NKG2A Inhibits Invariant NKT Cell Activation in Hepatic Injury[1]

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Activation of invariant NKT (iNKT) cells in the liver is generally regarded as the critical step for Con A-induced hepatitis, and the role of NK cell receptors for iNKT cell activation is still controversial. In this study we show that blockade of the NKG2A-mediated inhibitory signal with antagonistic anti-NKG2A/C/E mAb (20d5) aggravated Con A-induced hepatitis in wild-type, Fas ligand (FasL)-mutant *gld*, and IL-4-deficient mice even with NK cell and CD8 T cell depletion, but not in perforin-, IFN-γ-, or IFN-γ- and perforin-deficient mice. Consistently, 20d5 pretreatment augmented serum IFN-γ levels and perforin-dependent cytotoxicity of liver mononuclear cells following Con A injection, but not their FasL/Fas-dependent cytotoxicity. However, blockade of NKG2A-mediated signals during the cytotoxicity effector phase did not augment cytotoxic activity. Activated iNKT cells promptly disappeared after Con A injection, whereas NK1$^-$ iNKT cells, which preferentially expressed CD94/NKG2A, predominantly remained in the liver. Pretreatment with 20d5 appeared to facilitate disappearance of iNKT cells, particularly NK1$^-$ iNKT cells. Moreover, Con A-induced and α-galactosylceramide-induced hepatic injury was very severe in CD94/NKG2A-deficient DBA/2J mice compared with CD94/NKG2A-intact DBA/2JJcl mice. Overall, these results indicated that a NKG2A-mediated signal negatively regulates iNKT cell activation and hepatic injury. ***The Journal of Immunology,*** **2009, 182: 250–258.**

Invariant NKT (iNKT)[4] cells are a special T cell population coexpressing NK cell receptors and a TCR consisting of an invariant Vα14Jα18 chain (Vα24Jα15 in humans) and a semivariant TCR β-chain that is largely biased toward Vβ8.2 (Vβ11 in humans), Vβ2, and Vβ7 (1, 2). This TCR recognizes glycolipid Ags such as α-galactosylceramide (α-GalCer) and isoglobotrihexosylceramide presented on the MHC class I-like molecule, CD1d (1–3). One of most striking characteristics of iNKT cells is their ability to promptly secrete various cytokines, including both IFN-γ and IL-4, after their TCR ligation, and activated iNKT cells exert perforin- and Fas ligand (FasL) mediated-cytotoxicity (1, 2). iNKT cells distribute preferentially in the liver, and both iNKT cell-produced cytokines and their cytotoxic activity critically contribute to Con A-induced and α-GalCer-induced hepatitis (4–6).

NK cell receptors are thought to play roles in controlling iNKT cell responses because NK cell receptors control NK cell and conventional CD8 T cell activation when they interact with their cognate ligands (7, 8). Qa-1^b, a MHC class I-like molecule, can interact with CD94/NKG2 complexes that are mostly expressed in mice as inhibitory CD94/NKG2A (9–12). Thus, the Qa-1^b-CD94/NKG2A interaction is thought to play an inhibitory role in NK cell responses to self, particularly when interacting with other lymphoid cells (7–13). Although CD94/NKG2A, but not other CD94/NKG2 complexes, are expressed on iNKT cells, their function is still controversial (14, 15).

In this study we now demonstrate that pretreatment with antagonistic anti-NKG2A/C/E mAb (20d5) significantly augmented Con A-induced hepatic injury even in NK cell- and CD8 T cell-depleted wild-type (WT) mice, *gld* mice, and IL-4-deficient mice, but not in IFN-γ-, perforin-, or IFN-γ and perforin-deficient mice. Consistently, pretreatment with 20d5 augmented serum IFN-γ levels and perforin-mediated cytotoxicity of hepatic mononuclear cells (MNC). However, inhibition of NKG2A-mediated signals in the effector phase did not effect cytotoxic activity. NK1$^-$ iNKT cells preferentially expressed NKG2A and predominantly remained during Con A-induced hepatitis, whereas pretreatment with 20d5 augmented the disappearance of NKG2A-expressing iNKT cells in the liver. These results suggested that CD94/NKG2A negatively regulates iNKT cell responses to Con A and Con A-induced hepatitis, similarly as the CD94/NKG2A inhibitory effect on iNKT cell activation via TCR ligation by specific ligands (14, 15). Consistently, Con A-induced and α-GalCer-induced hepatic injury were severe in CD94/NKG2A-deficient DBA/2J mice compared with CD94/NKG2A-intact DBA/2JJcl mice. Thus, CD94/

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[4] Abbreviations used in this paper: iNKT, invariant natural killer T cell; ALT, alanine aminotransferase; ASGM-1, asialo GM1; AST, aspartate aminotransferase; B6, C57BL/6 (mice); CMA, concanamycin A; FasL, Fas ligand; α-GalCer, α-galactosylceramide; MNC, mononuclear cell; WT, wild type.

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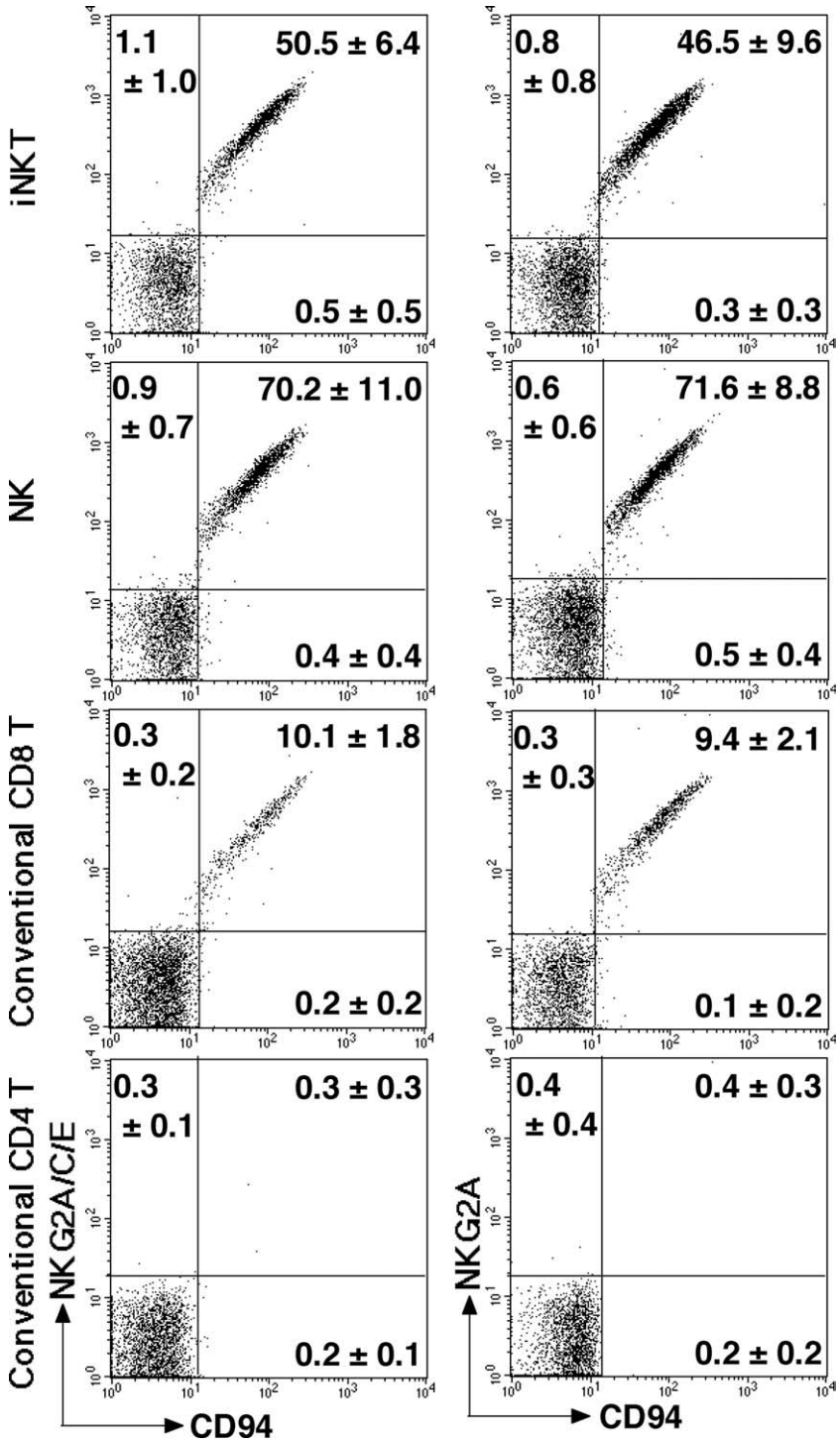

FIGURE 1. CD94/NKG2A, but not CD94/NKG2C or E, expression on hepatic iNKT, NK, and conventional CD8 T cells. Cell surface expression of CD94, NKG2A/C/E, and NKG2A was analyzed on electronically gated hepatic iNKT, NK, conventional CD8, and conventional CD4 T cells freshly isolated from B6 mice.

