Activation of Immature Hepatic NK Cells As Immunotherapy for Liver Metastatic Disease

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Activation of Immature Hepatic NK Cells As Immunotherapy for Liver Metastatic Disease


NK cells can identify and eliminate emerging tumors due to altered expression of activating and inhibitory ligands on aberrant cells, a process that is greatly enhanced following NK cell activation. As a principal site of both tumor metastases and immature NK cells, the liver represents a unique anatomic location in which activation of the innate immune system could provide substantial therapeutic benefit. We describe here the NK cell-dependent destruction of a primary hepatic tumor following infection with an attenuated intracellular bacterium derived from *Listeria monocytogenes*. NK cell-mediated immunity correlated with the ordered migration and maturation of NK cells within the liver. Cytolytic activity was partially dependent on NKG2D-mediated tumor cell recognition, but surprisingly was still effective in the absence of type I IFN. Significantly, NK cell-mediated destruction of a primary hepatic tumor in infected mice led to long-lived CD4- and CD8 T cell-dependent tumor-specific adaptive immunity. These findings establish that activation and differentiation of immature NK cells using complex microbial stimuli can elicit potent anti-tumor activity within the liver, promote cross-presentation of tumor-derived Ags leading to long-lived systemic anti-tumor immunity, and suggests a paradigm for clinical intervention of liver metastatic carcinoma. The Journal of Immunology, 2007, 179: 7376–7384.

Natural killer cells are critical effectors of the innate immune system that provide an early host response to invading pathogenic organisms as well as tumor cells, and play a pivotal role in bridging the innate and adaptive arms of the immune response (1, 2). The contribution of NK cells to the innate immune response relies on their ability to recognize and kill infected or malignant cells, as well as to produce soluble factors that attract and activate other immune effectors. Activation of NK cells by cytokines enhances both their potency and their sensitivity to stimulatory receptors, promoting recognition and killing of target cells using an array of killing mechanisms (3). Target cell recognition involves interpretation of multiple stimulatory and inhibitory signals. However, due to numerous diverse pathogen- or host-derived (i.e., malignancy) mechanisms, NK cells may be unable to stem disease progression to chronic infection or cancer.

Although our understanding of NK cell differentiation and maturation has improved substantially, many aspects of this process remain poorly defined. NK cells are derived from a common lymphoid progenitor found within the bone marrow (4). Phenotypically immature NK cells, expressing NK1.1, CD122, and TRAIL, but not CD49b (DX5) can be found in high frequency in the mouse liver (5, 6). Following infection with murine CMV, immature NK cells undergo maturation as characterized by the stepwise acquisition of CD49b (DX5), CD11b, and CD43 (5, 7). The potential of this immature hepatic NK cell reservoir to expand and mature following other inflammatory stimuli is not well understood. Although immature TRAIL+ NK cells have been implicated to confer partial anti-metastatic function in the liver, the capacity of maturing NK cells to exert tumoricidal activity within their local microenvironment has not been fully explored (6, 8). Given its central role as an immune response regulator, targeting the activation of immature NK cells within the liver represents a compelling therapeutic strategy to stimulate host innate and, ultimately, adaptive host defense mechanisms.

Hepatic metastatic carcinoma represents one clinical indication where NK cell function likely impacts progression of disease. The liver is the most common site of metastasis in patients with colorectal cancer (CRC) (9, 10), and is a common site of metastasis for other gastrointestinal malignancies, such as pancreatic cancer. Nearly one-quarter of the 135,000 annually diagnosed cases of CRC will develop metastasis exclusively in the liver (11). Interestingly, it has been observed that CRC patients with diseased livers have a relatively low incidence of liver metastasis (12–17). There is a significantly decreased incidence of liver metastases among individuals with CRC having concurrent infection with hepatitis B or C viruses (19, 20). Okuno and colleagues (18) demonstrated that activation of the innate immune system with recombinant cytokines could prevent liver metastases. This finding suggests that activation of innate immune effectors within the liver might inhibit metastasis in people. Taken together, these observations suggest that innate immune activation within the liver can prolong survival by limiting progression of metastases.

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* Abbreviations used in this paper: CRC, colorectal cancer; LLO, cytolysin listeriolysin O; DC, dendritic cell; MST, median survival time.

