potential in treatment of cancers (5, 6). These existing immunotherapies focus on activation of T cell responses in cancer patients. Unlike T cells, which recognize specific tumor Ags, NK cells recognize and lyse tumor cell targets independent of specific Ags, including tumor cells that lack expression of the MHC molecules. Therapies that promote NK cell activation might thus be useful in combination with T cell-stimulating immunotherapies. Our results in this study demonstrate use of a bacteria-derived polypeptide to stimulate NK cell activation and antitumor responses in vivo. Future efforts to further improve our understanding of how L1S promotes NK cell activation and antitumor responses could thus reveal new approaches to boost immune responses for therapy of cancer.
DISCLOSURES

The authors have no financial conflicts of interest.

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Supplementary Figure 1. Effects of L1S protein on cytokine secretion by cultured BMDCs and titration of L1S in vivo. BMDCs were cultured from B6 mice and treated with 30 µg/ml recombinant L1S. Supernatants were harvested at 24 h to quantify (A) IL-1β and (B) IL-18 using commercial ELISAs. (C) Titrated doses of L1S were given to intratracheally (i.t.) to groups of three B6 mice. Mice were harvested 24h later and lung single-cell suspensions stained to evaluate intracellular IFNγ production in gated CD3-NK1.1+ NK cells. In all cases, data depict mean± SEM for at least 3 samples per bar. (*) indicates p < 0.05 using ANOVA with Newman-Keuls comparison.
Supplemental Figure 2. Sample images and formula for normalization of tumor areas. Images of lungs were taken at harvest. Similar lung surfaces were imaged for each mouse. Images were analyzed using IFiji (imageJ) to calculate (A) total lung area as outlined in yellow and (B) Area of each tumor as outlined in yellow. (C) To normalize for differences in total measured lung surface area due to exclusion of shadowed areas, the indicated equation was used to calculate % metastatic area.

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\text{Proportion of Lung Surface Containing Metastasis} = \frac{\text{Tumor Metastasis Area}}{\text{Total Lung Area}}
\]
Supplemental Figure 3. Effects of i.p. L1S treatment on tumor mass recovered from a B16.F10 melanoma peritoneal tumor model. Mice were given tumor cells i.p. on d0 and a single i.p. inoculation with 500 µg PBS ± L1S on d5. Mice were sacrificed for analysis on d10. (A) Experimental timeline. (B) Total tumor mass recovered from the peritoneum was determined by weighing on a scale. The mean ± SEM of measurements from individual mice are plotted. Data are pooled from at least 3 experiments using 3 or more mice per experiment. (*) indicates p < 0.05 using t-test.