NKG2A is a major regulator of iNKT cells when activated via their TCR.

Materials and Methods

Mice

C57BL/6 (B6) WT mice and *gld* mice were obtained from Charles River Japan. B6 IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice, perforin-deficient (perforin$^{-/-}$) mice, IL-4-deficient (IL-4$^{-/-}$) mice, and DBA/2J lacking CD94 (10) were obtained from The Jackson Laboratory. B6 IFN-γ and perforin-deficient (IFN-γ/perforin$^{-/-}$) mice were bred at the Peter MacCallum Cancer Centre. DBA/2JJcl expressing CD94 were obtained from CLEA Japan. All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of Juntendo University (Tokyo, Japan), Niigata University (Niigata Japan), and Peter MacCallum Cancer Centre (East Melbourne, Australia).

Reagents

A synthetic form of α-GalCer was provided by Kirin Brewery and was dissolved in pyrogen-free PBS and i.p. injected to mice (6). PE-conjugated tetrameric CD1d molecules loaded with α-GalCer (α-GalCer/CD1d) were prepared as described (16). The anti-NKG2A/C/E mAb (20d5) and anti-CD8 mAb (53-6.7) were generated as described previously (14, 17). Control rat IgG and LPS were purchased from Sigma-Aldrich. A neutralizing anti-mouse FasL mAb (MFL3) was obtained from BD Biosciences. Concanamycin A (CMA), which inhibits perforin-mediated cytotoxicity (18), and anti-asialo GM1 (ASGM1) Ab were purchased from Wako Pure Chemicals.

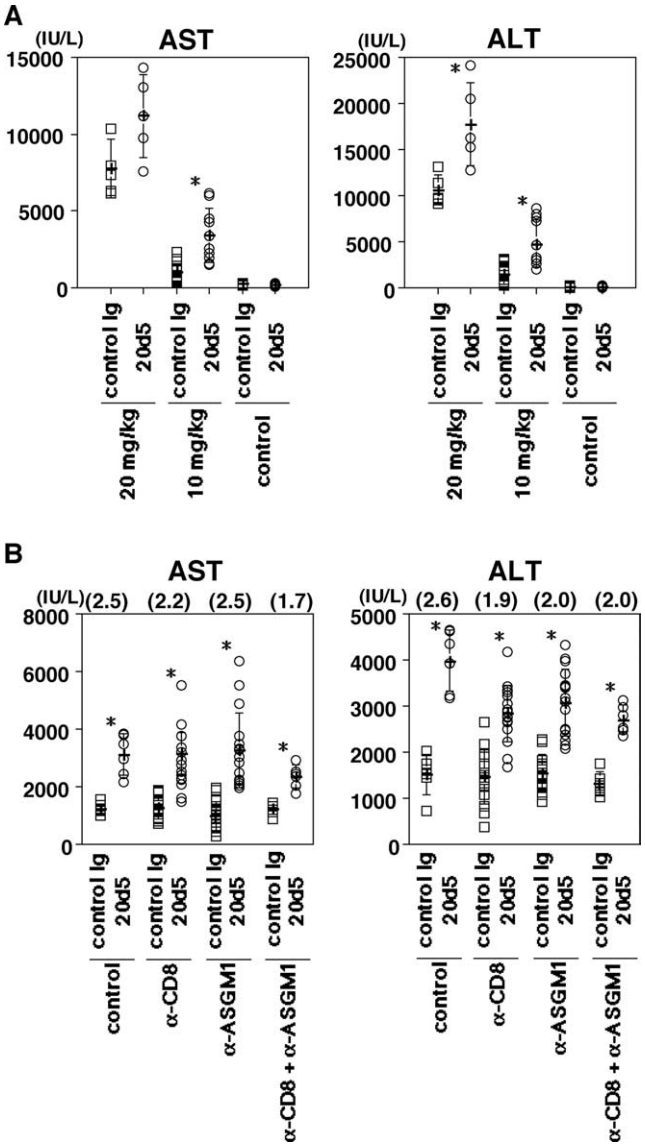

FIGURE 2. Augmented serum AST and ALT levels following Con A injection by NKG2A signal blockade. *A*, Mice were treated with i.p. injection of control Ig (□) or anti-NKG2A/C/E mAb (20d5) (○) 2 days before i.v. injection of the indicated amount of Con A. Serum samples were obtained from 5–20 mice in each group 16 h after Con A injection. *B*, Mice were i.p. treated with the indicated Abs (α-CD8 and α-ASGM1, where "α-" represents "anti-") to deplete NK cells or CD8 T cells 8 h before control Ig (□) or 20d5 (○) treatment. Two days later, these mice were i.v. injected with Con A (10 mg/kg). Serum samples were obtained from 5–20 mice in each group 16 h after Con A injection. The relative increasing ratio of AST or ALT following pretreatment with 20d5 compared with the treatment with control Ig is indicated in parentheses. Data are represented as the mean ± SD. *, $p < 0.05$ as compared with control Ig-treated mice. Similar results were obtained from three independent experiments.

Induction of Con A-induced hepatitis

Con A (Sigma-Aldrich) was dissolved in pyrogen-free PBS and i.v. injected into mice through the tail vein (5). In some experiments, mice were i.p. administered with 300 μg of anti-CD8 mAb and/or 100 μg anti-ASGM1 Ab 8 h before treatment with 300 μg of 20d5 or isotype-matched control Ig 2 days before Con A injection. Sera from individual mice were obtained 16 h after Con A or 24 h after α-GalCer injection. Serum aminotransferase (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) activities were measured by the standard photometric method using a Hitachi type 7350 automatic analyzer (Hitachi).

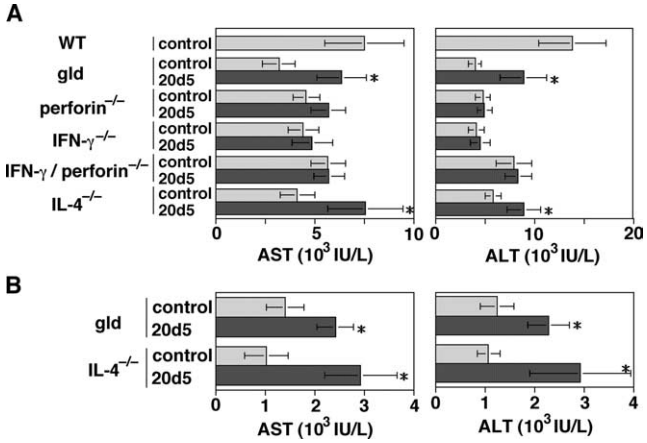

FIGURE 3. Augmentation of Con A-induced hepatic injury in NKG2A signal-blocked *gld* and IL-4-deficient mice. *A*, The indicated mice were i.p. treated with 20d5 2 days before Con A injection (10 mg/kg). Serum samples were obtained from 5–15 mice in each group 16 h after Con A injection. *B*, The indicated mice were i.p. administered with anti-ASGM1 Ab and anti-CD8 mAb 8 h before i.p. treatment with control Ig or 20d5. Two days later, mice were i.v. injected with Con A (10 mg/kg). Serum samples were obtained from 5–10 mice in each group 16 h after Con A injection. Data are represented as the mean ± SD. *, $p < 0.05$ as compared with control Ig-treated same strain mice. Similar results were obtained from three independent experiments.