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*Listeria monocytogenes* is a Gram-positive facultative intracellular pathogen known to induce robust innate and adaptive immunity (21–24). *L. monocytogenes* infects a broad range of cell types, although the liver and spleen represent the two predominant sites of bacterial replication in vivo (25). Once within the phagocytic vacuole, the pore forming cytolsin listeriolysin O (LLO) mediates bacterial escape into the host cell cytosol (26). Escape from the vacuole triggers a cascade of proinflammatory cytokines and chemokines, including IFN-α/β, MCP-1, IL-12p70, IL-18, and IFN-γ (27, 28). Innate immune activation, particularly production of IFN-γ, is necessary for early control of *L. monocytogenes* replication (23, 29). We previously described a strain of *L. monocytogenes*, *Lm* ΔactAΔinlB that is 1000-fold attenuated in mice compared with wild-type *L. monocytogenes*, due to deletion of the bacterial genes encoding internalin B (InlB) and ActA, which together promote infection of hepatocytes via the hepatocyte growth factor receptor and cell-to-cell spread within the liver (24). This attenuated strain retains the ability to stimulate potent innate and adaptive immunity, while exhibiting a safety profile compatible with use in a clinical setting.

In this report, we describe the therapeutic potential of liver-resident immature NK cells for the treatment of metastatic disease following infection with a live-attenuated intracellular bacterium. Our results demonstrate that treatment of mice bearing hepatic metastases with *Lm* ΔactAΔinlB confers NK cell-dependent antitumor immunity within the liver, and describe the host-pathogen interactions that mediate this effect. By determining the stepwise differentiation and population dynamics of NK cells within the liver and spleen following *Lm* ΔactAΔinlB infection, we show that both liver-resident immature and peripheral mature NK cells comprise the hepatic NK cell pool following infection. We demonstrate that this *Lm* ΔactAΔinlB-induced hepatic NK cell pool led to cross-presentation of relevant tumor rejection Ags, priming of a systemic tumor-specific adaptive T cell response, and long-term survival. Our findings establish the rationale for harnessing liver-resident immature NK cells to treat metastatic disease within the liver.

**Materials and Methods**

**Mice**

Female BALB/c and C57BL/6 mice 8–10 wk of age were purchased from Charles Rivers Laboratory. BALB/c:IFNARβ−/− mice were bred and maintained as previously described (30). All experiments involving mice followed protocols approved by the Animal Care and Use Committee of Cerus Corporation.

**Tumor cells**

CT26 (provided by Dr. Drew Pardoll) is an N-nitroso-N-methylurethane-induced murine colon adenocarcinoma cell line derived from BALB/c mice (31). Cells were maintained in culture in RPMI supplemented with 10% FBS and penicillin/streptomycin (50 U/ml). Liver-specific CT26 metastasis were established following the protocol described by Jain et al. (32).

**Bacteria and immunizations**

*Lm* ΔactAΔinlB is based on wild-type *L. monocytogenes* strain 10403s and is deleted of both actA and internalin B as previously described (24). The *Lm* ΔΔmy *L. monocytogenes* strain is unable to produce LLO, is unable to escape into the host cell cytosol, and is unable to grow in vivo (33). Heat-killed *Lm* ΔactAΔinlB was generated by heating bacterial suspensions at 70°C for 3 h with periodic mixing. Complete killing was verified by plating 1 × 1010 bacterial equivalents onto BHI agar. Bacteria were diluted in HBSS to the appropriate concentration (unless stated otherwise, 1 × 10⁷ CFU *Lm* ΔactAΔinlB, 3 × 10⁶ CFU *Lm* ΔΔmy, 3 × 10⁵ particles HK- *Lm* ΔactAΔinlB) and administered i.v. in a final volume of 200 μl. For tumor protection studies, vaccinations were given once a week for 3 wk starting 3 days after tumor implantation.

**Lymphocyte depletion**

BALB/c mice were injected i.p. with mAbs to CD4, CD8, or asialo-GM1 (depletes predominantly NK cells without depleting NK-T cells) (34) pre- and post-CT26 implantation to establish and maintain lymphocyte subset depletion during the treatment period. For GK1.5 and 2.43 (anti-CD4 and anti-CD8 Abs, respectively) mice were injected on days −12, −8, −5, −9, and 16 at 250 μg per dose. The anti-NK Ab (anti-asialo GM1) was injected on days −11, −8, −4, −9, and 16 at 100 μg per dose. Depletion was confirmed by flow cytometry 1 day before tumor cell implantation.

**Enumeration of CFU within spleen and liver**

Groups of 5 mice were immunized with 5 × 10⁷ CFU *Lm* ΔactAΔinlB, then sacrificed 12, 24, 48, or 72 h later and spleens and livers harvested. Tissues were homogenized in DPBS plus 0.1% Tween 20, followed by serial dilutions and plating on BHI agar.

**Processing of livers and spleens**

Liver leukocyte isolation was performed by dissociating the tissue through a 50 μm nylon filter, followed by separation of leukocytes and hepatocytes using 92.5% Percoll (GE Healthcare). Following centrifugation at 500 × g for 20 min, the leukocyte pellet was removed and erythrocytes lysed (ACl buffer; Sigma-Aldrich). Spleens were dissociated by needle dissection, followed by erythrocyte lysis, washing, and enumeration.

