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Invariant NKT Cells Are Required for Antitumor Responses Induced by Host-Versus-Graft Responses

Toshiki I. Saito, Hao Wei Li, and Megan Sykes

Based on clinical observations, we have previously shown in a murine model that recipient leukocyte infusion (RLI) induces a host-versus-graft reaction in mixed bone marrow chimeras and that rejection of donor cells leads to a specific antitumor response against recipient malignancies. This response is dependent on T cells and IFN-γ. We investigated the role of NKT cells (NKTs) in this phenomenon. Depletion of recipient NK1.1+ cells led to loss of an anti-tumor effect induced by RLI in mixed bone marrow chimeras. In recipients specifically lacking host invariant NKT cells (iNKTs), RLI did not induce an antitumor effect, indicating a critical role for recipient iNKTs. Conversely, specific activation of iNKTs enhanced the anti-tumor effect induced by RLI. Following RLI, recipient iNKTs, NK cells, dendritic cells (DCs), and CD8 T cells were activated. CD8 T cells were the major producers of IFN-γ. Lack of recipient iNKTs resulted in failure of activation of NK cells and DCs by RLI. Our studies demonstrate a central role for iNKTs in promoting RLI-induced anti-tumor effects and suggest that this pathway involved promotion of the activation of recipient NK cells and DCs. The Journal of Immunology, 2010, 185: 2099–2105.

In the course of clinical trials involving nonmyeloablative allogeneic hematopoietic stem cell transplantation as treatment for hematologic malignancies, surprising responses of refractory lymphomas and myelomas were observed in a significant fraction of patients who rejected the donor hematopoietic cell grafts (1). These results suggested that the anti-tumor effects might be mediated by host-derived cells. To analyze this surprising observation, we used the preclinical murine model that was the basis for the conditioning regimen in our clinical trials (2, 3) to test the hypothesis that host-versus-graft (HVG) reactions could lead to anti-tumor effects. We have shown that either spontaneous or induced rejection of engrafted donor cells indeed leads to tumor-specific responses. Some mice ultimately survive a lethal dose of host-type tumor when donor marrow is rejected, as observed in the clinical setting (4). This effect requires T cells and induces a tumor-specific cytotoxic response (5). Neither bystander killing nor cross-reactivity of TCR-recognizing allo-Ags and tumor-specific Ags is likely to be the mechanism of induction of these tumor-specific immune responses (5, 6). Thus, the mechanism by which alloresponses lead to tumor-specific responses is still unknown.
**Bone marrow transplantation**

Mixed chimerism was induced in female BALB/c mice using a non-myeloablative regimen as described (3). The regimen consists of in vivo T cell-depleting anti-CD4 (GK1.5) (1.76 mg/mouse) and anti-CD8 (2.43) (1.44 mg/mouse) mAbs administered i.p. on day −5, cyclophosphamide (Cytosan, Mead Johnson, Evansville, IN) 200 mg/kg i.p. on day −1, and 7 Gy thymic irradiation from a 60Co source on day 0. Donor bone marrow cells (BMCs) were harvested from B10.BR (H-2b) or B10.RII (H-2r) mice, and single-cell suspensions were prepared as described previously (3). Four to six hours after the thymic irradiation, 25 × 10^6 donor BMCs were injected i.v. through the tail vein.

To generate synchimeras in which only iNKTs lack the ability to produce IFN-γ, wild-type BALB/c mice (aged 11 wk) received lethal total body irradiation (8 Gy) and 10^6 BMCs each from Jax KO and IFN-γ KO BALB/c mice. Mice in a control group received BMCs from Jax KO and wild-type BALB/c mice. Eight weeks later, these synchimeras were used as recipients of nonmyeloablative conditioning and allogeneic (B10.RII) bone marrow transplantation (BMT).

**Recipient leukocyte infusions**

Recipient BALB/c spleen cell suspensions were prepared and administered i.v. at a dose of 30 × 10^6 splenocytes recipient/6–8 wk post allogeneic BMT, as described (4). Some groups received spleen cells from Jax.11+18+BALB/c or Jax18 KO BALB/c mice. Some groups received anti-NK1.1 mAb (PK136) to deplete NK1.1+ cells. Where indicated, some groups received 2 μg ofGalCer (ALX-306-027-M001, Alexis Biochemicals, Plymouth Meeting, PA) or vehicle (0.2% DMSO/PBS) i.p. three times every 4 d beginning 7 d post RLI.

