TCR-Dependent and -Independent Activation Underlie Liver-Specific Regulation of NKT Cells


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The fate of invariant NKT (iNKT) cells following activation remains controversial and unclear. We systematically examined how iNKT cells are regulated following TCR-dependent and -independent activation with α-galactosylceramide (αGC) or IL-18 plus IL-12, respectively. Our studies reveal activation by αGC or IL-18 plus IL-12 induced transient depletion of iNKT cells exclusively in the liver that was independent of caspase 3-mediated apoptosis. The loss of iNKT cells was followed by repopulation and expansion of phenotypically distinct cells via different mechanisms. Liver iNKT cell expansion following αGC, but not IL-18 plus IL-12, treatment required an intact spleen and IFN-γ. Additionally, IL-18 plus IL-12 induced a more prolonged expansion of liver iNKT cells compared with αGC. iNKT cells that repopulate the liver following αGC had higher levels of suppressive receptors PD-1 and Lag3, whereas those that repopulate the liver following IL-18 plus IL-12 had increased levels of TCR and ICOS. In contrast to acute treatment that caused a transient loss of iNKT cells, chronic αGC or IL-18 plus IL-12 treatment caused long-term systemic loss requiring an intact thymus for repopulation of the liver. This report reveals a previously undefined role for the liver in the depletion of activated iNKT cells. Additionally, TCR-dependent and -independent activation differentially regulate iNKT cell distribution and phenotype. These results provide new insights for understanding how iNKT cells are systemically regulated following activation. The Journal of Immunology, 2011, 186: 838–847.

In contrast to acute treatment that caused a transient loss of iNKT cells, chronic αGC or IL-18 plus IL-12 results in an inability to detect iNKT cells in the liver (4). However, the in vivo mechanisms underlying the regulation of iNKT cells by this treatment remain unclear. Whereas one report showed IL-12 depleted liver NKT cells with repopulation deriving from the bone marrow (10), another report found that during Listeria monocytogenes infection NK1.11# iNKT cells were reduced by internalizing NK1.11 receptor in an IL-12-dependent manner (11).

TCR engagement represents another major pathway of iNKT cell activation. In contrast to conventional T cells that recognize a variety of peptide Ags presented by MHC I or II receptors, iNKT cells have a more restricted TCR repertoire (12, 13) that recognizes glycolipids presented by CD1d receptor (14, 15). Most studies examining antigenic TCR activation of iNKT cells use α-galactosylceramide (αGC) (1). Studies examining iNKT cell fate following αGC activation report conflicting results. Early reports showed triggering of iNKT cells through the TCR with αGC and anti-CD3 induced a rapid loss of iNKT cells that was attributed to apoptosis (5, 10). In contrast, subsequent studies reported that iNKT cells downmodulate TCR following acute αGC activation, rendering them undetectable by standard flow cytometric methods (16–18).

In this study, we comprehensively examined iNKT cell regulation in lymphoid tissues following both acute and chronic TCR-dependent and -independent activation with αGC and IL-18 plus IL-12, respectively. We found a previously undefined role for the liver for depleting activated iNKT cells. Furthermore, we found differential reshaping of the iNKT cell phenotype that depended

Abbreviations used in this article: CD1d-tet, CD1 tetramer; C, cycle threshold; FCA, flow cytometric analysis; αGC, α-galactosylceramide; iNKT, invariant NKT; qPCR, quantitative PCR; splx, spleenectomized; thx, thymectomized; TNC, total number of cells; VC, vehicle control.

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on the nature of activation. These findings suggest a previously unappreciated adaptability of iNKT cells in response to micro-environmental signals through differential stimulation of their activating and/or regulatory cell surface receptors.

Materials and Methods

Mice and treatment approaches

Approval for the animal experimentation presented in this study was received from the Institutional Animal Care and Use Committee at the National Cancer Institute (Office of Laboratory Animal Welfare Assurance A4159-01). C57BL/6 mice were obtained from the Animal Production Area of the National Cancer Institute Cancer Research and Development Center. IFN-γ knockout mice (strain B6.129S7-Ifngtm1Ts/J) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were bred in a dedicated pathogen-free environment at the National Cancer Institute animal facility. All mice were between 8 and 16 wk of age at the start of the experiment.