**Flow cytometry**

Abs were purchased from eBioscience unless noted: CD3 (145-2C11), CD4 (GK1.5), CD8 (53–6.7), CD11b (M1/70), CD19 (MB19-1), CD43 (S7; BD Biosciences), CD45 (30–F11), CD45.1 (A20), CD45.2 (104), CD49b (DX5), CD69 (H1.2F3), NK1.1 (PK136), IFN-γ (XMGl2.2), and NK22 (CX5). Pan-RAE-1 (186107) and H60 (205326) were obtained from R&D Systems. Intracellular staining of IFN-γ was performed directly ex vivo using the Cytofix/Cytoperm kit (BD Biosciences). Samples were acquired using the FACSCanto flow cytometer (BD Biosciences). Cyto- kines were quantified from mouse serum using the CBA mouse inflammation kit (BD Biosciences).

**Adaptive transfer and NK cell homing**

NK cells were enriched from the spleens of C57BL/6 mice by magnetic separation (NK cell isolation kit; Miltenyi Biotec). 15 × 10⁶ cells were transferred to naive B6.SJL-Ptprca Pepcb recipients (35). Mice were given 1 × 10⁸ CFU *Lm* ΔactAΔinlB 24 h later. Organs were harvested 24 h after infection and the frequency of donor to recipient NK cells determined by flow cytometry.

**In vitro killing assays**

Dilutions of hepatic and splenic leukocytes were added to 1 × 10⁷ 51Cr-loaded YAC-1 or CT26 targets in 96-well plates. Before coculture, selected groups were incubated with anti-NKG2D (clone 191004; R&D Systems). Following 4 h of incubation, supernatant was transferred to 96-well filter plates. Plates enumerated using a standard 96-well liquid scintillation counter (Trilux Microbeta; PerkinElmer).

**Statistical analyses**

Tumor survival was analyzed by one-way ANOVA and two-tailed Student’s t test for statistical significance.

**Results**

*Lm* ΔactAΔinlB induces NK cell-dependent anti-tumor responses in the CT26 hepatic metastases model

We hypothesized that in metastatic liver cancer, activation of the endogenous immature NK cell pool would facilitate their phenotypic and functional development, yielding a pool of armed cytolytic effectors at the site of the tumor. To evaluate the anti-tumor activity of *Lm* ΔactAΔinlB in hepatic tumor-bearing mice, BALB/c mice were implanted on day 0 with 1 × 10⁶ CT26 tumor cells via hemispleen surgery to establish liver metastasis and treated i.v. with *Lm* ΔactAΔinlB on days 3, 10, and 17 (32). A dose-dependent increase in median survival of tumor-bearing mice following *Lm* ΔactAΔinlB treatment was observed over a dose range of 3 × 10⁴ to 3 × 10⁷ CFU (Fig. 1A). All control mice implanted with CT26 tumor cells succumbed to tumor burden by day 27 with a median survival time of 7377 The Journal of Immunology.
FIGURE 1. NK-dependent anti-tumor activity following *Lm* ΔactΔinlB infection. Female BALB/c mice were implanted with 1 × 10^6 CT26 cells via hemispleen surgery. Mice bearing hepatic metastases were injected on days 3, 10, and 17 with the indicated dose of *Lm* ΔactΔinlB. A, Mice bearing 3-day hepatic tumor metastases were treated with the indicated dose of *Lm* ΔactΔinlB (q weekly × 3) and monitored for survival. B, Mice were left intact or depleted in vivo of CD4^+^ T cells, CD8^+^ cells, or NK cells before tumor implantation and maintained by additional injections at 6 and 13 days following implantation. Mice were treated with *Lm* ΔactΔinlB (3 × 10^5^ CFU) on days 3, 10, and 17 following implantation of intrahepatic tumor cells. Data is represented as percent survival (n = 10/group). C, BALB/c mice were immunized with 5 × 10^6^ CFU *Lm* ΔactΔinlB. Leukocytes were harvested from spleens and livers for analysis 48 h later: NK cell frequency and CD69 expression in *Lm* ΔactΔinlB-treated BALB/c mice; expression of NKG2D on CD49b^+^CD69^+^CD3^-^ NK cells from liver and spleen of BALB/c mice (open histogram) relative to autofluorescence controls (shaded histogram) (D); expression of RAE-1, H60, and MHC class I by CT26 and YAC-1 (open histogram) relative to autofluorescence controls (shaded histogram) (E); killing of *^5^Cr*-loaded CT26 and YAC-1 targets by splenic and hepatic effector cells, with and without an NKG2D blocking Ab (F).