**A20 cell line**

A20 is a B cell leukemia/lymphoma of BALB/c origin (10). Cells were maintained in culture and administered i.v. at a lethal dose of 5 × 10^5 cells 7 d post RLI, as described elsewhere (4).

**Flow cytometric assessment of chimerism**

Chimerism in various lineages was analyzed at various times post BMT by flow cytometry, as described previously (3, 4). Nonspecific FcγR binding was blocked by anti-mouse FcγRIII receptor mAb 2.4G2 (II). Cells were labeled with anti-H-2D^b-FITC (clone 34-2-12; BD Biosciences, San Jose, CA) or anti-H-2K^d (clone 16-1-11, specific for H-2Kk, crossreacts with H-2r; BD Biosciences) mAbs 24 h prior to staining. T cells were stained for degranulation assays.

**Autoradiography for degranulation assays**

Cells were directly stained intracellularly (without ex vivo stimulation) with anti–IFN-γ or IL-4 mAbs. Each lineage was gated using the following Abs: DX5-FITC, anti–B220-PerCP/Cy5.5, anti–CD4-PE/Cy7, anti–CD8-allophycocyanin/Cy7 (BD Biosciences), H-2D^d–FITC (clone 34-2-12; BD Biosciences, San Jose, CA) labeled with allophycocyanin (National Institute of Allergy and Infectious Diseases Tetramer Facility, Germantown, MD). Flow cytometric data were analyzed with FlowJo (Tree Star, Ashland, OR). Relative percentages of donor cells were calculated as previously described (4).

**Intracellular staining and degranulation assay**

Cells were directly stained intracellularly (without ex vivo stimulation) with anti–granzyme B–PE or rat IgG–PE as isotype control (BD Biosciences). Cells were directly stained (without ex vivo stimulation) with anti–lysosomal-associated membrane protein (LAMP) 1 and LAMP2 mAb (BD Biosciences) for degranulation assays.

**IFN-γ and IL-4 secretion assay**

Mouse IFN-γ and IL-4 secretion. Detection kits were purchased from Miltenyi Biotec (Auburn, CA). Briefly, a bispecific conjugate of anti-CD45 Abs and anti-IFN-γ or IL-4 (IFN-γ or IL-4–specific Catch Reagent) is attached to the cell surface of all CD45+ leukocytes. The cells are then incubated for 45 min at 37°C in complete RPMI 1640 medium to allow cytokine secretion. The secreted IFN-γ or IL-4 binds to the IFN-γ Catch Reagent on the positive secreting cells. These cells can subsequently be labeled with a second anti-IFN-γ or IL-4–PE mAb for sensitive detection by flow cytometry. Each lineage was gated using the following Abs: DX5–FITC, anti–B220–PerCP/Cy5.5, anti–CD4–PE/Cy7, anti–CD8–allophycocyanin/Cy7 (BD Biosciences), H-2D^d–Pacific Blue (prepared in our laboratory at Massachusetts General Hospital), CD1d^a2–allophycocyanin, and CD3–FITC. Dead cells were gated out by 0.5 μg/mL DAPI (D8417, Sigma-Aldrich, St. Louis, MO)

**Statistics**

Descriptive data were analyzed with Prism 4.0 statistical software (GraphPad Software, San Diego, CA). All p values <0.05 were considered statistically significant. Group comparisons were made by nonparametric (Mann-Whitney) test. Survival curves were created with the Kaplan-Meier method, and the log-rank test was used to determine differences in survival between groups of mice.

**Results**

**Requirement for NK1.1+ cells for the anti-tumor effect induced by HVG reactions**

We have shown that RLI-derived CD8+ T cells are required to obtain maximum anti-tumor effects of RLI. Although host-derived CD4+ T cells contribute, RLI-derived CD4+ T cells and B cells are not required for the anti-tumor effect (5, 6). NK cells and NKTs are known to modulate the alloreponse (7) and shape acquired immune reactions (8, 12). Thus, we examined whether NK cells or NKTs are required for the anti-tumor effects induced by HVG reactions.

