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The function and regulation of natural killer T cells in inflammatory diseases

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Abstract

The function and regulation of natural killer T cells in inflammatory diseases

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Activated natural killer T (NKT) cells are a unique and relatively small subset of T cells that were originally defined to share properties of both conventional T and natural killer (NK) cells. NKT cells produce a broad range of cytokines, which determine immunomodulatory functions in various animal models. In this study, we identified a distinct subset of NKT cells which produce interleukin-17 (IL-17) in airway inflammation and determined regulation of NKT cell function by retinoic acid (RA) in liver inflammation.

IL-17, a proinflammatory cytokine secreted by activated T cells and neutrophils, stimulates granulopoiesis and infiltration of neutrophils and mediates recruitment of T cells into peripheral tissue. In chapter I, we show that IL-17 is also produced by a distinct population of NKT cells upon T cell receptor (TCR) stimulation. Administration of α-galactosylceramide (α-GalCer), a strong agonist of NKT cells, induces rapid IL-17 production by small population of NKT cells, mostly belonging to a population different from that of interleukin-4 (IL-4)- and (interferon-γ) IFN-γ-producing NKT cells. IL-17-producing NKT cells showed unresponsiveness after stimulation of α-GalCer as conventional NKT cells. During airway inflammation induced by pulmonary activation of NKT cells with α-GalCer, IL-17 contributes to the infiltration of neutrophils into the airway but has no effect on airway hyperreactivity (AHR). These results indicate that TCR stimulation induces IL-17 expression by a novel population of NKT cells and may help explain diverse NKT cell functions.

RA is a diverse regulator of immune response. Although it has been demonstrated that RA promotes NKT cell activation *in vitro* via increasing CD1d expression of antigen presenting cells (APCs), the direct effects of RA on NKT cell response *in vivo* and NKT cell-mediated liver injury were not elucidated. Treatment of RA ameliorated concanavalin A (Con A)-induced liver damage but not α-GalCer-

induced liver damage. The levels of IFN-γ and IL-4 in serum were reduced significantly by RA, though the tumor necrosis factor-α (TNF-α) levels were left unchanged in both liver injuries. Correlated with cytokine levels in serum, RA regulated production of IFN-γ, IL-4 but not TNF-α in NKT cells without influencing activation status. These regulations were also detected when liver mononuclear cells (MNCs) or NKT hybridoma cells alone were treated with Con A and α-GalCer in the presence of RA *in vitro*. The regulatory effect of RA on NKT cells was mediated by retinoic acid receptor-α (RAR-α) and RA reduced the phosphorylation of MAPK. Collectively, RA differentially modulates effector cytokine production of NKT cells in hepatitis and the suppressive effect of RA on hepatitis varies with pathogenic mechanisms of liver injury.

In conclusion, we established that TCR stimulation induces IL-17 expression by a novel population of NKT cells in airway inflammation and RA differentially modulates effector cytokine production of NKT cells in hepatitis. These findings suggest that diverse function and regulation of NKT cells in inflammatory diseases should be considered in clinical approaches.

Keywords: Natural killer T cells, NKT cells, IL-17, NK1.1, airway inflammation, neutrophil, Retinoic acid, ATRA, Con A-induced hepatitis, α-GalCer-induced hepatitis, cytokine.

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Abbreviations

NKT cell, natural killer T cell

IL-17, interleukin-17

α-GalCer, α-galactosylceramide

TCR, T cell receptor

AHR, airway hyperreactivity

Con A, concanavalin A

APCs, antigen presenting cells

RA, retinoic acid;

ATRA, all-trans retinoic acid

MAPK, mitogen-activated protein kinase

NK cell, natural killer cell

RALDH, retinaldehyde dehydrogenase

RAR, retinoic acid receptor; RXR, retinoic X receptor

ALT, alanine aminotransferase

FasL, Fas ligand

Treg, regulatory T cell.