(MST) of 23 days. In contrast, mice treated with *Lm* ΔactΔinlB survived significantly longer compared with vehicle alone. Even at the lowest tested bacterial dose, the MST was increased by 7 days, although all mice eventually succumbed to their tumor burden (p < 0.003). Higher doses of *Lm* ΔactΔinlB increased tumor-free survival with 20% of mice treated with 5 × 10^5^ CFU (MST 45 days) and 60% of mice treated with the highest dose (3 × 10^7^ CFU; MST undefined) surviving over 100 days post tumor implantation. *Lm* ΔactΔinlB administered at the optimal weekly 3-dose regimen (3 × 10^7^ CFU) provided therapeutic benefit and resulted in the long-term survival of greater than 100 days in 47% (81/174) of all treated mice in the CT26 liver metastases model (range 20–90% long-term survival, pooled data from 18 independent studies). Notably, unlike the protective effects observed in the liver metastatic model, *Lm* ΔactΔinlB did not provide a protective anti-tumor immune response in mice bearing CT26 lung metastases (data not shown), supporting the hypothesis that it is the immunologic milieu of the liver that facilitates anti-tumor immunity.

To identify the lymphocyte populations that were critical for mediating anti-tumor activity, mice were left intact or depleted of CD4^+^ cells, CD8α^+^ cells or asialo-GM1^+^ cells (predominantly depletes NK cells without depleting NK-T cells) (34) 1 wk before establishing intrahepatic metastases. Depletion of NK cells in untreated animals resulted in a slight acceleration in tumor growth (MST of 14 vs 21 days; p = 0.0226), in agreement with the well-described role of NK cells in tumor surveillance (Fig. 1B). Treatment with *Lm* ΔactΔinlB significantly increased the median survival (MST 45 days) and resulted in 40% of the mice surviving for >100 days. Therapeutic efficacy of *Lm* ΔactΔinlB was not impacted by depletion of CD8^+^ T cells in our treatment model. Interestingly, the median survival of CD4^+^ T cell depleted *Lm* ΔactΔinlB-treated mice was comparable to intact *Lm* ΔactΔinlB-treated mice (MST 47 days vs 45 days, respectively); however, there were no long-term survivors in this depleted cohort. In striking contrast, depletion of NK cells completely abrogated the therapeutic anti-tumor activity following *Lm* ΔactΔinlB treatment (Fig. 1B, □). Although in vivo depletion using asialo-GM1 predominantly depletes NK cells, it may also impact subsets of αβ T cells, γδ T cells, and myeloid cells. Nonetheless, depletion of CD4^+^ or CD8^+^ cells alone had no impact on long-term survival. Importantly, it was previously demonstrated that *L. monocytogenes*...
cannot directly infect CT26 tumor cells, minimizing the possibility that \textit{Lm} \textit{ΔactAΔinIB} acts directly upon the tumor (35). Taken together, these data suggest that treatment with \textit{Lm} \textit{ΔactAΔinIB} alone leads to NK cell-mediated anti-tumor immunity in mice bearing intrahepatic metastases.

Role of NKG2D in NK cell-mediated anti-tumor immunity
NK cell-mediated killing involves the interpretation of stimulatory and inhibitory signals. Defining the stimulatory ligands expressed by CT26 may explain how these cells are efficiently eliminated by \textit{Lm} \textit{ΔactAΔinIB}-activated NK cells in vivo. NKG2D ligands are attractive candidates because of their role in normal tumor immune surveillance and tumor cell killing in vivo (36, 37). In BALB/c mice treated with \textit{Lm} \textit{ΔactAΔinIB}, NK cells up-regulated CD69 and accumulated within the liver (Fig. 1C). Concurrently, NK cells within liver and spleen up-regulated surface NKG2D expression following \textit{Lm} \textit{ΔactAΔinIB} treatment (Fig. 1D). CT26 tumor cells express moderate levels of the NKG2D ligand H60, but lack RAE-1 (Fig. 1E). This profile contrasts expression by the prototypic NK cell target YAC-1, which expresses RAE-1 and H60, and only low levels of MHC class I. Regardless of the differential expression of NKG2D ligands, effectors isolated from the liver or spleen 48 h after \textit{Lm} \textit{ΔactAΔinIB} treatment demonstrated potent cytolytic activity toward both CT26 and YAC-1 targets (Fig. 1F). Although NK cells dominate the liver following \textit{Lm} \textit{ΔactAΔinIB} treatment, other cells capable of immediate NKG2D-mediated effector function, such as γδ T cells, could contribute to the observed ex vivo cytotoxicity. Nevertheless, liver-derived effectors were consistently superior to those from the spleen at killing CT26 and YAC-1 targets. Blockade of NKG2D reduced CT26-directed cytotoxicity by ~40% while YAC-1 killing was reduced ~15%. Thus, \textit{Lm} \textit{ΔactAΔinIB} treatment induced NKG2D-dependent and -independent target cell recognition and killing.