Flow cytometric analysis

MNC were prepared as described (5). Cells were first preincubated with anti-mouse CD16/32 (2.4G2) mAb to avoid nonspecific binding of mAbs to FcγR. Surface expression of CD94, NKG2A^{B6} and NKG2A/C/E on iNKT cells, NK cells, and conventional CD8 T cells and conventional CD4 T cells was analyzed on electronically gated TCR β^+ α-GalCer/CD1d tetramer$^+$ cells, TCR β– NK1.1$^+$ cells, α-GalCer/CD1d tetramer$^-$CD8$^+$ cells, and α-GalCer/CD1d tetramer$^-$CD4$^+$ cells in B6 mice by four-color flow cytometry using a FACSCalibur apparatus (BD Bioscience). Surface expression of FasL on iNKT cells, NK cells, and conventional CD8 T cells was analyzed on electronically gated TCR β^+ α-GalCer/CD1d tetramer$^+$ cells, TCR β^-NK1.1$^+$ cells, TCR β^+ α-GalCer/CD1d tetramer$^-$CD8$^+$ cells by four-color flow cytometry using a FACSCalibur. Surface expression of NKG2A, CD28, and ICOS on NK1.1$^-$ iNKT cells and NK1.1$^+$ iNKT cells were analyzed on electronically gated TCRβ^+ α-GalCer/CD1d tetramer$^+$NK1.1$^-$ cells and TCRβ^+ α-GalCer/CD1d tetramer$^+$NK1.1$^+$ cells by four-color flow cytometry using a FACSCalibur. Surface molecules were stained with FITC-, PE-, and allophycocyanin-conjugated anti-mouse NK1.1 mAb (PK136), FITC- or allophycocyanin conjugated anti-mouse CD8 mAb (53-6.7), allophycocyanin-conjugated anti-mouse CD4 mAb (RM4-5), PE-Cy5.5- or allophycocyanin-conjugated anti-mouse TCR β mAb(H57-597), FITC-conjugated anti-mouse CD94 mAb (18d5), biotin-conjugated anti-mouse FasL (CD95L, CD178) mAb (MFL3), biotin-conjugated anti-mouse NKG2A^{B6} mAb (16a11), biotin-conjugated anti-mouse NKG2A/C/E mAb (20d5), biotin-conjugated anti-mouse CD28 mAb (37.51), biotin-conjugated anti-mouse IOCS (CD278) mAb (7E.17G9), FITC-conjugated anti-mouse CD3 mAb (145-2C11), FITC-, PE-, PE-Cy5.5-, allophycocyanin-, or biotin-conjugated isotype-matched control mAbs, PE-conjugated α-GalCer/CD1d, and PE-Cy5.5- or allophycocyanin-conjugated streptavidin. All Abs and streptavidins were purchased from eBioscience.

ELISA

IFN-γ in the sera was determined by using mouse IFN-γ-specific ELISA kits (OptEIA; BD Bioscience) according to the manufacturer's instructions.

Cytotoxicity assay

Cytotoxic activity was tested against FasL-sensitive and NK cell-sensitive YAC-1S cells, FasL-resistant and NK cell-resistant B16 cells, or B6 LPS blast cells by a standard 4-h ^{51}Cr release assay as previously described (5). LPS blast cells were prepared as previously described (19). Effector cells (hepatic and splenic MNC) were prepared from mice 6 h after the i.p. injection of Con A. Some mice were i.p. administered with 300 μg of

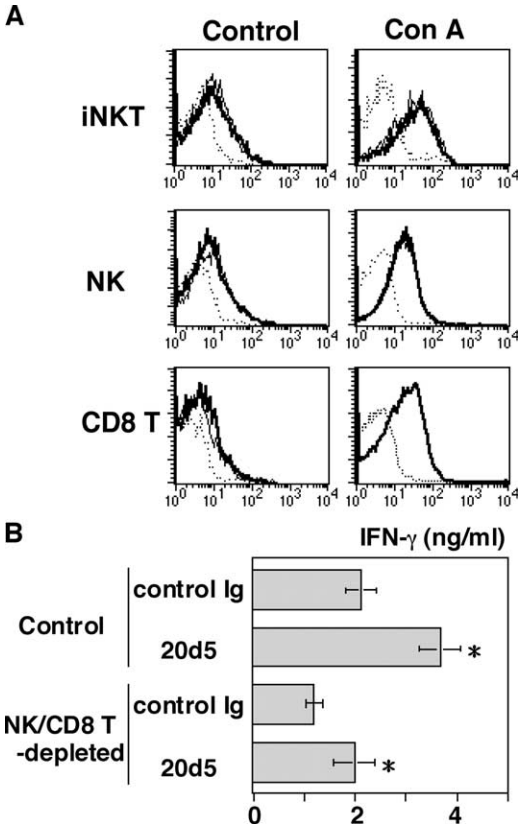

FIGURE 4. Augmentation of serum IFN-γ levels by NKG2A signal blockade in Con A-injected mice. *A*, FasL expression on hepatic MNC populations after Con A injection. Liver MNC were obtained 4 h after Con A injection and then FasL expression was analyzed on electronically gated iNKT cells, NK cells, and conventional CD8 T cells. The dotted lines indicate the staining with isotype-matched control Ig, the thin lines indicate the staining with anti-FasL mAb in control Ig-treated mice, and the thick lines indicate the staining with anti-FasL mAb in 20d5-treated and Con A-injected mice. The staining with isotype-matched control Ig in Con A-injected mice and 20d5-treated and Con A-injected mice was identical. *B*, Serum IFN-γ levels in Con A-injected mice. Mice were treated with i.p. injection of control Ig or 20d5 2 days before i.v. injection with Con A (10 mg/kg). NK cells and CD8 T cells were depleted by Ab treatment 8 h before 20d5 treatment in some mice. Serum samples were obtained from five mice in each group 12 h after Con A injection, and serum IFN-γ levels were examined with ELISA. Data are represented as the mean ± SD. *, $p < 0.05$ as compared with control Ig-treated mice. Similar results were obtained from three independent experiments.