MNCs, mononuclear cells

ELISA, enzyme-linked immunosorbent assay

FACS, fluorescence-activated cell sorter

MACS, macnetic-activated cell sorter

IFN-γ, interferon-γ

IL-4, interleukin-4

TNF-α, tumor necrosis factor-α

i.p., intraperitoneally

i.v., intravenously

SD, standard deviation

Introduction

NKT cells are a unique and relatively small subset of T cells that were originally defined to share properties of both conventional T and NK cells [1, 2]. The development and glycolipid antigen recognition of NKT cells depend upon CD1d, an MHC class I-like molecule [3]. Within hours of stimulation by α-GalCer, a strong agonist isolated from a marine sponge, NKT cells secrete a large number of cytokines that can modulate immune responses in diverse animal models, including those involved in tumor metastasis, autoimmune diseases, and bacterial or parasitic infections [4-9].

In chapter I, we examined the properties and role of IL-17-producing NKT cells. We found that a distinct population of NKT cells produced IL-17 when they encountered α-GalCer in a CD1d-dependent manner and that they differed from IL-4- and IFN-γ-producing NKT cells. Although IL-17 produced by NKT cells contributed to infiltration of neutrophils into lung in α-GalCer-induced airway inflammation, it had no effect on the development of airway hyperreactivity.

In chapter II, we observed that RA regulates differentially NKT cell-mediated hepatitis and cytokine production from NKT cell. Our results suggest selective

regulatory effects and the therapeutic potential of RA on NKT cell-dependent diseases.

CHAPTER I

A distinct subset of natural killer T cells produces IL-17, contributing to airway infiltration of neutrophils but not to airway hyperreactivity

I.1. Summary

Activated natural killer T (NKT) cells produce a broad range of cytokines, including IL-4 and IFN-γ, that determine immunomodulatory functions in various animal models. In this report, we show that a well-known proinflammatory cytokine, IL-17 is also produced by a distinct population of NKT cells upon TCR stimulation. Administration of α-galactosylceramide (α-GalCer), a strong agonist of NKT cells, induces rapid IL-17 production by small population of NKT cells, mostly belonging to a population different from that of IL-4- and IFN-γ-producing NKT cells. IL-17-producing NKT cells showed unresponsiveness after stimulation of α-GalCer as conventional NKT cells. During airway inflammation induced by pulmonary activation of NKT cells with α-GalCer, IL-17 contributes to the infiltration of neutrophils into the airway but has no effect on airway hyperreactivity (AHR). These results indicate that TCR stimulation induces IL-17 expression by a novel population of NKT cells and may help explain diverse NKT cell functions.

I.2. Introduction

Although NKT cells can be divided by their phenotypic markers, such as CD4 and NK1.1, it is not clear if phenotypically distinct subsets have different functions. Interestingly, it has been demonstrated that double-negative NKT cells more efficiently reject tumor than do $CD4^+$ NKT cells [10]. On the other hand, a TCR component can divide NKT cells into two functionally separate populations - Vα14Jα18 and non-Vα14Jα18, and the latter can suppress tumor surveillance [11]. However, more investigations are needed to determine the function of each cell subset [12].

IL-17, a proinflammatory cytokine secreted by activated T cells and neutrophils, stimulates granulopoiesis and infiltration of neutrophils and mediates recruitment of T cells into peripheral tissue [13, 14]. Many researchers have studied the role of IL-17 in both innate and adaptive immunity. γδ T cells, which are responsible for innate immunity in peripheral tissue, secrete IL-17 in an IL-23-dependent manner during *Mycobacterium tuberculosis* infections [15]. It has been reported that IL-17-producing T cells that developed following vaccination enhance the recruitment of IFN-γ-producing effector T cells during recall responses against *M. tuberculosis* [14]. Moreover, IL-17 from Th-17 and IL-23 are considered new targets that are

critically involved in the pathogenesis of diverse autoimmune diseases in mice, especially experimental autoimmune encephalomyelitis [16]. IL-17 production was observed in NKT-like cells in adhesion molecule-deficient mice for homeostatic regulation of neutrophil generation in an IL-23-dependent manner [17]. Recently, $NK1.1^-$ NKT cells were shown to produce IL-17 by TCR stimulation and to mediate neutrophilia in lung [18].