First, MCs were generated using nonmyeloablative conditioning with injection of fully allogeneic BMCs. One month post allogeneic BMT, we confirmed stable donor chimerism in the peripheral blood. Host-type whole splenocytes were then infused as the RLI to deliberately induce rejection 6–8 wk post allogeneic BMT. Finally, host-type tumor cells were injected 1 wk post RLI to mimic minimal residual disease, and animals were followed as described (Fig. 1) to assess anti-tumor responses (4).

To distinguish the possible contribution of host-derived NK cells and NKTs from that of RLI-derived NK cells and NKTs, we made use of an NK1.1+ congenic BALB/c mouse line (13) in which NK cells and NKTs express the NK1.1 molecule and can be depleted by anti-NK1.1 mAb. Recipients of NK1.1+ cell-depleted RLI (n = 9) showed similar tumor survival compared with recipients of NK1.1+ cell-replete RLI (n = 9) (median survival time [MST] 52 versus 52 d respectively; p = 0.68, Fig. 1A). In contrast, NK1.1+ cell-depleted chimeras that received RLI (n = 8) had a shorter survival (MST 35.5 d) than NK1.1+ cell-replete chimeras that received NK/NK-Tetramer negative RLI (n = 10) (MST 68 d; p < 0.01), and no anti-tumor effect was observed in the NK1.1+ cell-depleted recipients (Fig. 1B). Studies to determine the expression of NK1.1 on NK and iNKTs in NK1.1+ BALB/c mice (Supplemental Fig. 1A, 1B) and the efficacy of depletion by anti-NK1.1 mAb showed that the mAb depleted 50–80% of NK cells and 70–90% of iNKTs (Supplemental Fig. 1C). These results showed for control chimeras (Δ; n = 6), chimeric recipients of RLI (○; n = 6), and chimeric recipients of NK1.1+ RLI and anti-NK1.1 Ab (●; n = 9). B. Survival is shown for control chimeras (Δ; n = 6), NK1.1+ chimeric recipients of tumor plus RLI (○; n = 8), and NK1.1+ chimeric recipients of tumor plus RLI and anti-NK1.1 Ab (●; n = 8). The groups in A and B were prepared in a single experiment. The experiment was performed three times with similar results.

**FIGURE 1.** Effect on anti-tumor effects of RLI of depletion of NK1.1+ cells in the recipient chimera or in the RLI. A. Survival is shown for control chimeras (Δ; n = 6), chimeric recipients of RLI (○; n = 6), and chimeric recipients of NK1.1+ RLI and anti-NK1.1 Ab (●; n = 9). B. Survival is shown for control chimeras (Δ; n = 6), NK1.1+ chimeric recipients of tumor plus RLI (○; n = 8), and NK1.1+ chimeric recipients of tumor plus RLI and anti-NK1.1 Ab (●; n = 8). The groups in A and B were prepared in a single experiment. The experiment was performed three times with similar results.

CY, cyclophosphamide; TCD, T cell depletion; TII, thymic irradiation.
show that NK cells and/or NKTs in the recipients are essential for the anti-tumor effect of RLI, whereas NK cells and/or NKTs in the RLI are dispensable.

Requirement for iNKTs for the anti-tumor effect of RLI
To distinguish the possible role of NKTs, we used Jα18 KO mice that are deficient in iNKTs. Jα18 KO chimeric recipients of Jα18 KO-derived RLI (n = 12) demonstrated shorter survival than wild-type chimeric mice receiving wild-type RLI (n = 11; MST 41.0 versus 67.0 d; p < 0.01), and no survival benefit of Jα18 KO RLI was observed in Jα18 KO recipients (p = 0.93) (Fig. 2A). Survival of Jα18 KO chimeric recipients of wild-type RLI (n = 19; MST 49 d) was similar to that of Jα18 KO chimeric recipients receiving Jα18 KO-derived RLI (p = 0.18). In contrast, wild-type chimeric mice receiving Jα18 KO RLI (n = 17; MST 61 d) showed longer survival than Jα18 KO chimeric recipients of Jα18 KO-derived RLI (p < 0.01) (Fig. 2B). Analysis of chimerism showed that the lack of recipient iNKTs did not affect the rejection of donor cells induced by RLI (Fig. 2C), indicating that the loss of anti-tumor effects was not due to altered HVG reactions. These results show that host but not RLI-derived iNKTs are essential for the anti-tumor effect induced by RLI. The data are consistent with the observation that only host- and not RLI-derived NK1.1+ cells are required for the anti-tumor effect of RLI.