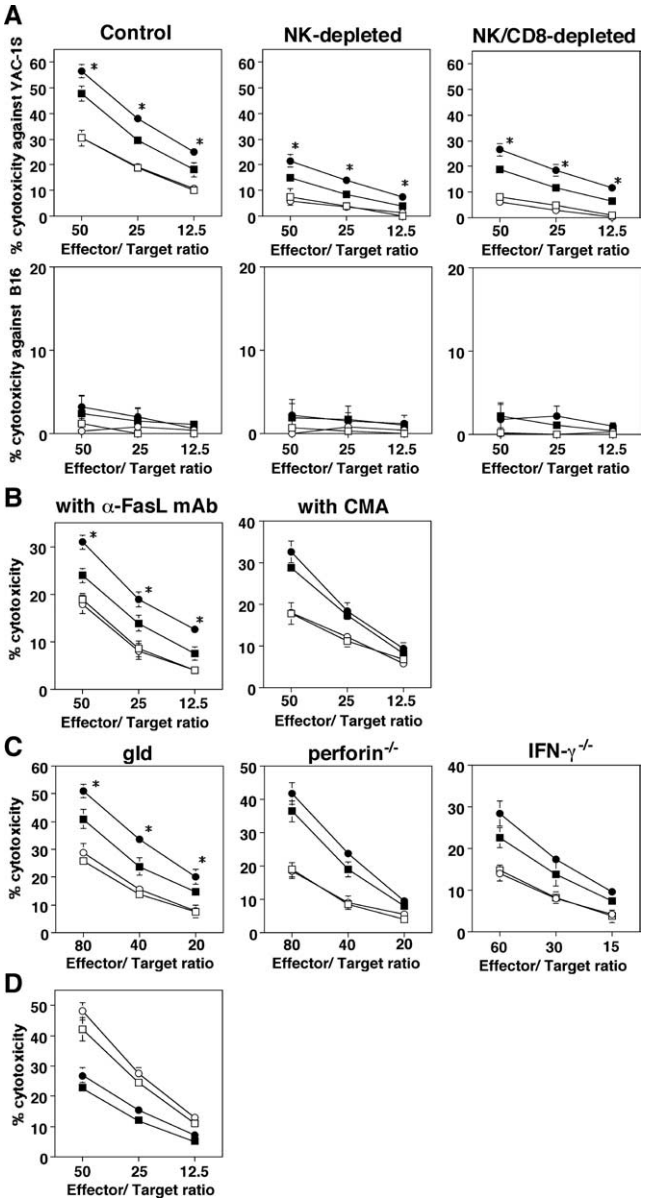

FIGURE 5. *A–C*, Blockade of NKG2A-mediated signals preferentially augments perforin-mediated cytotoxicity of hepatic MNC control, NK cell-depleted, and NK cell- and CD8 T cell-depleted WT (*A*), WT (*B*), FasL-mutant *gld* mice, perforin-deficient, and IFN-γ-deficient mice (*C*) were treated with control Ig (squares) or 20d5 (circles) 2 days before PBS (open symbols) or Con A (10 mg/kg) (closed symbols) injection. Hepatic MNC were prepared 6 h after Con A injection and cytotoxicity was analyzed against NK cell-sensitive and FasL-sensitive YAC-1S cells (*A–C*) and NK-resistant and FasL-resistant B16 cells (*A*). Cytotoxic activity was examined in the presence or absence of anti (α)-FasL mAb or perforin-inhibiting CMA (*B*). *D*, Effect of blockade of the NKG2A-mediated signal in the effector phase. Hepatic MNC were prepared from WT mice 6 h after Con A injection and cytotoxic activity was analyzed against B6 LPS blast cells in the presence (circles) or absence (squares) of 20d5. Cytotoxic activity was also examined with (closed symbols) or without (open symbols) anti-FasL mAb. Data are represented as the mean ± SD of triplicate samples. *, $p < 0.05$ as compared with control Ig-treated mice. Similar results were obtained from three independent experiments.

isotype-matched control Ig or anti-NKG2 mAb 2 days before Con A injection. Specific cytotoxicity was calculated as previously described (5). In some experiments, the cytotoxicity assay was performed in the presence of anti-FasL (MFL-3) mAb (10 μg/ml), anti-NKG2A/C/E mAb (20d5) (10 μg/ml), and/or CMA (50 nM).

Statistical analysis

Data were analyzed by a two-tailed Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Aggravation of hepatic injury following blockade of the CD94/NKG2A-mediated signals in Con A-induced hepatitis

It has been reported that activated iNKT cells play critical roles in Con A-induced hepatic injury (4, 5), and we reported that a NKG2A-mediated signal negatively regulated iNKT cell activation by specific ligands (14). As previously reported using spleen and liver MNC (7–12, 14), CD94/NKG2A complexes, but not other CD94/NKG2 complexes, were expressed on iNKT cells, NK cells, and conventional CD8 T cells, whereas conventional CD4 T cells did not express any CD94/NKG2 complex (Fig. 1). These

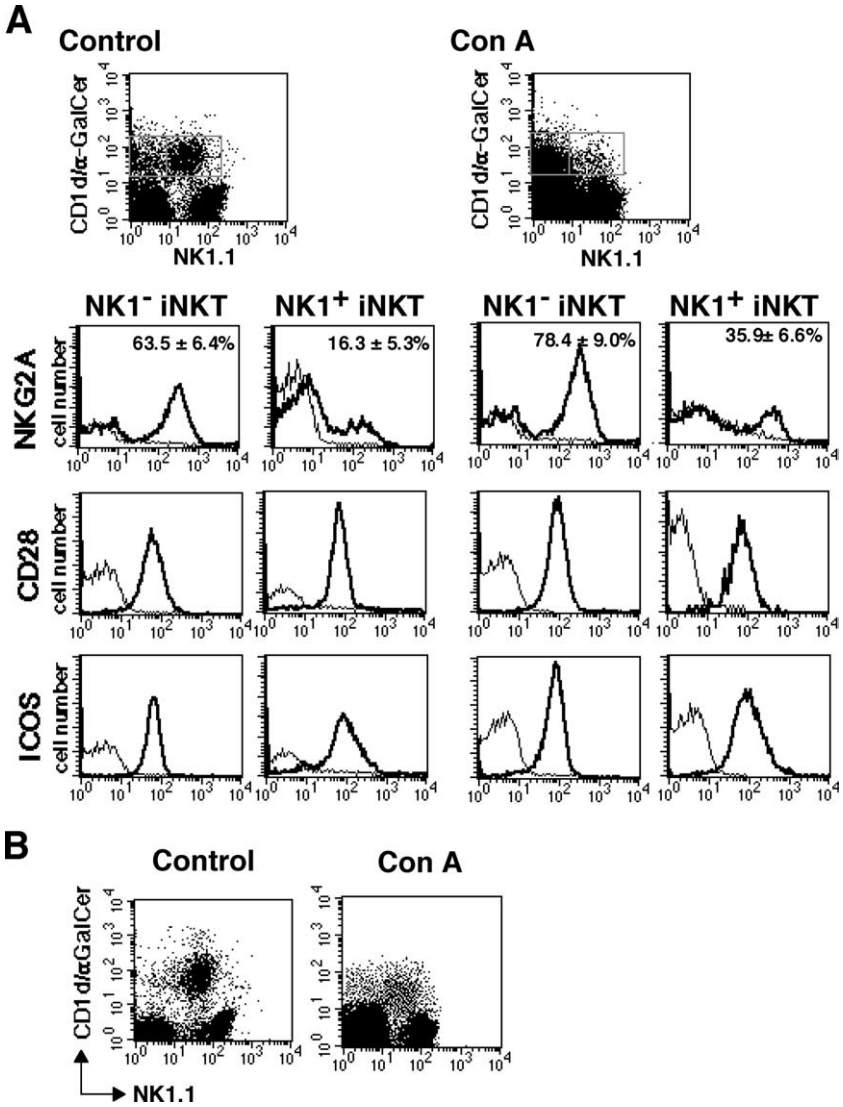

FIGURE 6. NK1$^-$ iNKT cells preferentially remain in the liver of Con A-injected mice due to their NKG2A expression. *A*, Cell surface expression of NKG2A, CD28, and ICOS on hepatic NK1$^+$ and NK1$^-$ α-GalCer/CD1d $^+$ iNKT cells. Hepatic MNC were harvested from control- and Con A-treated mice (10 mg/kg) 4 h after i.v. injection. Cell surface expression of the indicated molecules was analyzed on electronically gated hepatic NK1$^+$ and NK1$^-$ α-GalCer/CD1d $^+$ iNKT cells. The analysis gates are indicated as the squares with gray line in the dot plots panels. Thin lines indicate the staining with isotype-matched control Ig, and thick lines indicate the staining with the respective mAb. *B*, Disappearance of iNKT cells following Con A injection in 20d5 pretreated mice. Mice were treated with 20d5 2 days before i.v. injection of Con A (10 mg/kg). Hepatic MNC were harvested 4 h after Con A injection. Control shows the results of hepatic MNC isolated 4 h after PBS injection in 20d5-pretreated mice.

results clearly suggested that antagonistic anti-NKG2A/C/E mAb (20d5) treatment blocks only the CD94/NKG2A-mediated inhibitory signal and not any potential activating signal. Thus, we examined the role of NKG2A-mediated signals in Con A-induced hepatic injury following treatment with 20d5.