In this study, we examined the properties and role of IL-17-producing NKT cells. We found that a distinct population of NKT cells produced IL-17 when they encountered α-GalCer in a CD1d-dependent manner and that they differed from IL-4- and IFN-γ-producing NKT cells. Although IL-17 produced by NKT cells contributed to infiltration of neutrophils into lung in α-GalCer-induced airway inflammation, it had no effect on the development of airway hyperreactivity. These findings suggest the possibility of a unique subset of NKT cells that regulates the immune system under some conditions.

I.3. Materials and Methods

Mice and immunization

We obtained six-week-old female C57BL/6 and Balb/c mice from Charles River Laboratories. $CD1d^{-/-}$ mice (kindly provided by Dr. S. Park., Korea University, Seoul, Korea) were bred under specific pathogen-free conditions in the experimental animal facility of the College of Pharmacy, Seoul National University (Seoul, Korea). All of the experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University. For α-GalCer stimulation *in vivo*, mice were treated with 2 μg of α-GalCer intravenously.

α-GalCer

α-GalCer was synthesized by coupling phytosphingosine with hexacosanoic acid following the previously reported protection/deprotection and galactosylation scheme [19]. α-GalCer, kindly provided by Dr. S. Kim (Seoul National University, Seoul, Korea), was dissolved in 0.5% Tween 20 in PBS, which was used as vehicle in all experiments.

Cytokine ELISA

To evaluate cytokine production, splenocytes were cultured at 5 x 10^6 cells/ml with α-GalCer or vehicle at 100 ng/ml. Culture supernatants were harvested, and the concentration of IL-17 was measured using a set of anti-IL-17 mAbs (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

RNA isolation and RT-PCR

To assess the mRNA transcription level of IL-17, total RNA was isolated using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) in accordance with the user's manual. For reverse transcription PCR (RT-PCR), 1.5 μg of total RNA was reverse transcribed into cDNA by oligo (dT) priming using M-MLV RT (Invitrogen Life Technologies) according to the manufacturer's instructions. Sense and antisense primers used in the PCR were as follows: for IL-17, 5'-CAC CCA GCA CCA GCT GAT-3' and 5'-AAT CAA TAG CAC GAA CTG-3' and for GAPDH, 5'-TTA GCA CCC CTG GCC AAG G-3' and 5'-CTT ACT CCT TGG AGG CCA TG-3'. Equal aliquots of RT-PCR product were electrophoresed on 1.5% agarose and stained with ethidium bromide.

Cell sorting

$CD3^-$, $CD3^+$, $CD3^{int}$/α–GalCer-loaded CD1d $dimer^+$ cells were sorted using a FACSAria® system (BD Biosciences) after staining with FITC-conjugated anti-CD3 antibody in combination with PE-labeled α-GalCer-loaded CD1d dimer.

Intracellular cytokine staining

Two hours after immunization with α-GalCer, single cells were prepared by passing minced spleens through a 70-μm nylon mesh and then RBC was lysed with ammonium chloride. Intracellular staining was performed with BD Cytofix/Cytoperm Plus (BD Biosciences) according to the manufacturer's instructions. To stain the surface marker, mouse IgG1-conjugated CD1d dimer, PE-labeled anti-mouse IgG1, PE/Cy7-labeled anti-NK1.1 mAb, PE/Cy7-labeled anti-CD4 mAb, PE/Cy7-labeled anti-B220 mAb, and FITC-conjugated TCRβ (all from BD Biosciences) were used. After permeabilization, intracellular staining was performed with PE/Cy7-conjugated IL-4 mAb (BD Biosciences), APC-conjugated IFN-γ mAb, and APC-conjugated IL-17 mAb (BioLegend, San Diego, CA, USA). Stained cells were analyzed with FACSCalibur (BD Biosciences) and CellQuest Pro software (BD Biosciences).