Enhancement of anti-tumor effect through iNKT-specific activation
Because iNKTs are essential for the anti-tumor effect of RLI and influence levels of chimerism, we examined the effect of activating iNKTs by administering αGalCer. A course of 2 μg αGalCer on days 56, 60, and 64 (i.e., beginning 7 d post RLI) was given i.p. to chimeras and RLI recipients. iNKTs were critical for RLI-induced anti-tumor immunity, IFN-γ and IL-4 when they are activated, we measured secretion of these cytokines by iNKTs in various organs in MCs 7 d post RLI. As shown in Fig. 4, a significantly higher percentage of IFN-γ- and IL-4–producing iNKTs was detected in the BM, liver, and spleen of MCs receiving RLI compared with MCs not receiving RLI at 7 d post RLI (n = 3 per group; p < 0.05). These data show that HVG reactions triggered by RLI given to MCs resulted in the systemic activation of iNKTs.

Because our previous work showed a critical role for IFN-γ in the RLI-induced anti-tumor effect (5), we sought to determine whether IFN-γ secreted by iNKTs was critical for their role in this phenomenon. To this end, we created synchimeras in which the recipient iNKTs were specifically ablated by reconstituting lethally irradiated BALB/c recipients with a mixture of BMCs from syngeneic Jα18 KO and IFN-γ–KO BALB/c mice. These recipients later underwent allogeneic BMT to become MCs. Six weeks following the allogeneic BMT, these MCs received RLI followed by inoculation of A20 tumor cells 1 wk later. In the control group of chimeric recipient mice that had been initially reconstituted with BMCs from Jα18 KO and wild-type rather than IFN-γ KO mice, RLI induced a significant anti-tumor effect (n = 9; MST 47 d) compared with MCs not receiving RLI (n = 9; MST 35 d; p < 0.05) (Fig. 5). These data indicated that HVG reactions triggered by RLI given to MCs resulted in the systemic activation of iNKTs.

Production of IFN-γ by activated CD8 T cells following RLI
When we used IFN-γ–deficient mice as BMT recipients and RLI donors, the anti-tumor effect was completely abrogated (5), showing that IFN-γ is essential for this effect. In addition, depletion of CD8 T cells following RLI prevented the increase in serum IFN-γ levels associated with RLI, indicating that the induced serum IFN-γ increase was dependent upon CD8 T cells (5). However, it
is not clear which cell type produced IFN-γ following RLI. Although recipient iNKTs were activated and produced IFN-γ following RLI, iNKT-derived IFN-γ was not required for RLI-induced anti-tumor immunity. Thus, to further identify the cell types producing IFN-γ following RLI, we performed sequential flow cytometric analyses.

BMCs, liver, spleen, and lymph node cells were harvested from control chimeras and RLI recipients on days 0 (3 h post RLI), 1, 2, 5, 7, 9, 14, and 21 post RLI. Then, without any stimulation in vitro, IFN-γ secretion was measured using an FCM-based IFN-γ secretion assay. We also examined serum IFN-γ levels at the same time points and measured IFN-γ mRNA levels in FACS-sorted cells on days 1 and 8 post RLI.

Robust IFN-γ secretion was observed from days 7–14 post RLI administration mainly by host CD8+ T cells (Fig. 6A). Kinetic studies showed that IFN-γ production by CD8 T cells in spleen, liver, and BM peaked at days 9, 7, and 14 post RLI, respectively. In addition, a greater peak percentage of IFN-γ-producing CD8 T cells was observed in the liver and BM compared with spleen (Fig. 6B). Similar kinetics of IFN-γ upregulation were detected at the mRNA level (Fig. 6C). There was no increase in IFN-γ mRNA at early time points (i.e., 3 h to 2 d post RLI in sorted iNKTs, NKs, or CD8 T cells) (Fig. 6C) or in the serum before day 7 (Supplemental Fig. 2). Together, these results show that host-type CD8+ T cells are major IFN-γ producers following RLI. Moreover, IFN-γ secretion from CD8+ T cells from Jα18 KO chimeric recipients of Jα18 KO-derived RLI was similar to that of wild-type chimeric recipients of wild-type RLI (Fig. 6D), indicating that iNKTs do not play a critical role in promoting IFN-γ production by CD8 T cells. Together, our data are consistent with a role for iNKTs in the anti-tumor effect of RLI that does not include IFN-γ secretion or induction of its secretion by CD8 T cells.