Serum AST and ALT levels were substantially augmented after Con A injection in the 20d5-pretreated mice compared with control Ig-pretreated mice (Fig. 2*A*). These results suggested that NKG2A inhibited Con A-induced hepatic injury possibly by inhibiting lymphocyte activation in response to Con A. NK cells, iNKT cells, and conventional CD8 T cells expressed CD94/NKG2A and were suggested to contribute to Con A-induced hepatic injury (4, 5, 20–22); thus, we next examined the impact of 20d5 treatment on Con A-induced hepatic injury in CD8 T cell- and/or NK cell-depleted mice. The elevation of serum AST and ALT levels by 20d5 pretreatment was highest in control Ig-treated mice, but 20d5 pretreatment also significantly increased serum AST and ALT levels in CD8 T cell- and/or NK cell-depleted mice (Fig. 2*B*). Thus, the NKG2A-mediated signal inhibits Con A-induced hepatic injury by suppressing iNKT cell activation, although conventional CD8 T cell and NK cell activation would be also reduced by the NKG2A-mediated signal.

FasL or IL-4 deficiency does not diminish the augmentation of Con A-induced hepatic injury by blockade of the NKG2A-mediated signal

It has been reported that iNKT cell-mediated cytotoxicity (FasL- and perforin-dependent killing) and iNKT cell-producing cytokines (IFN-γ and IL-4) critically contribute to Con A-induced hepatic injury (4, 5). Thus, we next examined the effect of 20d5 pretreatment on Con A-induced hepatic injury in several gene-deficient mice. Pretreatment with 20d5 augmented the elevation of serum ALT and AST levels in FasL-mutant *gld* mice and IL-4-deficient mice after Con A injection (Fig. 3*A*), even when NK cells and CD8 T cells were depleted (Fig. 3*B*). However, 20d5 pretreatment did not demonstrate any effect upon ALT and AST levels in Con A-injected perforin-, IFN-γ-, and IFN-γ and perforin-deficient mice (Fig. 3*A*). Thus, the NKG2-mediated signal inhibited Con A-induced hepatic injury by suppressing IFN-γ production and perforin-mediated killing.

Augmentation of Con A-induced IFN-γ production but not FasL expression by blockade of the NKG2A-mediated signal in vivo

FasL expression was augmented on hepatic iNKT cells, NK cells, and conventional CD8 T cells after Con A injection, although FasL

expression levels on iNKT cells were higher compared with those on NK cells or conventional CD8 T cells (Fig. 4*A*). 20d5 pretreatment did not modify FasL expression on iNKT cells, NK cells, or conventional CD8 T cells in both control and Con A-treated mice (Fig. 4*A*). Serum IFN-γ and IL-4 levels were also examined, because we have reported that NKG2A-mediated signals negatively regulate IFN-γ and IL-4 production from ligand-activated iNKT cells (14). IL-4 was not detected at any time points after Con A injection even in the 20d5-pretreated mice (data not shown); however, serum IFN-γ level was increased by 20d5 pretreatment in Con A-injected mice (Fig. 4*B*). NK cell and CD8 T cell depletion reduced the serum IFN-γ level after Con A injection in concert with the contribution of NK cells and CD8 T cells to Con A-induced hepatic injury. Con A-induced serum IFN-γ elevation was augmented by 20d5 pretreatment in NK cell- and CD8 T cell-depleted WT mice (Fig. 4*B*), suggesting the inhibitory role of NKG2A in IFN-γ production by Con A-activated iNKT cells. These results indicated that the NKG2A-mediated signal inhibits IFN-γ production by iNKT cells, NK cells, and conventional CD8 T cells, but not FasL expression on these cells during Con A-induced hepatic injury.

Augmentation of perforin-mediated cytotoxicity of liver MNC from Con A-injected mice by anti-CD94/NKG2A mAb treatment

We then examined the effect of the NKG2A-mediated signal on the cytotoxic activity of hepatic MNC using NK cell- and FasL-sensitive YAC-1S cells and NK cell- and FasL-resistant B16 cells. Cytotoxic activity of hepatic MNC against YAC-1S cells was augmented 6 h after Con A injection in WT mice, and 20d5 pretreatment increased the cytotoxic activity of hepatic MNC in Con A-injected mice, but not control mice (Fig. 5*A*). Con A-induced cytotoxic activity was reduced by NK cell depletion; however augmentation of cytotoxicity by 20d5 pretreatment was observed in NK cell-depleted and NK cell- and CD8 T cell-depleted mice, suggesting that NK cells and iNKT cells mainly exerted cytotoxic activity after Con A injection. B16 cells were still resistant to the cytotoxicity exerted by hepatic MNC 6 h after Con A injection. The cytotoxic activity of hepatic MNC from 20d5- and Con A-treated mice was significantly higher compared with that from Con A-treated mice even when FasL-mediated cytotoxicity was inhibited by anti-FasL mAb (Fig. 5*B*). However blockade of perforin-mediated killing by CMA diminished the augmentation of cytotoxic activity by 20d5 pretreatment (Fig. 5*B*). Consistently, 20d5 pretreatment significantly augmented cytotoxic activity of hepatic MNC from *gld* mice, but not that of perforin-deficient mice, following Con A injection (Fig. 5*C*). Significant augmentation of cytotoxic activity by 20d5 pretreatment was not found in the experiments using IFN-γ-deficient mice (Fig. 5*C*). 20d5 pretreatment did not affect the cytotoxic activity of hepatic MNC in the absence of Con A injection. 20d5 did not have any effect on cytotoxic activity of hepatic MNC against Qa-1^b-expressing B6 LPS blast cells when 20d5 was added during the cytotoxicity assay (Fig. 5*D*). Similar results were obtained in the experiments using splenic MNC (data not shown); however, cytotoxic activity was weak compared with that of liver MNC, probably due to a lower number of iNKT and NK cells in spleen. Taken together, these results indicated that the NKG2A-mediated signal inhibited perforin-mediated cytotoxicity due to inhibition of IFN-γ production responding to Con A, and that NKG2A-mediated signals contribute little to cytotoxic activity during the effector phase.

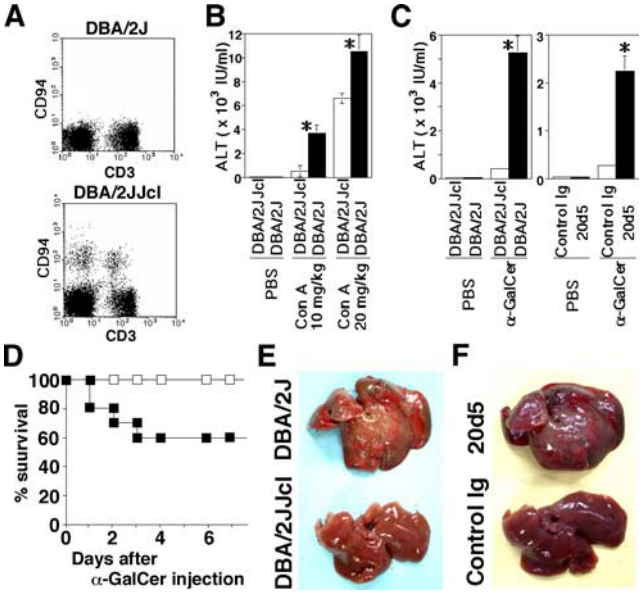

FIGURE 7. Deficiency of CD94/NKG2A in DBA/2 mice augments Can A-induced and α-GalCer-induced hepatic injury. *A*, Impaired CD94 expression in DBA/2J, but not DBA/2JJcl, mice. Hepatic MNC were freshly isolated from the indicated DBA/2 mice substrains, and CD94 expression was examined by flow cytometric analysis. Similar results were obtained using freshly isolated splenic MNC. *B*, Serum ALT levels in CD94/NKG2A-deficient DBA/2J and CD94/NKG2A-intact DBA/2 JJcl mice after Con A injection. Mice were i.v. treated with the indicated amounts of Con A, and sera were collected 16 h after injection. Serum samples were obtained from three mice in each group. *, $p < 0.05$ as compared with DBA/2JJcl mice treated with same amount of Con A. *C*, Serum ALT levels of α-GalCer-injected DBA/2 mice. Serum samples were obtained from five mice in each group 24 h after α-GalCer (2 μg) i.p. injection. Some DBA/2JJcl mice were treated with i.p. injection of control Ig or 20d5 2 days before i.p. injection of α-GalCer (*right panel*). Data are represented as the mean ± SD of triplicate samples. *, $p < 0.05$ as compared with DBA/2JJcl mice (*left panel*) or control Ig-treated mice (*right panel*). *D*, Survival rate of α-GalCer-injected NKG2A-deficient DBA/2J (■) and NKG2A-intact DBA/2JJcl (□) mice. *E*, Hepatic damage in α-GalCer-injected NKG2A-deficient DBA/2J and NKG2A-intact DBA/2JJcl mice. Photos show the liver of either substrain of DBA/2 mice 24 h after i.p. injection of α-GalCer. *F*, Hepatic damage in α-GalCer-injected control Ig- or 20d5-pretreated DBA/2JJcl mice. Photos show the liver of DBA/2JJcl mice pretreated with control Ig or 20d5 24 h after i.p. injection of α-GalCer. Similar results were obtained from three independent experiments.