Induction of AHR and analysis of bronchoalveolar lavage fluid

α-GalCer was administered intranasally (1.5–2 μg in 50 μl) to mice anesthetized with ketamine and xylazine. Vehicle was administered to control groups. Airway responsiveness was assessed 24 h later as described [20]. AHR was calculated in enhanced pause (Penh). To neutralize IL-17, 50 μg of anti-IL-17 mAb (R&D Systems, Minneapolis, MN, USA) was injected i.p. 2 h before α-GalCer administration. Three hours after methacholine challenge test, mice were killed and BAL cells were obtained and analyzed as previously described [21]. Differential cell counts were performed by counting at least 300 cells on cytocentrifuged preparations followed by staining with Diff-Quick (Dade Behring, Dudingen, Switzerland).

Statistics

Results were expressed as the means ± SEM. Student's *t* test was used to examine differences between groups.

I.4. Results

IL-17-producing NKT cells are distinguishable from conventional NKT cells.

NKT-like cells have been reported to produce IL-17 in an IL-23-dependent manner to modulate the homeostasis of neutrophils in adhesion molecule–deficient mice [17]. Recently, NK1.1^{-} NKT cells has been demonstrated to produce IL-17 by α-GalCer stimulation [18]. To confirm these data, splenocytes from naïve C57BL/6 mice and CD1d$^{-/-}$ mice were stimulated *in vitro* with α-GalCer. As a result, IL-17 was detected in the culture supernatant of splenocytes from wild-type but not from CD1d$^{-/-}$ mice (Figure I-1A). We next sought to identify the splenocyte population responsible for IL-17 production after stimulation with α-GalCer. Two hours after treatment with α-GalCer, splenocytes were sorted into three populations – CD3^{-}, CD3^{+}, and CD3^{int}/α-GalCer-CD1d^{+} cells – and their levels of IL-17 mRNA expression were examined. Whereas CD3^{int}/α–GalCer-CD1d^{+} cells, which have the phenotype of NKT cells from α–GalCer-treated mice, primarily expressed IL-17 mRNA, the others produced only negligible levels of IL-17 (Figure I-1B). The expression levels of the IL-17 protein were investigated by culturing splenocytes 2 h after α-GalCer treatment without any additional stimulation, followed by

intracellular cytokine staining. Consistent with the findings for mRNA expression, α-GalCer-CD1d^{+} cells primarily produced IL-17 (Figure I-1C). In Balb/c mice, the treatment of α-GalCer also induced IL-17 production from NKT cells (data not shown).

Because IL-17-producing NKT cells represented only 2.28% of total NKT cells, far less than IFN-γ- and IL-4-producing NKT cells (60-70%) (Figure I-1C), we next tried to determine whether IL-17-producing NKT cells are the same population as conventional NKT cells producing IFN-γ and IL-4. To shed light on this issue, 2 h after treatment with α-GalCer, splenocytes were prepared and IL-17 and other cytokines were assessed by intracellular cytokine staining. There was nearly complete overlap by IFN-γ-producing and IL-4-producing NKT cells, but not for IL-17-producing and IFN-γ- or IL-4-producing NKT cells (Figure I-2A). These results indicate that IL-17-producing NKT cells can be distinguished from IFN-γ- and IL-4-producing conventional NKT cells—at least during the early response after TCR stimulation.

To characterize IL-17-producing NKT cell subset, we analyzed the expression of CD4 and NK1.1 on IL-17-producing NKT cells. Two hours after treatment with α-GalCer, splenocytes were prepared and IL-17 and other cytokines were assessed by intracellular cytokine staining. IFN-γ, IL-4, and IL-17 were detected in both of

$CD4^+$ and $CD4^-$ NKT cells (Figure I-2B). On the other hand, whereas IFN-γ and IL-4 were produced by both $NK1.1^+$ and $NK1.1^-$ NKT cells, IL-17 was produced primarily by $NK1.1^-$ NKT cells (Figure I-2C), a finding consistent with results of a previous study [18]. However, because IL-17 producing NKT cells barely overlapped with conventional NKT cells and $NK1.1^-$ NKT cells also include considerable IL-4 and IFN-γ-producing NKT cells, our observation indicates that it is inappropriate to define NK1.1 as a marker of IL-17 producing NKT cells.