Murine rIL-12 was purchased from PeproTech (Rocky Hill, NJ), murine rIL-18 was kindly provided by GlaxoSmithKline (Upton Merion, PA), and oGC was obtained from Kirin Brewery or purchased from Alexis Biochemicals. IL-12 or IL-18 stock aliquots of cytokines were diluted with HBSS containing 0.1% (v/v) sterile-filtered C57BL/6 normal serum. For acute treatment, mice were injected i.p. on day 0 with vehicle control (VC; HBSS containing 0.1% C57BL/6 normal serum), IL-12 (0.5 μg), and IL-18 (0.3 μg), or oGC (1 μg). For chronic treatment, mice were given VC or IL-18 plus IL-12 on days 0–4 and 8–10 or oGC on days 0, 2, 4, 8, and 10 via i.p. injection. The general caspase inhibitor Q-Val-Asp(non-omethylated)-OPH (Q-VD-OPh) (Sigma-Aldrich) was dissolved in DMSO at a concentration of 20 mM, and was administered 20 mg/kg.

Cell isolation

To isolate liver leukocytes, mice were euthanized. The liver was flushed with 10 ce cold HBSS through the portal vein. The liver(s) was removed, placed in a stomacher bag (VWR International) containing 25 ml cold wash buffer (HBSS containing 0.1% BSA [Sigma-Aldrich] and 0.5 mM EDTA [Invitrogen]), and disrupted for 30 s with a stomacher 80 (Seward, West Sussex, U.K.) set for 30 s on medium setting. The disrupted liver was collected and washed with wash buffer by centrifugation at 325 × g for 10 min at 4 °C. The resulting pellet was resuspended in 40% Percoll (VWR International, Suwanee, GA) and then underlaid with 80% Percoll. Percoll solutions were made by diluting Dulbecco’s PBS-Percoll mixture (1 part 10X Dulbecco’s PBS to 9 parts Percoll) with DMEM (Mediatech, Manassas, VA) to the proper concentration. The Percoll gradient was centrifuged at room temperature for 25 min at 850 × g. The leukocytes were collected at the interface and washed twice in cold wash buffer.

Isolation of lung leukocytes was done by mincing the lung with sharp curved iris scissors and then shaking the minced tissue in 25 ml complete RPMI 1640 media containing 700 U/ml collagenase type I (Worthington) and DNase 1 (0.5 mg/ml; Sigma-Aldrich) for 30 min at 37°C. The resulting pellet was resuspended in 40% Percoll (VWR International, Suwanee, GA) and then underlaid with 80% Percoll. Percoll solutions were made by diluting Dulbecco’s PBS-Percoll mixture (1 part 10X Dulbecco’s PBS to 9 parts Percoll) with DMEM (Mediatech, Manassas, VA) to the proper concentration. The Percoll gradient was centrifuged at room temperature for 25 min at 850 × g. The leukocytes were collected at the interface and washed twice in cold wash buffer.

Bone marrow cells were recovered by flushing the femurs and tibias. Leukocytes were isolated from themys, spleen, and lymph nodes (pooled inguinal and axillary) by mechanical disruption through a filter-bag mesh (Fisher Scientific) placed in a stomacher bag (VWR International) containing 25 ml cold wash buffer (HBSS containing 0.1% BSA [Sigma-Aldrich] and 0.5 mM EDTA [Invitrogen]), and disrupted for 30 s using a stomacher 80 (Seward, West Sussex, U.K.) set for 30 s on medium setting. The disrupted liver was collected and washed with wash buffer by centrifugation at 325 × g for 10 min at 4 °C. The resulting pellet was resuspended in 40% Percoll (VWR International, Suwanee, GA) and then underlaid with 80% Percoll. Percoll solutions were made by diluting Dulbecco’s PBS-Percoll mixture (1 part 10X Dulbecco’s PBS to 9 parts Percoll) with DMEM (Mediatech, Manassas, VA) to the proper concentration. The Percoll gradient was centrifuged at room temperature for 25 min at 850 × g. The leukocytes were collected at the interface and washed twice in cold wash buffer.

Lymphocyte counts were determined from the isolated leukocytes using a Sysmex KX-21 (Roche, Indianapolis, IN) automated cell counter.

Flow cytometric analysis

Data were collected using a LSRII Special Order System equipped with solid state blue (488 nm), red (640 nm), and violet (405 nm) lasers, and then analyzed using FlowJo Express v3 (De Novo software). Appropriately titrated mAbs against mouse CD4 (FITC or Pacific Blue; clone RM4-5), NK1.1 (PE or PerCP-Cy5.5; clone PK136), Lag3 (PE; clone eBio907B7), ICOS (PE; clone 7E.17G9), PD-1 (Fic; 343), and CD28 (PerCP/Cy5.5; clone 37.51) were purchased from eBioscience; CD45 (Pacific Orange; clone 30-F11), PD-1 (Fic; J43), and CD28 (PerCP/Cy5.5; clone PK136), Lag3 (PE; clone eBioC9B7W), ICOS (PE; clone 37.51) and CD8 (PE; clone 53–5.3) were purchased from eBioscience; CD1d tetramer (CD1d-tet) loaded with PBS-57 was prepared by the National Institute of Allergy and Infectious Diseases MH C Tetramer Core Facility (Atlanta GA). Cytox/ Cytoperm fixation/permeabilization kit (BD Pharmingen) was used for intracellular staining of caspase 3.