NKG2A$^+$ iNKT cells preferentially remain in liver after Con A injection

As we previously reported (5), the percentage of iNKT cells in the liver MNC quickly decreased after Con A injection (17.7 ± 3.5% in control mice and 3.3 ± 0.6% 6 h after Con A injection). NK1$^-$ iNKT cells preferentially remained in the liver compared with NK1$^+$ iNKT cells after Con A injection (NK1$^+$ iNKT cells: 10.9 ± 1.8% in control mice and 1.0 ± 0.6% in Con A-injected mice; NK1$^-$ iNKT cells: 3.8 ± 1.5% in control mice and 2.3 ± 0.6% in Con A-injected mice) (Fig. 6*A*). Interestingly, NKG2A was expressed preferentially on NK1$^-$ iNKT cells, and the NKG2A-expressing population increased in both NK1$^-$ and NK1$^+$ iNKT cells after Con A injection (Fig. 6*A*). Almost all iNKT cells expressed the costimulatory molecule receptors CD28 and IOCS (Fig. 6*A*), which were reported to contribute to iNKT cell activation (23, 24) and Con A responses (25). Moreover, pretreatment with 20d5 appeared to facilitate the disappearance of iNKT cells, particularly enhancing the disappearance of NK1$^-$

iNKT cells (0.6 ± 0.5% in 20d5-pretreated and Con A-injected mice) and keeping the iNKT cell populations similar to those found in normal mice ($NK1^+$ iNKT cells > $NK1^-$ iNKT cells) (Fig. 6*B*). These results indicated that NKG2A-mediated signaling inhibits activation-induced disappearance of iNKT cells responding to Con A.

Augmentation of Con A-induced and α-GalCer-induced and hepatic injury in CD94/NKG2A-deficient mice

We finally examined the effect of genetic deficiency in CD94/NKG2A-mediated signaling on hepatic injury induced by iNKT cell activation. In concert with a previous report (10), DBA/2J mice were defective in CD94 expression whereas expression of CD94 was observed in DBA/2JJcl mice (Fig. 7*A*). Con A-induced hepatic injury was more obvious in DBA/2J mice compared with DBA/2JJcl mice (Fig. 7*B*). Moreover, when these two substrains of DBA/2 mice were treated with i.p. injection of α-GalCer, CD94-deficient DBA/2J mice suffered more severe hepatic damage compared with CD94-intact DBA/2JJcl mice, and 40% of these died of hepatic injury just 3 days following α-GalCer injection (Fig. 7*C*, *left panel*, *D*, and *E*). 20d5 pretreatment augmented hepatic injury induced by α-GalCer in DBA/2JJcl mice (Fig. 7*C*, *right panel*, and *F*) similarly as that induced by Con A in B6 mice. Taken together, these results clearly demonstrated that a CD94/NKG2A-mediated signal negatively regulates hepatic injury induced by Con A and the iNKT cell-specific ligand α-GalCer.

Discussion

iNKT cells preferentially distribute to liver and have been demonstrated to play critical roles in antitumor immune responses and protection from infectious disease (1, 2). It has been reported that activation of hepatic iNKT cells triggers hepatic injury following iNKT cell specific ligand, α-GalCer, or Con A injection (4–6), and NK cells and conventional CD8 T cells may also play roles (20, 22). In this study we reported that blockade of a NKG2A-mediated inhibitory signal by mAb (20d5) resulted in the augmentation of Con A-induced serum IFN-γ elevation and hepatic injury even when NK cells and CD8 T cells were depleted. Serum AST and ALT levels were augmented by 20d5 pretreatment in WT, *gld*, and IL-4 deficient mice, but not in IFN-γ- or/and perforin-deficient mice. Consistently, 20d5 pretreatment augmented perforin-mediated killing, but not FasL expression or FasL/Fas-mediated killing, and this augmentation of perforin-mediated killing was not observed in Con A-treated, IFN-γ-deficient mice. Moreover, cytotoxic activity was not augmented when 20d5 was added to the cytotoxicity assay. Thus, CD94/NKG2A contributes to Con A-induced hepatic MNC activation, particularly iNKT cell activation, to produce IFN-γ that enhanced perforin-mediated cytotoxic activity. In concert, impaired CD94/NKG2A in the DBA/2 mice substrain resulted in more severe α-GalCer-induced and Con A-induced hepatic injury.

Previous studies have shown that iNKT cells disappear quickly after activation by anti-CD3 mAb, iNKT cell-specific ligands, Con A, and IL-12 treatment due to activation-induced cell death (5, 26, 27) and/or down-regulation of cell surface functional molecules (TCR, costimulatory molecule receptors, and NK cell receptors) (14, 15, 28–30). Regardless, the disappearance of iNKT cells is thought to be an early sign of iNKT cell activation. We have thus demonstrated the following: 1) Con A injection decreased iNKT cells in vivo; 2) NKG2A-expressing $NK1^-$ iNKT cells preferentially remained following Con A injection; 3) the NKG2A-expressing population increased among both $NK1^-$ and $NK1^+$ iNKT cells after Con A injection; and 4) 20d5 pretreatment appeared to facilitate the disappearance of iNKT cells, particularly NKG2A-expressing $NK1^-$ iNKT cells, after Con A injection. These results suggested that NKG2A inhibited the iNKT cell response to Con A. In concert, we have reported that CD94/NKG2A negatively controls iNKT cell activation by the iNKT cell-specific ligands α-GalCer and OCH (14). Inhibitory Ly49 receptors, which recognize MHC class I directly, have also been reported to suppress α-GalCer-induced iNKT cell activation (31). CD94/NKG2A is more frequently expressed on iNKT cells than Ly49 (14, 15); thus, CD94/NKG2A may play a more dominant role in regulating iNKT cell activation than Ly49 and may maintain self-tolerance of iNKT cells and help to avoid any pathogenic effect of iNKT cell activation (32, 33). Further, by using TAP-deficient mice in vitro, CD94/NKG2A was reported to play no role in splenic iNKT cell activation by α-GalCer (15). Thus, the magnitude of the inhibitory effect of CD94/NKG2A-mediated signaling on iNKT cells might be variable among iNKT cell subsets, activation status, various tissues, and their microenvironments, and/or the experimental conditions (34).

Con A-induced hepatitis is a popular model of autoimmune hepatitis, and the hepatic injury is transient (35, 36). Fas/FasL-mediated killing, perforin-mediated killing, IFN-γ, IL-4, and TNF-α were reported to play a role in Con A-induced hepatic injury (4, 5, 20, 21, 35, 37–39). We have reported that Qa-1^b, a ligand for CD94/NKG2A, is inducible by IFN-γ (14). It has been shown that CD94/NKG2A expressed on NK cells and conventional $CD8^+$ T cells suppressed their activation (7–9, 12). Thus, IFN-γ-mediated Qa-1^b up-regulation may be a negative feedback mechanism for overactivation of NK cells, iNKT cells, and conventional CD8 T cells, and resulting in transient hepatic injury. It will be interesting to explore the role of CD94/NKG2A in other iNKT cell-mediated biological responses, anticancer activity, or infectious diseases using Qa-1^b-deficient (40) or CD94-deficient (10) mice.