NK cell accrual within the liver is independent of sustained hepatic bacterial burden
The inability to detect NK1.1$^+$ cells in the BALB/c mouse makes characterization of NK cell maturation difficult in this mouse strain. To facilitate a comprehensive study of NK cell development during \textit{Lm} \textit{ΔactAΔinIB} infection, we performed a series of experiments using the C57BL/6 mouse strain, where NK1.1$^+$CD49b$^-$ immature NK cells can be identified within the liver. As shown previously (24), deletion of internalin B from \textit{Lm} \textit{ΔactA} limits direct infection of hepatocytes, limits hepatocyte damage, and results in the spleen serving as the predominant site of bacterial infection (Fig. 2A). Nonetheless, as seen in the BALB/c strain, NK cell numbers remain elevated within the liver, but not the spleen following immunization of C57BL/6 mice (Fig. 2B). NK cells from \textit{Lm} \textit{ΔactAΔinIB}-treated mice produced IFN-γ (Fig. 2C) and transiently up-regulated CD69 (Fig. 2D) in both tissues following immunization. To characterize the lytic activity of NK cells following \textit{Lm} \textit{ΔactAΔinIB} treatment, isolated leukocytes were cultured with $^{51}$Cr-loaded YAC-1 targets. Increased lytic activity was measurable within the liver and spleen 24 h post immunization (Fig. 2E), and continued to increase over 72 h. YAC-1-specific killing activity within the liver was 50–100% greater than that within the spleen at all time points, although some of this difference might be attributed to the discrepancy in NK cell frequency between the two tissues. Thus, although \textit{Lm} \textit{ΔactAΔinIB} is rapidly cleared from the liver, NK cell frequency and potency remained consistently elevated over 72 h. Importantly, the change in NK cell frequency, activation state, cytokine production, and cytolytic activity was consistent between the BALB/c and C57BL/6 mouse.

FIGURE 2. NK cell number and function following bacterial infection. C57BL/6 mice were immunized with $1 \times 10^7$ CFU \textit{Lm} \textit{ΔactAΔinIB}. Spleens and livers were harvested at 24, 48, and 72 h for analysis: enumeration of \textit{Lm} \textit{ΔactAΔinIB} within the spleen and liver (A); frequency of NK1.1$^+$CD3$^-$ NK cells within spleen and liver (B); direct ex vivo expression of intracellular IFN-γ by NK1.1$^+$CD3$^-$ NK cells (C); expression of CD69 by NK1.1$^+$CD3$^-$ NK cells (D); in vitro killing of $^{51}$Cr-loaded YAC-1 targets by liver and spleen derived effectors (E).
Maturation and expansion of immature hepatic NK cells

Immature NK cells within the liver undergo differentiation and expansion following viral infection (5). We hypothesized that a similar differentiation takes place following Lm \( \Delta actA\Delta inlB \) treatment. To characterize NK cell expansion within the liver during infection with an intracellular bacterium, we followed the acquisition of distinct markers of NK cell differentiation as described previously (5, 7). This approach allowed us to monitor the total leukocyte population (CD45\(^+\)) and identify both immature and mature cells of the NK lineage (NK1.1\(^-\)CD19\(^-\)CD3\(^-\)).

We followed the stepwise acquisition of CD49b (DX5), CD11b, and CD43 in the hepatic NK1.1\(^-\) NK cell population in C57BL/6 mice. Twenty-four hours post infection, >90% of NK cells (~20% of total WBCs) expressed CD49b (Fig. 3A). Interestingly, the majority of these NK cells were also CD11b\(^{high}\) and CD43\(^{high}\), a phenotype consistent with terminally differentiated NK cells. By forty-eight hours post infection, a CD11b\(^{high}\) CD43\(^{low}\) NK cell population dominated. The CD11b\(^{high}\) CD43\(^{low}\) NK cells continued to accumulate in the liver 72 h after treatment. The increase of CD49b\(^+\) CD11b\(^{high}\) CD43\(^{low}\) NK cells coincided with sustained cytolytic activity 48–72 h after Lm \( \Delta actA\Delta inlB \) treatment (Fig. 2E).

Thus, treatment with Lm \( \Delta actA\Delta inlB \) led to the rapid appearance of fully mature NK cells in the liver, followed by the accumulation of large numbers of NK cells with an intermediate maturation phenotype.