Cell proliferation was detected by injecting mice i.p. with BrdU (Sigma-Aldrich) dissolved in PBS at 100 ng/ml. BrdU incorporation was detected using a BrdU flow kit (BD Pharmingen) and analyzed by flow cytometric analysis (FCA).

Assessing relative changes in NKT cell concentration from mouse tissue using real-time PCR

Mice were treated acutely or chronically with VC, oGC, or IL-12 plus IL-12, and leukocytes were isolated from liver, spleen, lung, lymph node, bone marrow, and thymus tissues, as described above. Genomic DNA was isolated using a DNeasy kit (Qiagen), according to the manufacturer’s protocol for cultured animal cells. Genomic DNA was probed for Vα14/18 TCR gene rearrangement (target gene) (18) and 18S or actin (reference gene) using TaqMan primers and universal master mix all purchased from Applied Biosystems, and analyzed on an ABI 7300 real-time PCR system (Applied Biosystems). The relationship of the iNKT cell concentration (Vα14/18) with respect to total cell number (18S or actin) was calculated using the average Δ cycle threshold (Ct) method (ABI system software) from each sample run in triplicate. The Equation 2 was applied to express relative levels of Vα14/18 with respect to 18S or actin in linear form. Because treatments can modulate total cell numbers in each tissue that may mask absolute changes in the level of Vα14/18 signal, we multiplied 2−ΔΔCt by the TCN from each tissue to determine Vα14/18 levels in each tissue. The fold change relative to the VC was calculated using the equation:

\[ \frac{2^{\Delta C_{\text{TCN}}}}{2^{\Delta C_{\text{TCN,VC}}}} \times \text{TCN}_{\text{treatment}} \]

SEM was determined from six biological replicates from two separate experiments.

Statistical analysis

The p values were determined by the nonparametric Mann-Whitney U test using GraphPad Prism 5 software for experiments with five or more samples and Student t tests for samples size of <5. The data were considered significant if the two-tailed test was p < 0.05.

Results

IL-18 plus IL-12 and oGC-induced loss of iNKT cell detection was not dependent on caspase 3 activity

To compare TCR-dependent and -independent regulation of iNKT cells, we treated mice i.p. with oGC or IL-18 plus IL-12 and examined the modulation of iNKT cells from liver and spleen at 2 and 24 h using FCA of cells stained with PBS-57–loaded CD1d-tet. A reduction in detection of absolute iNKT cell numbers was observed at 2 h in the liver with both treatments (Fig. 1A). In contrast, no loss of iNKT cells was detected at 2 h in the spleen. By 24 h, iNKT cell detection was reduced in both liver and spleen with both treatments. NK1.1 flow cytometric subset analysis revealed IL-18 plus IL-12 reduced NK1.1+ iNKT cell detection in both liver and spleen by 24 h similar to IL-12 activation (11) (Fig. 1A, 1B). Consistent with previous reports (5, 19), we found oGC reduced the detection of all iNKT cells in the liver. However, in the spleen we found oGC was comparable to IL-18 plus IL-12 for the reduction of only NK1.1+ iNKT cell detection.