CD94/NKG2A is reported to inhibit activation of NK cells and conventional CD8 T cells (7–9, 12), and both cells are reported to play a substantial role in Con A-induced hepatic injury (20, 22). Activated NK cells might be mainly responsible for cytotoxic activity rather than iNKT cells, because sustained IFN-γ production and cytotoxicity following iNKT cell activation were mediated by NK cells secondarily activated by IFN-γ derived from iNKT cells and IL-12 derived from dendritic cells (27, 41, 42). Hence, elevation of serum AST, ALT, and IFN-γ levels and cytotoxic activity by 20d5 pretreatment in Con A-injected mice were reduced by CD8 T cell and/or NK cell depletion. It is also possible that conventional CD4 T cells play a role in IFN-γ production and FasL-mediated cytotoxicity induced by Con A injection (43). However, conventional CD4 T cells do not express CD94/NKG2A and FasL does not play a major role in augmented cytotoxicity following 20d5 treatment. Thus, augmentation of hepatic injury, serum IFN-γ level, and cytotoxic activity by 20d5 pretreatment in NK cell- and CD8 T cell-depleted mice clearly indicated augmentation of iNKT cell activation by blockade of the CD94/NKG2A-mediated inhibitory signal. CD94/NKG2A possibly plays inhibitory roles in lymphocyte activation (including iNKT cells, NK cells, and conventional CD8 T cells) when iNKT cells are activated by Con A and their specific ligands in vivo. However, aggravation of α-GalCer-induced hepatic injury by CD94 deficiency was higher when compared with that of Con A-induced hepatic injury in DBA/2 mice, and this result suggests a dominant inhibitory role of CD94/NKG2A on activated iNKT cells rather than NK cells and conventional CD8 T cells.

In this study we showed that hepatic $NK1^-$ iNKT cells preferentially express NKG2A and that blockade of NKG2A-mediated inhibitory signals accelerated activation of $NK1^-$ iNKT cells

rather than NK1$^+$ iNKT cells and augmented Con A- and α-GalCer-induced hepatic injury. It was recently reported that NK1$^-$ iNKT cells, but not NK1$^+$ iNKT cells, produce IL-17 that plays a critical role in the onset and progression of some cell-mediated autoimmunity and immune-mediated inflammatory diseases (44–46). Con A-induced hepatic injury is thought to be a cell-mediated immunoinflammatory disease similar to human autoimmune hepatitis (36). Thus, selective enhancement of IL-17-producing NK1$^-$ iNKT cell activation might also play a critical role in the augmentation of Con A- and α-GalCer-induced hepatic injury by 20d5 treatment as well as augmentation of IFN-γ production and perforin-mediated cytotoxicity by iNKT cells, NK cells, and conventional CD8 T cells. Further studies are now ongoing to reveal the role of IL-17 in iNKT cell-mediated hepatic injury, although examination of activated iNKT cells is technically difficult due to their prompt disappearance.

CD94/NKG2A blockade might be also applicable to improve the antitumor effect of α-GalCer in humans. In addition to an antitumor effect, α-GalCer has been shown to protect mice against infections and autoimmune diseases (1, 2, 47, 48). Therefore, the blockade of CD94/NKG2A may be also applied to improve the therapeutic effect of α-GalCer in these diseases. However, it has been also shown that α-GalCer occasionally induces tissue pathologies (6, 32, 33) and exacerbated autoimmune diseases depending on the model and/or administration protocol (49). In these cases, signal induction through inhibitory NK cell receptors including CD94/NKG2A might prevent tissue damage. Therefore, further studies are needed to determine the optimal protocol to modulate NK cell receptor-mediated signals in iNKT cell-targeting therapy for the safe treatment of tumor, infections, and autoimmune diseases.

Disclosures

The authors have no financial conflict of interest.