Within the spleen, a reproducible decrease in CD49b\(^+\) CD11b\(^{high}\) CD43\(^{high}\) cells was observed 24 h post infection. By 48 h, the appearance of CD49b\(^+\) CD11b\(^{high}\) CD43\(^{low}\) NK cells within the spleen suggested that maturing NK cells were migrating from the liver or bone marrow, because the immediate precursor, immature CD49b- NK cells, are scarce within this tissue. Together, these experiments demonstrated that the liver-specific increase in NK cells following Lm \( \Delta actA\Delta inlB \) infection is consistent with the maturation and expansion of immature NK cells within this tissue.

We hypothesized that the rapid increase in the number of fully mature NK cells within the liver resulted from their migration from peripheral tissues. To test this possibility, we transferred fully mature, terminally differentiated NK cells (CD49b\(^+\) CD11b\(^{high}\) CD43\(^{high}\)) from the spleens of naive CD45.2\(^+\) donor mice (C57BL/6) into naive CD45.1\(^+\) (B6.SJL) recipient mice (Fig. 4A). We treated these mice with Lm \( \Delta actA\Delta inlB \) and enumerated the transferred CD49b\(^+\) CD11b\(^{high}\) CD43\(^{low}\) NK cells within the liver and spleen. Twenty-four hours post treatment, both donor and recipient NK cells responded by upregulating surface CD69 expression (Fig. 4B). Donor-derived mature NK cells (CD45.2\^high\ CD49b\^+\ CD11b\^low\ CD43\^high\) demonstrated a greater than 2-fold increase within the liver while subsequently decreasing within the spleen (Fig. 4C), suggesting that the early appearance of CD49b\(^+\) CD11b\(^{high}\) CD43\(^{low}\) NK cells within the liver was due to their migration from peripheral tissues into the liver.

Potent anti-tumor efficacy requires escape from the vacuole, but not type I IFN signaling

Entry of L. monocytogenes into the cytosol elicits numerous proinflammatory cytokines, including IFN-\( \beta \) (27). Type I IFNs are potent activators of NK cell cytolytic function, and we questioned whether they were also critical for the anti-tumor efficacy of Lm \( \Delta actA\Delta inlB \) (38). To test this possibility, mice lacking the type I IFN\( \alpha \)/\( \beta \) receptor (BALB/c IFN\( \alpha \)/\( \beta \)\(^{-/-}\)) were implanted with hepatic CT26 tumors and three days later treated with Lm \( \Delta actA\Delta inlB \). Surprisingly, BALB/c IFN\( \alpha \)/\( \beta \)\(^{-/-}\) mice demonstrated significant anti-tumor efficacy following treatment with Lm \( \Delta actA\Delta inlB \) (Fig. 5A). The median survival for NT and Lm \( \Delta actA\Delta inlB \)-treated BALB/c IFN\( \alpha \)/\( \beta \)\(^{-/-}\) mice was 16 and 47
days, respectively. Furthermore, the overall survival of BALB/c.IFNα/βR−/− mice treated with Lm ΔactΔinIB was not statistically different from BALB/c mice treated with the same Lm ΔactΔinIB regimen. Therefore, type I IFN signaling was not absolutely required for the anti-tumor activity induced by Lm ΔactΔinIB.

To understand the necessity for bacterial entry into the cytosol for therapeutic benefit, tumor-bearing mice were immunized with live Lm ΔactΔinIB, heat-killed (HK) Lm ΔactΔinIB, or L. monocytogenes unable to produce LLO (Lm Δhly, unable to escape the phagocytic vacuole). Treatment of mice with Lm ΔactΔinIB resulted in the long-term survival of 80% for the duration of this study (Fig. 5B). In contrast, median survival and the number of long-term survivors was significantly reduced in groups treated with HK-Lm ΔactΔinIB or Lm Δhly relative to Lm ΔactΔinIB-treated mice. Both HK and Lm Δhly treatment improved MST relative to untreated animals, which is expected, given the interaction of these bacteria with host pattern recognition receptors on the cell surface and within the phagosome. Nonetheless, treatment with bacteria unable to enter the host cell cytosol did not translate to long-term survival. This finding suggests that bacterial entry into the cytosol is critical for the induction of a potent intrahepatic anti-tumor response (Fig. 5B), but not due to the actions of type I IFN (Fig. 5A).

IFN-γ production and accumulation of hepatic NK cells are not dependent on type I IFN

We characterized NK cells within the spleen and liver of BALB/c and BALB/c.IFNα/βR−/− mice following treatment with Lm ΔactΔinIB. Similar to Lm ΔactΔinIB-treated BALB/c mice, BALB/c.IFNα/βR−/− mice demonstrated a 3- to 4-fold increase in CD49b+ NK cells within the liver (Fig. 6A), but not within the spleen. NK cell IFN-γ production was independent of type I IFN signaling (Fig. 6B). In contrast, CD69 up-regulation was almost entirely dependent upon type I IFN signaling.