Previous studies found HIV (20, 21) and hepatitis C virus (22) infections caused the loss of iNKT cells in humans. Furthermore, lymphocytic choriomeningitis virus infection of mice caused the loss of iNKT cells by caspase 3-mediated apoptosis (23, 24). To determine whether the loss of iNKT cells following oGC or IL-18 plus IL-12 was due to caspase 3-mediated apoptosis, mice were treated with VC, oGC, or IL-18 plus IL-12, and leukocytes were isolated from the livers and spleens at 2 and 4 h for oGC and 4, 6, and 8 h for VC and IL-18 plus IL-12 treatment groups. Isolated leukocytes were surface stained with CD1d-tet and intracellularly stained with anti-active caspase 3 (Fig. 2A). No change in active caspase 3 was detected in iNKT cells from the liver and spleen of
mice treated with αGC for 2 and 4 h despite >50% loss by 2 h and an almost complete loss of liver iNKT cells by 4 h. In addition, no change in active caspase 3 was detected in iNKT cells from the liver and spleen of mice treated with IL-18 plus IL-12 for 4, 6 (data not shown), and 8 h (Fig. 2A). In contrast, control liver leukocytes cultured in the presence of 5 μM staurosporine for 4 h had significant levels of intracellular active caspase 3, signifying these cells were undergoing apoptosis (25) (Fig. 2B). As a second approach to determine whether liver iNKT cells were cleared by apoptosis, mice were pretreated for 2 h with broad-spectrum caspase inhibitor Q-VD-OPh to block caspase-mediated apoptosis (26) and then treated with VC, αGC, and IL-18 plus IL-12. iNKT cells from the liver of treated mice were quantitated using FCA of isolated liver leukocytes that stain CD1-tet. As shown in Fig. 2C, the caspase inhibitor Q-VD-OPh was unable to prevent the loss of iNKT cells following αGC treatment at 2 h or IL-18 plus IL-12 treatment at 8 h. Thus, activation of iNKT cells did not induce detectable levels of active caspase 3, and blocking broad-spectrum capases was unable to prevent the loss of iNKT cells, suggesting this loss was by a mechanism other than caspase 3-mediated apoptosis.

IL-18 plus IL-12 and αGC induced the loss of iNKT cells selectively in the liver

Because iNKT cells were differentially regulated in spleen and liver following αGC or IL-18 plus IL-12 treatment of mice, we
systemically examined their regulation in tissues where iNKT cells are known to reside or accumulate. Mice were treated with VC, aGC, or IL-18 plus IL-12, and leukocytes were isolated from liver, spleen, bone marrow, lung, thymus, and lymph nodes. The absolute number of iNKT cells was then determined by FCA of cells that stain with CD1d-tet (Fig. 3A). All tissues examined, except lymph nodes, showed a decrease in CD1d-tet–visualized iNKT cells following aGC and IL-18 plus IL-12 treatment. Instead of apoptosis, treatment of mice with aGC has been shown to cause TCR internalization that renders iNKT cells undetectable by flow cytometric techniques (16–18). Thus, to rigorously investigate iNKT cell regulation, we compared the fold change of genomic levels of Vα14 Jα18 TCR gene arrangement (specific to iNKT cells) using quantitative PCR (qPCR) with the fold change in CD1d-tet–visualized iNKT cells using FCA. Treatment of mice with aGC reduced, at 24 h, the level of CD1d-tet–visualized iNKT cells in lung as well as previously reported tissues, including liver, spleen, bone marrow, and thymus, but not lymph nodes (16, 17), as determined by FCA (Fig. 3B). IL-18 plus IL-12 also decreased the number of detectable iNKT cells in all of the examined tissues except lymph nodes. However, qPCR analysis revealed the liver was the only tissue that had a significant reduction in the level of iNKT cell-specific Jα14 Vα18 TCR gene arrangement following either IL-18 plus IL-12 or aGC treatment (Fig. 3B). Notably, for both qPCR and FCA, we evaluated the fold change per organ by standardizing the quantitation with the overall cell number to avoid treatment-induced changes in total cell numbers that would mask specific iNKT cell changes.

Taken together, these results reveal that the liver microenvironment is distinct from other organ sites for the depletion of activated iNKT cells. Whereas acute activation with aGC or IL-18 plus IL-12 caused iNKT cell detection to be lost by TCR internalization in most organs, iNKT cells in the liver were almost entirely depleted with aGC and substantially depleted with IL-18 plus IL-12 treatments.

Expansion of iNKT cells in the liver following aGC, but not IL-18 plus IL-12 treatment requires an intact spleen and IFN-γ Treatment of mice with aGC initiates a well-defined sequence of iNKT modulation, beginning with rapid disappearance at day 1, followed by expansion by day 3, and finally a Bim-dependent contraction by approximately day 5 (27). To determine whether IL-18 plus IL-12 treatment induces expansion and contraction of liver iNKT cells similar to aGC, mice were treated with VC, aGC, or IL-18 plus IL-12, and then leukocytes were isolated from the liver at days 0, 1, 3, 5, and 7. Both IL-18 plus IL-12 and aGC treatment decreased iNKT cells at day 1, followed by expansion by day 3 as compared with VC. However, liver iNKT cell levels from aGC-treated mice returned to baseline by day 5, whereas IL-18 plus IL-12–treated mice maintained peak levels of liver iNKT cells that persisted at least through day 7 (Fig. 4A, left panel).