α-GalCer induces anergy of IL-17-producing NKT cells.

Parekh et al. [22] recently reported that NKT cells become unresponsive after a single treatment of α-GalCer and that they are impaired in the secretion of cytokines after a second treatment with α-GalCer. Because the anergic response to α-GalCer could be an intrinsic property of NKT cells, we tested whether IL-17-producing cells become unresponsive upon α-GalCer stimulation by measuring IL-17 production in splenocytes from α-GalCer-retreated mice. After α-GalCer was injected into mice that had been treated with α-GalCer 3 days, 7 days, or 1 month earlier, production of IL-17 and other cytokines was assessed by intracellular cytokine staining. NKT cells from vehicle-treated mice produced IL-17, but splenocytes from mice treated 3 or 7 days earlier with α-GalCer produced

negligible levels of IL-17 and other cytokines (Figure I-3A). Although some IL-17-producing NKT cells were observed in splenocytes from mice treated 1 month earlier with α-GalCer, their levels did not represent a critical improvement over those in vehicle-treated mice. To confirm these data by assessing IL-17 secretion, splenocytes from mice treated 1 month earlier with α-GalCer or vehicle were prepared and stimulated with α-GalCer. Two days later, we observed that little IL-4 and IFN-γ were secreted by splenocytes of α-GalCer-pretreated mice. And also less IL-17 was detected in the supernatant of splenocytes from α-GalCer-pretreated than from vehicle-pretreated mice (Figure I-3B). These results demonstrate that IL-17-producing NKT cells are hyporesponsive after α-GalCer stimulation, suggesting that IL-17-producing cells share intrinsic properties with IL-4- and IFN-γ-producing NKT cells in anergy induction by pre-activation.

IL-17 from NKT cells activated by α-GalCer contributes to the infiltration of neutrophils into airway but has no effect on airway hyperreactivity.

Glycolipids like α-GalCer induce AHR and airway inflammation by activating pulmonary NKT cells when administered intranasally [23]. To evaluate the effect of IL-17 from activated NKT cells on lung inflammation, we blocked IL-17 by

using a mAb during the α-GalCer-induced AHR. We observed that AHR was developed upon intranasal administration of α-GalCer (Figure I-4A). After neutralizing IL-17 by injecting anti-IL-17 mAb into mice 2 h before challenge, there were significantly fewer neutrophils in bronchoalveolar lavage fluid but there was no effect on the induction of AHR (Figure I-4). These results indicate that IL-17 produced from activated NKT cells is not critical for the development of AHR but affects neutrophilia in lung.

Figures

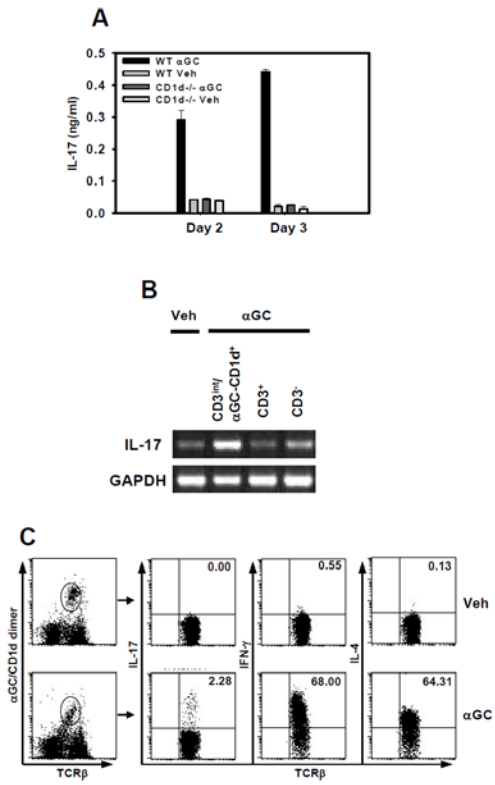

Figure I-1. NKT cells produce IL-17 upon α-GalCer stimulation.