We next characterized serum cytokines following treatment with Lm ΔactΔinIB. Both BALB/c and BALB/c.IFNα/βR−/− mice were treated with Lm ΔactΔinIB or Lm Δhly, and serum was collected 4 and twenty-four hours later. Four hours post treatment, high levels of IL-12p70 and MCP-1 were present in the serum (Fig. 6C). Four hour IL-12p70 and MCP-1 production was independent of cytosolic entry by Lm ΔactΔinIB and slightly reduced in the absence of type I IFN signaling. By twenty-four hours, MCP-1 production was dependent upon both cytosolic entry and type I IFN, while IL-12p70 required cytosolic entry, but was suppressed by type I IFN signaling. Serum IFN-γ peaked at twenty-four hours, and was dependent upon cytosolic entry but not type I IFN. Thus, in the absence of type I IFN signaling, NK cells still accumulated within the liver and produced IFN-γ following administration of Lm ΔactΔinIB.

Treatment with Lm ΔactΔinIB induces tumor-specific memory in long-term survivors

As presented in Fig. 1, the mechanism of intrahepatic anti-tumor activity induced by Lm ΔactΔinIB is NK cell dependent. To evaluate whether systemic tumor-specific T cell immunity was generated during the course of Lm ΔactΔinIB-induced tumor rejection, long-term survivors (>100 days post tumor cell implant) combined from several therapeutic tumor experiments were re-challenged subcutaneously with 2 × 10^7 CT26 tumor cells. As shown in Fig. 7A, 21 of 33 mice (64%) from the Lm ΔactΔinIB-treated long-term survivors were completely protected from the lethal challenge with CT26 tumor cells at a distal site as compared with
following immunization; BALB/c.IFNR−/− requires cytosolic entry, but is type I IFN independent. BALB/c
A/H9004 group.
indicated cytokines. Data represent the mean and SD of 5 mice per

FIGURE 6. Production of IFN-γ following Lm ΔactAΔinlB immunization requires cytosolic entry, but is type I IFN independent. BALB/c and BALB/c.IFNR−/− mice were immunized with 1 × 10⁷ CFU Lm ΔactAΔinlB. Splen and livers were harvested 24 h later for analysis. A, Frequency of CD49b+CD3− NK cells within the spleen and liver following immunization; B, expression of CD69 and IFN-γ by CD49b+CD3− NK cells within the liver and spleen following immunization. Data represent the mean and SD of 3 animals per group; C, serum samples were collected 4 and 24 h post immunization with 1 × 10⁷ CFU Lm ΔactAΔinlB or 3 × 10⁶ CFU ΔinlB Lm and assayed for the indicated cytokines. Data represent the mean and SD of 5 mice per group.

1 of 20 (5%) of the naive controls. To determine whether resis-
tance to tumor challenge was T cell dependent, a subset of long-
term survivors was depleted of either CD4+ T cells, CD8+ T cells or NK cells before CT26 rechallenge. In contrast to the primary anti-tumor response that was completely dependent on NK cells, the secondary anti-tumor response was independent of NK cell activity, but dependent on both CD4+ and CD8+ T cells. Mice that did develop tumors did so at a significantly reduced tumor growth rate when compared with naive mice (Fig. 7B). On day 60 post CT26 rechallenge, nine tumor-free mice were then challenged with Renca cells, a BALB/c syngeneic renal cell carcinoma, in the op-

Discussion
In these studies, we demonstrated the differentiation of NK cells within the liver following bacterial infection, and suggest that the presence of these cells facilitates destruction of hepatic metastases. Treatment with Lm ΔactAΔinlB led to NK cell-dependent anti-
tumor immunity in a murine model of hepatic metastatic cancer. Survival correlated with the maturation of hepatic NK cells, and with the recruitment of mature NK cells from the periphery. Long-
term survivors challenged subcutaneously with the same tumor demonstrated long-lived systemic anti-tumor immunity that was dependent upon CD8+ and CD4+ T cells. A mechanism involving the NK cell-mediated immune destruction of a primary hepatic tumor, subsequent cross-presentation of tumor-derived Ag, prim-
ing of tumor-specific T cells, and finally long-term adaptive im-
munity is supported by our results. We propose that the proximity of hepatic metastases to the primary reservoir of immature NK cells makes activation of the innate immune system an attractive therapeutic strategy.