Previous reports have proposed that aGC causes iNKT cells to simultaneously internalize TCR receptors and proliferate, rendering their expansion undetectable to conventional staining of cell surface molecules. Not until day 3 is iNKT cell expansion revealed by TCR re-expression (16–18). However, results presented in this study (Fig. 3) demonstrate that iNKT cells are depleted in the liver following aGC and IL-18 plus IL-12 treatment, suggesting proliferating resident liver iNKT cells are not likely the major source of expansion, but instead, more likely the result of recruitment from other sites. Two of the possible sites from which iNKT cells may be recruited are the spleen, which hosts a large number of iNKT cells, and the thymus, which is the site of iNKT cell development. To test these hypotheses, we analyzed iNKT cell expansion in aGC and IL-18 plus IL-12–treated mice that had...
been previously thymectomized (thx) or splenectomized (splx). We found expansion of iNKT cells at day 3 was not compromised in thx mice treated with αGC, consistent with a previous report (17), or with IL-18 plus IL-12 (Fig. 4 A, right panel). In contrast, splx mice did not expand iNKT cells following αGC treatment and only partially increased iNKT cell numbers with IL-18 plus IL-12 treatment as compared with respectively treated sham control mice (Fig. 4 A, center panel). This finding is consistent with recruitment either from the spleen or via the spleen as the source of iNKT cell expansion in the liver following αGC treatment. Because IL-18 plus IL-12-induced expansion was only partially blocked in splx mice, this suggests at least some of the increase in response to this type of activation could be due to proliferating resident iNKT cells (Fig. 4 A, center panel). Additionally, the bone marrow could contribute to the expansion of liver iNKT cells as this site has been previously reported to be a source for IL-12–induced expansion of liver NKT cells (defined by CD3+ NK1.1+ cells) (10).

Because NK1.1−iNKT cells persist in the liver following IL-18 plus IL-12 treatment, and their expansion only partially requires an intact spleen, we hypothesized that a portion of iNKT cell expansion could arise from NK1.1−iNKT proliferation. To test this hypothesis and determine whether αGC induces the recruitment of proliferating NK1.1−iNKT cells, we treated splx and sham control mice with αGC or IL-18 plus IL-12, and then on days 1 and 3 leukocytes were isolated from the liver and total iNKT cell numbers, or on day 3 NK1.1+ and NK1.1+ iNKT cell numbers were determined, as described in Fig. 1. Bars depict mean ± SEM representing nine individual animals from three independent experiments.

![Figure 4](http://www.jimmunol.org/)
to proliferating iNKT cells recruited to the liver via the spleen. However, splx mice treated with IL-18 plus IL-12 were virtually identical to sham control mice in the proportion of proliferating iNKT cells (red dots) were visualized by side and forward flow cytometry scatter, respectively, on back gated lymphocytes that stained double positive for CD1d-tet and CD45 markers. The dot plots shown are representative of nine animals from three independent experiments. B. Expression levels of iNKT cell effector and regulatory surface markers. Leukocytes from the liver of mice treated with vc, αGC, or IL-18 plus IL-12 were isolated at 2 h, day 1, and day 3, and cells were stained with mAbs for indicated surface markers and iNKT cell markers, as described in A. The line graph represents the mean ± SEM of six individual animals from two independent experiments.

Both αGC and IL-18 plus IL-12 induce IFN-γ that in turn can upregulate adhesion molecules, chemokine, and chemokine receptors necessary for recruitment (28, 29). Thus, we investigated whether IFN-γ was a possible effector cytokine for αGC-induced hepatic recruitment of iNKT cells. Wild-type and IFN-γ−/− mice were treated with αGC and IL-18 plus IL-12 on day 0, and the total number of liver iNKT cells was determined on days 1 and 3, and NK1.1+ and NK1.1− iNKT cells were determined on day 3. Neither NK1.1+ nor NK1.1− iNKT cells expanded in IFN-γ−/− mice following αGC treatment (Fig. 4C). However, no difference in iNKT cell expansion at day 3 was observed between wild-type and IFN-γ−/− mice treated with IL-18 plus IL-12. Collectively,

FIGURE 5. Phenotype of iNKT cells from the liver is differentially reshaped by αGC versus IL-18 plus IL-12 treatment of mice. A. Leukocytes were isolated from the liver of mice 3 d after treatment with VC, αGC, or IL-18 plus IL-12. Size and granularity of iNKT cells (red dots) were visualized by side and forward flow cytometry scatter, respectively, on back gated lymphocytes that stained double positive for CD1d-tet and CD45 markers. The dot plots shown are representative of nine animals from three independent experiments. B. Expression levels of iNKT cell effector and regulatory surface markers. Leukocytes from the liver of mice treated with vc, αGC, or IL-18 plus IL-12 were isolated at 2 h, day 1, and day 3, and cells were stained with mAbs for indicated surface markers and iNKT cell markers, as described in A. The line graph represents the mean ± SEM of six individual animals from two independent experiments.