(A) Splenocytes from naive C57BL/6 mice or CD1d$^{-/-}$ mice were stimulated with α-GalCer (100 ng/ml) or vehicle as a control. The supernatants were collected at indicated time points and IL-17 was measured by ELISA. (B) Mice were injected with 2 μg of α-GalCer or vehicle; 2 h later the mouse splenocytes were sorted into CD3^{-}, CD3^{+}, or CD3^{int}/α-GalCer-CD1d^{+} populations. mRNA expression of IL-17 in each population was detected using RT-PCR. (C) Mice were injected with 2 μg of α-GalCer (lower panel) or vehicle (upper panel); 2 h later the splenocytes were

cultured with Brefeldin A for 2 h and intracellular cytokines in $B220^{-}TCR\beta^{int}/\alpha$-GalCer-CD1d-$dimer^{+}$ cells were detected by flow cytometry. Results are representative of three separate experiments.

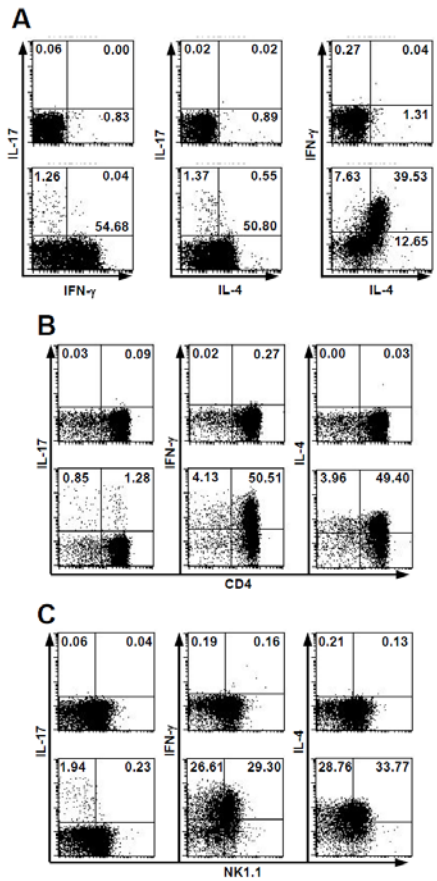

Figure I-2. IL-17-producing NKT cells are distinct populations that differ from conventional NKT cell.

(A) Mice were injected with 2 μg of α-GalCer (lower panel) or vehicle (upper panel); 2 h later the splenocytes were cultured with Brefeldin A for 2 h. Intracellular cytokines in TCRβ^{int}/α-GalCer-CD1d-dimer^{+} cells were detected by flow cytometry. (B, C) The cells were stained with anti-CD4 (B) or anti-NK1.1 (C) and intracellular cytokines in TCRβ^{int}/α-GalCer-CD1d-dimer^{+} cells were

detected by flow cytometry. Results are representative of three separate experiments.