The presence of immature NK cells in the adult liver of mice has been only recently appreciated, and their biological role in bacte-
rial infection and tumor immunity is not well defined (5). Similar to previously described results during infection with murine cyto-
egegalovirus, we observed that Lm ΔactAΔinlB induced a rapid maturation of immature hepatic NK cells, characterized by the acquisition of CD49b and CD11b (5). In contrast to MCMV, Lm ΔactAΔinlB infection resulted in the majority of hepatic NK cells exhibiting the fully mature, CD49b+CD11b+CD3− phenotype 24 h after infection. 48–72 h postinfection, the liver was domi-
nated by less mature CD49b+CD11b−CD3− phenotype NK cells. This result suggested a concerted response to bacterial infection con-
sisting of an initial migration of mature NK cells toward the liver, followed by the maturation and expansion of resident immature NK cells. The reproducible decrease in splenic NK cell numbers, in addition to adoptive transfer studies using mature NK cells en-
riched from the spleens of congenic mice, supported the hypothesis that following destruction of the hepatic tumor by NK cell-mediated cytotoxicity, long-lived systemic tumor-spe-
cific cellular immunity is induced, protecting animals from a sub-
sequent lethal tumor challenge.
results suggesting a role for MCP-1 in attracting innate and adaptive immune effectors into the liver (35, 39). Future studies will investigate the cellular and molecular constituents of this programmed NK cell response, as well as the ability of other innate immune activators to elicit a similar program.

A role for type I IFN in NK cell activation, recruitment and cytolytic activity has been well described (40). Similar to viral infection, infection with *L. monocytogenes* induces production of type I IFN (27). Although *L. monocytogenes* interacts with multiple TLRs on the cell surface and within the vacuole, the IRF3-dependent, MyD88/Trif-independent production of IFN-β occurs only upon cytosolic access in the *L. monocytogenes*-infected host cell (22, 27, 41). The well-described role of type I IFNs in NK cell activation, recruitment and cytolytic activity suggested a role for these cytokines in the induction of NK cell-mediated anti-tumor immunity following treatment with *Lm ΔactAΔinlB* (40, 42, 43). Interestingly, while cytotoxic entry of *L. monocytogenes* was required for optimal anti-tumor immunity, as poor efficacy was observed in mice treated with an LLO-deficient *Lm* strain or heat-killed *Lm* (*Lm Δhly* or HK-*Lm ΔactAΔinlB*, respectively), signaling through the type I IFN receptor was not (Fig. 5). Furthermore, while the anti-tumor response following treatment with *Lm Δhly* or HK-*Lm ΔactAΔinlB* was significantly poorer than the response to *Lm ΔactAΔinlB*, it was greater than that observed in vehicle-treated control animals. We suspect this effect is related to the early cytokine/chemokine burst observed when injecting high numbers of bacterial particles (Fig. 6C, 4 h time point). So although bacteria unable to access the cytosol could elicit a minimal amount of anti-tumor activity, only those bacteria able to grow within the host cell cytosol led to consistently robust anti-tumor immunity. Although this finding does not exclude a role for type I IFN-dependent activation of NK cells, it suggests that *L. monocytogenes* can elicit NK cell activation, migration, and cytotoxicity via multiple pathways.

NK cells are activated by a number of cytokines, including IFN-α/β, IL-2, IL-12p70, and IL-18. The composition of this cytokine milieu specifically impacts how NK cells recognize and kill target cells. Smyth and colleagues demonstrated that IL-2 activated NK cells recognize targets via NKG2D and kill in a perforin-dependent manner, while IL-18 activated NK cells could recognize targets independent of NKG2D, and used a FasL-dependent method of killing (44). *L. monocytogenes* infection leads to production of numerous cytokines, including IL-12p70 and IL-18 (45). It is therefore not surprising that infection with an intracellular bacterium activates NK cells to recognize and kill targets by multiple mechanisms (Fig. 1). Regulation of IL-12 by type I IFN complicates interpretation of any findings in type I IFN receptor-deficient mice. At high, but physiologically relevant levels, type I IFN has been shown to negatively regulate IL-12 production (46). As shown in this investigation, high levels of serum IL-12p70 24 h post immunization corroborated the strong suppressive effect of type I IFN on IL-12p70 secretion during *L. monocytogenes* infection (Fig. 6C). High levels of IL-12p70 in type I IFN receptor deficient mice may compensate for the lack of type I IFN, leading to increased activation and cytolytic activity necessary for anti-tumor immunity. Future studies will be needed to understand NK cell function in the absence of both type I IFN and IL-12, as well as the cytokine mechanisms used by *L. monocytogenes*-activated NK cells.

Together these data support immunomodulatory agents targeting the liver immature NK reservoir as a general therapeutic strategy for hepatic metastatic disease. Toward testing these hypotheses, we are presently evaluating the safety and tolerability of CRS-100, clinical formulations of the *Lm ΔactAΔinlB* strain, in a Phase I clinical trial under a U.S. IND application with study subjects who have carcinoma and hepatic metastases.

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