References

1. Kronenberg, M., and L. Gapin. 2002. The unconventional lifestyle of NKT cells. *Nat. Rev. Immunol.* 2: 557–568.
2. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Vα14 NKT cells in innate and acquired immune response. *Annu. Rev. Immunol.* 21: 483–513.
3. De Libero, G., and L. Mori. 2005. Recognition of lipid antigens by T cells. *Nat. Rev. Immunol.* 5: 485–496.
4. Kaneko, Y., M. Harada, T. Kawano, M. Yamashita, Y. Shibata, F. Gejyo, T. Nakayama, and M. Taniguchi. 2000. Augmentation of Vα14 NKT cell-mediated cytotoxicity by interleukin 4 in an autocrine mechanism resulting in the development of concanavalin A-induced hepatitis. *J. Exp. Med.* 191: 105–114.
5. Takeda, K., Y. Hayakawa, L. Van Kaer, H. Matsuda, H. Yagita, and K. Okumura. 2000. Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc. Natl. Acad. Sci. USA* 97: 5498–5503.
6. Osman, Y., T. Kawamura, T. Naito, K. Takeda, L. Van Kaer, K. Okumura, and T. Abo. 2000. Activation of hepatic NKT cells and subsequent liver injury following administration of α-galactosylceramide. *Eur. J. Immunol.* 30: 1919–1928.
7. Lanier, L. L. 2005. NK cell recognition. *Annu. Rev. Immunol.* 23: 225–274.
8. Vivier, E., and N. Anfossi. 2004. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. *Nat. Rev. Immunol.* 4: 190–198.
9. Vance, R. E., A. M. Jamieson, and D. H. Raulet. 1999. Recognition of the class Ib molecule Qa-1^b by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J. Exp. Med.* 190: 1801–1812.
10. Vance, R. E., A. M. Jamieson, D. Cado, and D. H. Raulet. 2002. Implications of CD94 deficiency and monoallelic NKG2A expression for natural killer cell development and repertoire formation. *Proc. Natl. Acad. Sci. USA* 99: 868–873.
11. Vance, R. E., J. R. Kraft, J. D. Altman, P. E. Jensen, and D. H. Raulet. 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1^b. *J. Exp. Med.* 188: 1841–1848.
12. Lu, L., M. B. Werneck, and H. Cantor. 2006. The immunoregulatory effects of Qa-1. *Immunol. Rev.* 212: 51–59.
13. Noble, A., Z. S. Zhao, and H. Cantor. 1998. Suppression of immune responses by CD8 cells. II. Qa-1 on activated B cells stimulates CD8 cell suppression of T helper 2 responses. *J. Immunol.* 160: 566–571.
14. Ota, T., K. Takeda, H. Akiba, Y. Hayakawa, K. Ogasawara, Y. Ikarashi, S. Miyake, H. Wakasugi, T. Yamamura, M. Kronenberg, et al. 2005. IFN-γ-mediated negative feedback regulation of NKT-cell function by CD94/NKG2. *Blood* 106: 184–192.
15. Uldrich, A. P., N. Y. Crowe, K. Kyparissoudis, D. G. Pellicci, Y. Zhan, A. M. Lew, P. Bouillet, A. Strasser, M. J. Smyth, and D. I. Godfrey. 2005. NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *J. Immunol.* 175: 3092–3101.
16. Matsuda, J. L., O. V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C. R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 192: 741–754.
17. Takeda, K., N. Yamaguchi, H. Akiba, Y. Kojima, Y. Hayakawa, J. E. Tanner, T. J. Sayers, N. Seki, K. Okumura, H. Yagita, and M. J. Smyth. 2004. Induction of tumor-specific T cell immunity by anti-DR5 antibody therapy. *J. Exp. Med.* 199: 437–448.
18. Kataoka, T., N. Shinohara, H. Takayama, K. Takaku, S. Kondo, S. Yonehara, and K. Nagai. 1996. Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J. Immunol.* 156: 3678–3686.
19. Takeda, K., and G. Dennert. 1993. The development of autoimmunity in C57BL/6 *lpr* mice correlates with the disappearance of natural killer type 1-positive cells: evidence for their suppressive action on bone marrow stem cell proliferation, B cell immunoglobulin secretion, and autoimmune symptoms. *J. Exp. Med.* 177: 155–164.
20. Watanabe, Y., M. Morita, and T. Akaike. 1996. Concanavalin A induces perforin-mediated but not Fas-mediated hepatic injury. *Hepatology* 24: 702–710.
21. Toyabe, S., S. Seki, T. Iiai, K. Takeda, K. Shirai, H. Watanabe, H. Hiraide, M. Uchiyama, and T. Abo. 1997. Requirement of IL-4 and liver NK1$^+$ T cells for concanavalin A-induced hepatic injury in mice. *J. Immunol.* 159: 1537–1542.
22. Wald, O., I. D. Weiss, H. Wald, H. Shoham, Y. Bar-Shavit, K. Beider, E. Galun, L. Weiss, L. Flaishon, I. Shachar, et al. 2006. IFN-γ acts on T cells to induce NK cell mobilization and accumulation in target organs. *J. Immunol.* 176: 4716–4729.
23. Hayakawa, Y., K. Takeda, H. Yagita, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *J. Immunol.* 166: 6012–6018.
24. Kaneda, H., K. Takeda, T. Ota, Y. Kaduka, H. Akiba, Y. Ikarashi, H. Wakasugi, M. Kronenberg, K. Kinoshita, H. Yagita, and K. Okumura. 2005. ICOS costimulates invariant NKT cell activation. *Biochem. Biophys. Res. Commun.* 327: 201–207.
25. Perrin, P. J., T. A. Davis, D. S. Smoot, R. Abe, C. H. June, and K. P. Lee. 1997. Mitogenic stimulation of T cells reveals differing contributions for B7-1 (CD80) and B7-2 (CD86) costimulation. *Immunology* 90: 534–542.
26. Eberl, G., and H. R. MacDonald. 1998. Rapid death and regeneration of NKT cells in anti-CD3ε- or IL-12-treated mice: a major role for bone marrow in NKT cell homeostasis. *Immunity* 9: 345–353.
27. Hayakawa, Y., K. Takeda, H. Yagita, S. Kakuta, Y. Iwakura, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Critical contribution of IFN-γ and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of α-galactosylceramide. *Eur. J. Immunol.* 31: 1720–1727.
28. Wilson, M. T., C. Johansson, D. Olivares-Villagomez, A. K. Singh, A. K. Stanic, C. R. Wang, S. Joyce, M. J. Wick, and L. Van Kaer. 2003. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. *Proc. Natl. Acad. Sci. USA* 100: 10913–10918.
29. Crowe, N. Y., A. P. Uldrich, K. Kyparissoudis, K. J. Hammond, Y. Hayakawa, S. Sidobre, R. Keating, M. Kronenberg, M. J. Smyth, and D. I. Godfrey. 2003. Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. *J. Immunol.* 171: 4020–4027.
30. Harada, M., K. Seino, H. Wakao, S. Sakata, Y. Ishizuka, T. Ito, S. Kojo, T. Nakayama, and M. Taniguchi. 2004. Down-regulation of the invariant Vα14 antigen receptor in NKT cells upon activation. *Int. Immunol.* 16: 241–247.
31. Maeda, M., S. Lohwasser, T. Yamamura, and F. Takei. 2001. Regulation of NKT cells by Ly49: analysis of primary NKT cells and generation of NKT cell line. *J. Immunol.* 167: 4180–4186.
32. Ito, K., M. Karasawa, T. Kawano, T. Akasaka, H. Koseki, Y. Akutsu, E. Kondo, S. Sekiya, K. Sekikawa, M. Harada, et al. 2000. Involvement of decidual Vα14 NKT cells in abortion. *Proc. Natl. Acad. Sci. USA* 97: 740–744.
33. Tupin, E., A. Nicoletti, R. Elhage, M. Rudling, H. G. Ljunggren, G. K. Hansson, and G. P. Berne. 2004. CD1d-dependent activation of NKT cells aggravates atherosclerosis. *J. Exp. Med.* 199: 417–422.
34. Crowe, N. Y., J. M. Coquet, S. P. Berzins, K. Kyparissoudis, R. Keating, D. G. Pellicci, Y. Hayakawa, D. I. Godfrey, and M. J. Smyth. 2005. Differential antitumor immunity mediated by NKT cell subsets in vivo. *J. Exp. Med.* 202: 1279–1288.
35. Mizuhara, H., E. O'Neill, N. Seki, T. Ogawa, C. Kusunoki, K. Otsuka, S. Satoh, M. Niwa, H. Senoh, and H. Fujiwara. 1994. T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6. *J. Exp. Med.* 179: 1529–1537.
36. Nicoletti, F., R. Di Marco, P. Zaccone, A. Salvaggio, G. Magro, K. Bendtzen, and P. Meroni. 2000. Murine concanavalin A-induced hepatitis is prevented by interleukin 12 (IL-12) antibody and exacerbated by exogenous IL-12 through an interferon-γ-dependent mechanism. *Hepatology* 32: 728–733.
37. Tagawa, Y., K. Sekikawa, and Y. Iwakura. 1997. Suppression of concanavalin A-induced hepatitis in IFN-$\gamma^{-/-}$ mice, but not in TNF-$\alpha^{-/-}$ mice: role for IFN-γ in activating apoptosis of hepatocytes. *J. Immunol.* 159: 1418–1428.
38. Ksontini, R., D. B. Colagiovanni, M. D. Josephs, C. K. Edwards, III, C. L. Tannahill, C. C. Solorzano, J. Norman, W. Denham, M. Clare-Salzler,

S. L. MacKay, and L. L. Moldawer. 1998. Disparate roles for TNF-α and Fas ligand in concanavalin A-induced hepatitis. *J. Immunol.* 160: 4082–4089.

39. Tagawa, Y., S. Kakuta, and Y. Iwakura. 1998. Involvement of Fas/Fas ligand system-mediated apoptosis in the development of concanavalin A-induced hepatitis. *Eur. J. Immunol.* 28: 4105–4113.
40. Hu, D., K. Ikizawa, L. Lu, M. E. Sanchirico, M. L. Shinohara, and H. Cantor. 2004. Analysis of regulatory CD8 T cells in Qa-1-deficient mice. *Nat. Immunol.* 5: 516–523.
41. Kitamura, H., K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, et al. 1999. The natural killer T (NKT) cell ligand α-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* 189: 1121–1128.
42. Carnaud, C., D. Lee, O. Donnars, S. H. Park, A. Beavis, Y. Koezuka, and A. Bendelac. 1999. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J. Immunol.* 163: 4647–4650.
43. Hahn, S., R. Gehri, and P. Erb. 1995. Mechanism and biological significance of CD4-mediated cytotoxicity. *Immunol. Rev.* 146: 57–79.
44. Michel, M. L., A. C. Keller, C. Paget, M. Fujio, F. Trottein, P. B. Savage, C. H. Wong, E. Schneider, M. Dy, and M. C. Leite-de-Moraes. 2007. Identification of an IL-17-producing NK1.1^{neg} iNKT cell population involved in airway neutrophilia. *J. Exp. Med.* 204: 995–1001.
45. Coquet, J. M., S. Chakravarti, K. Kyparissoudis, F. W. McNab, L. A. Pitt, B. S. McKenzie, S. P. Berzins, M. J. Smyth, and D. I. Godfrey. 2008. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4$^-$NK1.1$^-$ NKT cell population. *Proc. Natl. Acad. Sci. USA* 105: 11287–11292.
46. Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* 25: 821–852.
47. Smyth, M. J., and D. I. Godfrey. 2000. NKT cells and tumor immunity—a double-edged sword. *Nat. Immunol.* 1: 459–460.
48. Smyth, M. J., N. Y. Crowe, Y. Hayakawa, K. Takeda, H. Yagita, and D. I. Godfrey. 2002. NKT cells—conductors of tumor immunity? *Curr. Opin. Immunol.* 14: 165–171.
49. Mars, L. T., J. Novak, R. S. Liblau, and A. Lehuen. 2004. Therapeutic manipulation of iNKT cells in autoimmunity: modes of action and potential risks. *Trends Immunol.* 25: 471–476.