FIGURE 6. αGC modulation of cell surface markers is differentially regulated on resident versus recruited liver iNKT cells. Sham control or splx mice were treated with VC, αGC, or IL-18 plus IL-12, and 3 d later leukocytes were isolated from the liver, and (A) size and granularity of iNKT cells was determined as described in Fig. 5A, and (B) CD28, TCR, Lag3, PD-1, ICOS, and NK1.1 expression levels were determined, as described in Fig. 5B. The dot plots shown are a representative experiment of three animals that was repeated with similar results. The dot plot represents the mean ± SEM of three individual animals.
these data indicate qualitatively different mechanisms exist between TCR and cytokine activation-induced expansion of liver iNKT cells. \( \alpha \)GC and IL-18 plus IL-12 activation causes divergent phenotypic reshaping of liver iNKT cells

Because \( \alpha \)GC and IL-18 plus IL-12 treatments induced the proliferation of NK1.1\(^{+} \) iNKT cells (Fig. 4B), we examined whether these treatments induced other phenotypic changes in liver iNKT cells. Liver iNKT cell size and granularity were evaluated 3 d after treatment with VC, \( \alpha \)GC, or IL-18 plus IL-12. \( \alpha \)GC caused a substantial increase in iNKT cell size and granularity compared with VC and IL-18 plus IL-12 (Fig. 5A). We next investigated activation-induced changes in cell surface markers known to regulate T cell functions at 2, 24, and 72 h. IL-18 plus IL-12 treatment caused a pronounced increase in iNKT cell TCR and ICOS levels by day 1, demonstrating this treatment induces iNKT cells with an effector phenotype (Fig. 5B). As previously reported (30) and we report in this study, \( \alpha \)GC caused upregulation of costimulatory receptors ICOS and CD28. However, the increase in these molecules was in conjunction with an immediate decrease in TCR levels, an increase in the suppressive surface molecule Lag3, and, as previously reported, PD-1 expression (31).

We also analyzed CD40L expression by iNKT cells, because this signaling pathway is a major effector mechanism by which \( \alpha \)GC induces a proinflammatory cytokine cascade (32). Interestingly, IL-18 plus IL-12 did not induce significant levels of CD40L, whereas \( \alpha \)GC transiently induced CD40L expression that was lost as PD-1 and Lag3 molecules increased. Collectively, these data demonstrate \( \alpha \)GC or IL-18 plus IL-12 treatments differentially regulate activating and inhibitory surface molecules on iNKT cells in the liver microenvironment, causing a shift in phenotype.

\( \alpha \)GC, but not IL-18 plus IL-12, treatment requires an intact spleen for maximal induction of inhibitory and activating coreceptors

We next compared the activation phenotype of resident versus recruited liver iNKT cells following \( \alpha \)GC or IL-18 plus IL-12 treatment of sham control and splx mice. Sham control and splx mice were treated with \( \alpha \)GC, and on day 3 the size and granularity of liver iNKT cells were analyzed by flow cytometry. \( \alpha \)GC treatment of sham control mice increased iNKT cell size and granularity in sham control mice, but not in splx mice (Fig. 6A), suggesting iNKT cells recruited to the liver are activated differentially compared with iNKT cells resident to the liver. We therefore compared cell surface marker expression on resident and recruited iNKT cells from the liver 3 d after treatment with \( \alpha \)GC and IL-18 plus IL-12 (Fig. 6B). Surface expression levels of TCR, ICOS, and NK1.1 were comparable on liver iNKT cells from IL-18 plus IL-12–treated splx and sham control mice (Fig. 6B). In contrast, iNKT cells from splx mice treated with \( \alpha \)GC had reduced expression levels of CD28, PD-1, and ICOS and slightly more NK1.1 expression compared with sham control mice treated with \( \alpha \)GC. Thus, recruitment via the spleen following \( \alpha \)GC, but not IL-18 plus IL-12, treatment is needed to repopulate the liver with iNKT cells expressing maximal levels of activating and inhibitory receptors.