To further characterize the activation profiles of CD8 T cells, we examined additional activation molecules. Consistent with the IFN-γ secretion profile, increased proportions of CD8+ T cells from chimeras receiving RLI showed the effector memory CD62L−CD44high phenotype 7 d post RLI (Fig. 7A, 7B). Elevated granzyme B production was detected in CD8+ T cells in the RLI recipients' spleens 9 d post RLI (Fig. 7C). We also measured degranulation via expression of LAMP1 and LAMP2 and detected degranulation of CD8+ T cells in the spleen 9 d post RLI (Fig. 7C). Studies of granzyme A, IL-17F, IL-17A, Fas ligand, and TRAIL mRNA in splenic iNKTs 1 and 8 d post RLI showed no elevations of any of these transcripts (data not shown).

**iNKTs are required for RLI-induced DCs and NK cell activation**

iNKTs have been shown to affect immune responses by enhancing the activation of DCs (14, 15) and NK cells (16, 17). Thus, to elucidate the mechanisms by which recipient iNKTs promoted RLI-induced anti-tumor immunity, we assessed the effects of recipient iNKTs on DCs and NK cell activation following RLI. As shown in Fig. 8, on day 7 post-RLI, activation of splenic DCs (Fig. 8A) and BM (Fig. 8B) NK cells was detected by significantly increased expression of CD69 (n = 3 per group, RLI versus no RLI; p < 0.05). Similarly, activation of DCs was also induced by RLI, as the expression of CD86 was significantly enhanced (p < 0.05). Activation of NK cells and DCs was significantly enhanced (p < 0.05). Therefore, HVG responses induced by RLI in MCs resulted in the activation of both NK cells and DCs. However, increased expression of CD69 on NK cells and CD86 on DCs was not induced by RLI in Jα18 KO MCs (Fig. 8). These data indicate that recipient iNKTs promote NK cell and DC activation following RLI.

**Discussion**

Administration of RLI to established MCs represents a novel strategy for separating graft-versus-host disease and graft-versus-tumor effects (4–6). However, the mechanisms of the anti-tumor effect of RLI are currently unclear. Previously, we have shown that CD8 T cells, IFN-γ, and rejection of donor cells are critical for the generation of anti-tumor immunity (4, 5). These studies led us to hypothesize that the potent systemic HVG alloresponses mediated by RLI create a proinflammatory environment that culminates in anti-tumor effects. However, the possible role of innate immune cells in this process was unclear. In this study, we demon-
strate that recipient iNKTs play a critical role in the RLI-induced anti-tumor effect.

Host CD8+ T cells in stable BM chimeras are tolerant to donor Ags (2). We have previously shown that nontolerant CD8+ T cells in the RLI are essential for the anti-tumor effect of RLI (5) and that RLI plus tumor administration generates tumor-specific cytotoxicity (5) and immune memory (T. I. Saito and M. Sykes, unpublished observations). Depleting CD8+ T cells in vivo or ex vivo from RLI abrogates the anti-tumor effect in recipients with normal iNKTs (5). However, we show in this study that recipient iNKT deficiency also abrogates the anti-tumor effect, even in the presence of functional CD8+ T cells. Moreover, specific activation of iNKTs led to enhancement of the anti-tumor effect of RLI. Because iNKTs do not express CD8, these data show that both iNKTs and conventional CD8+ T cells are required for RLI to induce maximum anti-tumor effects.

Although iNKTs have been reported to downmodulate alloresponses (7, 18) and to promote CD8+ T cell-dependent anti-tumor effects (8), this is the first demonstration of an interaction between alloreactive CD8+ T cells and iNKTs that culminates in anti-tumor effects.

We demonstrate that administration of RLI to MCs induces systemic activation of recipient iNKTs, as iNKTs in BM, liver, and spleen all showed expression of IFN-γ 7 d following RLI. Recent studies of iNKT activation in the setting of microbial infection have revealed that iNKTs can be directly activated by microbe-derived foreign Ags loaded into CD1d molecules or by simultaneous stimulation by cytokines derived from DCs activated by microbes and their cognate self-Ags. This second mode of activation requires the participation of both cytokines and self-Ags recognized by iNKTs. In a third setting, activation of iNKTs can dominantly be driven by cytokines, such as IL-12 and IL-18 (19). In our model, it can be excluded that the activation of iNKTs was driven by foreign cognate Ags, as RLI would not introduce any cognate foreign Ags recognized by iNKTs. However, the second or the third modes described above might explain iNKT activation in our model. In the setting of tumor immunity, it has been shown that cytokines alone are sufficient to activate iNKTs (20–22). Further studies will require examination of these cytokines. In addition, it has been well established that cytokines that can activate iNKTs are involved in alloresponses (23, 24). When MCs receive RLI, the nontolerant donor-reactive T cells initiate a potent systemic alloresponse against the donor cells. We hypothesize that the alloresponse results in the production of proinflammatory cytokines, including IL-12 and possibly IL-18. These cytokines, either alone or with the engagement of self-Ags recog-
nized by iNKTs, activate recipient iNKTs in multiple tissues, which in turn mediate their downstream effects.

Multiple studies have demonstrated that iNKTs can potentiate immune responses by enhancing activation of DCs (14, 15) and NK cells (16, 17). Although the roles of NK cells and DCs in RLI-induced anti-tumor immunity are unclear at this point, our data demonstrate that iNKTs are required for the RLI-induced activation of NK and DCs. As depletion of NK1.1+ cells, which include NK cells and NKTs, can abrogate RLI-induced anti-tumor immunity, NK cells may also play a role. This possibility is currently under investigation. Similarly, the role of DCs in RLI-induced anti-tumor immunity is unclear currently. However, we have obtained preliminary data showing that depletion of recipient-derived DCs abrogates RLI-induced anti-tumor effects (T. I. Saito and M. Sykes, unpublished observations). In addition, enhanced activation of DCs following RLI, as shown by increased expression of CD86, supports our hypothesis that HVG reactions induced by RLI promote activation of DCs, facilitating the cross-presentation of tumor Ags to CD8 T cells. Taken together, the observed role of iNKTs in promoting NK cell and DC activation following RLI provides a possible mechanism for the promotion of anti-tumor immunity by recipient iNKTs in this model.

Induction of anti-tumor immunity by RLI did not require iNKT-derived IFN-γ. Because iNKTs in the recipient and not those in the RLI are critical for anti-tumor effects, the lack of a role for iNKT-derived IFN-γ is consistent with previous studies showing that RLI-derived IFN-γ is more important than recipient-derived IFN-γ in promoting anti-tumor effects (5). Although iNKT-derived IFN-γ has been reported to mediate a stimulatory effect on NK cells and DCs (16, 17), CD40L expression by iNKTs may have similar effects (25, 26), and its role is currently under investigation. In addition to the lack of a role for iNKT-derived IFN-γ in our model, we show that iNKTs are not required for the induction of IFN-γ production by CD8 T cells following RLI. These results underscore the importance of alloreactive CD8 T cells as the major producer of RLI-induced IFN-γ. Our previous data showed that depletion of CD8 T cells following RLI led to loss of the serum IFN-γ increase seen at day 7 post RLI (5), suggesting that CD8 T cells were the main producers or facilitated the production of IFN-γ.

In summary, our studies have revealed a critical role for recipient iNKTs in RLI-induced anti-tumor immunity. These current findings not only advance our understanding of the mechanisms of RLI-induced anti-tumor effects, but also demonstrate that the anti-tumor effect of RLI can be augmented by using specific activators of iNKTs. The iNKT agonist αGalCer has been found to be safe in clinical trials (27–29). Therefore, more extensive efforts are warranted to further elucidate the mechanisms by which iNKTs promote the anti-tumor effects of RLI.
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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental data

**Figure S1**

Expression of NK1.1 on NK and iNKT cells and efficacy of NK and iNKT cell depletion by anti-NK1.1 antibody. (A) Proportions of NK cells (NK1.1+ DX5+) and NKT cells (CD1d<sup>tet+</sup>) among live B220-negative cells in the liver and the spleen are shown. (B) Expression of CD3 and NK1.1 on CD1d<sup>tet+</sup> NKT cells are shown. (C) Depletion of NK and NKT cells in the liver and the spleen by anti-NK1.1 mAb or anti-asialoGM1 is shown. One representative of two samples in each group is shown.

**Figure S2**

Serum IFN-γ level following RLI. Serum IFN-γ level in MC receiving RLI (filled symbols) and no RLI (open symbols) was measured at different time points following RLI.