Chronic treatment with \( \alpha \)GC or IL-18 plus IL-12 induces a profound and systemic iNKT cell depletion that requires the thymus for repopulation of the liver

Because \( \alpha \)GC and IL-18 plus IL-12 treatment causes depletion of liver iNKT cells, we tested whether continual removal of
iNKT cells in the liver induced by chronic αGC or IL-18 plus IL-12 treatment would exhaust systemic iNKT cells. Using qPCR and FCA, we examined changes in iNKT cells in the liver, spleen, bone marrow, lung, thymus, and lymph nodes of mice treated chronically with αGC on days 0, 2, 4, 8, and 10 or IL-18 plus IL-12 on days 0–4 and 8–10. In all tissues examined, chronic IL-18 plus IL-12 treatment profoundly reduced iNKT cell number by the end of treatment as evaluated by both FCA and qPCR analysis (Fig. 7A). Similarly, chronic αGC treatment reduced iNKT cells in all tissues examined, but only a minor reduction was observed in the thymus using qPCR analysis. After cessation of treatment with either agent at day 10, the liver was slowly repopulated with iNKT cells, and levels comparable to those of untreated mice were reached between days 40 and 80 (Fig. 7B). However, the rate by which iNKT cells repopulated the liver varied with treatment. Whereas liver iNKT cell numbers recovered from chronic αGC treatment by day 43, it took until day 80 before these cells recovered from chronic IL-18 plus IL-12 treatment, suggesting that the signaling mechanisms induced by IL-18 plus IL-12 and αGC differentially impact iNKT cell renewal/development (Fig. 7B).

Early studies using surrogate markers presented evidence that NKT cells could undergo extrathymic development (33–35). With the advent of CD1d-tet loaded with iNKT cell Ags, it was reported that development of iNKT cells occurs in the thymus (36–38). To test whether the iNKT cells that repopulate the liver after the systemic depletion induced by chronic treatment with either agent are newly developed cells in the thymus (36), we compared the efficiency of the repopulation in thx and sham control mice. Unlike sham-treated mice, iNKT cells from thx mice treated chronically with αGC or IL-18 plus IL-12 did not repopulate the liver by day 80 (Fig. 7B), demonstrating that thymic development is required to repopulate the liver with iNKT cells following the depletion induced by either agent.

Discussion

In this study, we identify a previously undefined and unique role for the liver in the elimination of iNKT cells following acute activation with αGC or IL-18 plus IL-12. Following αGC-induced depletion of iNKT cells in the liver, our data demonstrated subsequent iNKT cell expansion was likely through the recruitment of iNKT cells via the spleen, whereas IL-18 plus IL-12 expansion was only partially dependent on the spleen. Furthermore, we show cells that expand in the liver following αGC or IL-18 plus IL-12 treatment of mice had divergent phenotypes. Finally, we found whereas acute treatment of mice induced transient depletion of liver iNKT cells, chronic αGC or IL-18 plus IL-12 treatment caused a systemic and persistent depletion of iNKT cells that required an intact thymus for subsequent repopulation of the liver.

Initially, it was reported that iNKT cells undergo apoptosis following activation with αGC, anti-CD3, or IL-12 (5, 10). More recently, several elegant studies proposed that upon activation with αGC, iNKT internalize their TCR instead of undergoing apoptosis and rapidly expand (16–18). Our data provide evidence that iNKT cells are differentially regulated following activation depending on the microenvironment in which they reside. Our data show that treatment of mice with αGC induces an almost complete loss of iNKT cells, whereas IL-18 plus IL-12 induce a partial loss of iNKT cells specifically in the liver. However, TCR internalization by iNKT cells occurs in all other tissues tested except the lymph node, as determined by FCA and qPCR analysis. Two of the studies that reported TCR internalization by activated iNKT cells focused on iNKT cells from the spleen, which is in agreement with our findings. Similar to our results, using CD1d-tet staining, Crowe et al. (16) found liver iNKT cells disappeared following αGC activation and expanded 3 d later. However, in an apparent contrast to our results, they proposed the loss of liver iNKT cells following αGC treatment of mice was due to receptor internalization (16). This discrepancy is most likely due to the use of intracellular staining with NK1.1 and αβTCR markers used in that study to detect NKT cells with internalized receptors following αGC activation, whereas our study used qPCR analysis of genomic levels of Vα14 Jα18 TCR gene arrangement. NK1.1 and αβTCR markers are not always synonymous with iNKT cells and can be expressed by activated T cells and noninvariant NKT cells (16, 28, 37, 39, 40). The use of these markers can be further complicated by the fact that αGC activation of iNKT cells by dendritic cells can cause bystander activation and proliferation of T cells (41, 42). Thus, the proliferation of NK1.1+ αβTCR+ cells, and the IFN-γ production by cells that have their αβTCR internalized, could be an αGC-induced bystander effect on T cells or noninvariant NKT cells. However, it is likely that liver iNKT cells are internalizing their TCR following αGC activation, but as a precursor to elimination.

From data presented in this work, we propose a more complex model in which the liver can be an important site for the elimination of activated iNKT cells. Consistent with our model, it has been shown that the liver also eliminates activated CD8 T cells (43). In this process, activated CD8 T cells are trapped in the liver primarily due to ICAM interactions and subsequently cleared by apoptosis. Similar to this process, iNKT cells were also shown to undergo migratory arrest in the liver following αGC and IL-18 plus IL-12 treatment of mice (44). These results suggest unique mechanisms exist in the liver that immobilize activated iNKT cells with subsequent eradication. Our model, in which activated iNKT cells are eliminated by liver-specific mechanisms, would explain why studies examining iNKT cells in vitro found no apoptosis in response to αGC activation. Additionally, reports that found NKT cells are relatively resistant to apoptosis (45) are consistent with our finding that the loss of iNKT cells appears to be independent of caspase 3-mediated apoptosis.

Reinterpretation of the current paradigm of iNKT cell regulation is further supported by data obtained with αGC-induced expansion of iNKT cells. Treatment of mice with αGC induces an expansion of NK1.1+ iNKT cells that becomes apparent at day 3. Whereas previous studies proposed that αGC-induced expansion was from local proliferation of iNKT cells that had internalized TCR, we clearly show that full expansion of liver NK1.1+ iNKT cells requires an intact spleen and IFN-γ, suggesting recruitment is the mechanism for iNKT cell expansion. Recruitment of iNKT cells to the liver has been previously shown in a study that found sulfatide activation of type II NKT cells caused the recruitment of iNKT cells to the liver in an IL-12– and MIP-2–dependent manner (46). In contrast to αGC treatment, we found acute IL-18 plus IL-12 treatment-induced expansion of iNKT cells was independent of IFN-γ and only partially dependent on an intact spleen, demonstrating a mechanism distinct from that induced by αGC. A possible explanation for this difference is that IL-18 plus IL-12 removes only a subset of iNKT cells, unlike αGC treatment that removes all subsets of liver iNKT cells. IL-18 plus IL-12 causes the loss of the NK1.1+ subset of iNKT cells either through receptor internalization, as previously reported (11, 16), or through targeted deletion of this subset. Therefore, expansion of iNKT by IL-18 plus IL-12 could be due to proliferation of the remaining iNKT cells.

Differences between αGC and IL-18 plus IL-12 activation of iNKT cells are further demonstrated at the contraction phase. Following αGC-induced expansion, iNKT cell numbers contract.
to baseline around day 5 (27). In contrast, IL-18 plus IL-12–induced expansion of iNKT cells remained at maximal levels out to at least day 7. Taken together, these results indicate that TCR-dependent and -independent activation induce disparate programmed responses in iNKT cells and illustrate the potential to reshape effector or regulatory functions of these cells. This difference was further highlighted by the shift in iNKT cell phenotype. IL-18 plus IL-12 treatment induced the expansion of liver iNKT cells that have higher levels of TCR and ICOS, both of which can enhance effector functions (47, 48), whereas αGC-induced TCR reduction and increased suppressive molecules PD-1 and Lag3 are shown to inhibit iNKT cell proliferation and function (31, 49). Differences between αGC and IL-18 plus IL-12 for the induction of various inhibitory and activating receptors could be due to feedback of specific cytokines that these stimuli may differentially induce. For example, IL-18 plus IL-12 preferentially induces iNKT cells to produce IFN-γ (50), whereas αGC induces iNKT cells to produce numerous other cytokines such as IL-4, IL-17, and IL-13 (51–54). Thus, iNKT cell phenotype can be reshaped with type of activation.

Whereas acute activation by αGC and IL-18 plus IL-12 caused a transient loss in liver iNKT cells, we found that chronic stimulation with these agents caused a systemic loss of iNKT cells that required an intact thymus for repopulation of the liver. Repopulation of the liver following chronic αGC treatment of mice occurred with faster kinetics than did IL-18 plus IL-12, which is probably due to αGC and IL-18 plus IL-12 targeting iNKT cells at different stages of development. Whereas IL-18 plus IL-12 was shown to disrupt developing cells at an early CD4+/CD8+ double-negative stage (3, 55), αGC targets iNKT cells that have acquired the properly rearranged TCR, after differentiating from a CD4+ CD8+ cell precursor. Furthermore, our data show iNKT cell reduction of thymic iNKT cells by chronic αGC was less than by chronic IL-18 plus IL-12 treatment.

The data presented in this study show acute or chronic activation of iNKT cells has distinctly different effects on the fate of iNKT cells that is determined by the liver microenvironment. Additionally, chronic TCR-dependent and -independent inflammatory signals can reshape iNKT cell phenotype and frequency.

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Disclosures

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References