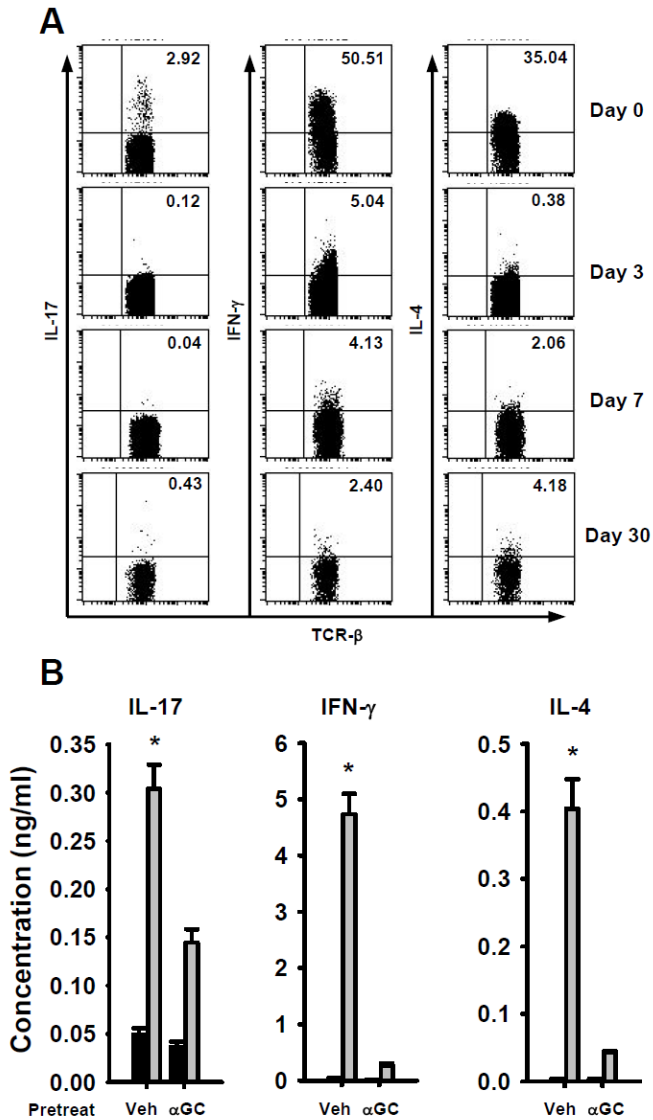

Figure I-3. α-GalCer stimulation induces hyporesponsiveness of IL-17-producing NKT cells.

(A) After an initial injection of α-GalCer, mice were reinjected with 2 μg of α-GalCer at the indicated time point; 2 h later cytokines were detected in splenic

NKT cells by intracellular cytokine staining. (B) After α-GalCer-pretreated mice were sacrificed at day 30, the splenocytes obtained were stimulated *in vitro* with 100 ng/ml α-GalCer (gray bar) or vehicle (black bar). Two days later, cytokine levels in supernatant were measured by ELISA. Results are representative of two separate experiments. *, $P<0.005$.

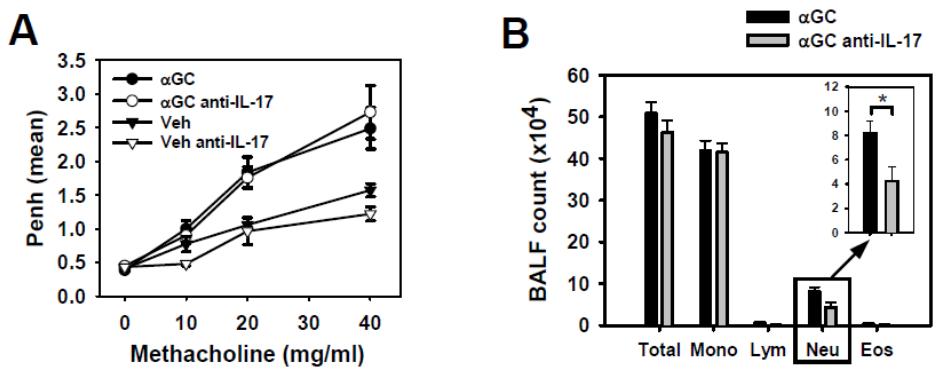

Figure I-4. IL-17 induced by pulmonary NKT cell activation mediates airway infiltration of neutrophils but has no effect on airway hyperreactivity.

Balb/c mice were challenged with 1.5 μg of α-GalCer intranasally in the presence or absence of anti-IL-17 neutralizing antibody. (A) 24 h after challenge, the methacholine response was measured. (B) Cells in BAL fluid from each mouse were differentially counted 3 h after methacholine test. Blockade of IL-17 had no effect on the development of AHR but reduced the infiltration of neutrophils into airway. *, $P<0.05$

I.5. Discussion

As IL-17 is increasingly linked to various disease processes, it is essential to define its source. We and others showed that NKT cells produce IL-17 upon TCR stimulation, suggesting that they could be one source of the cytokine [18]. NKT cells have been divided into several subsets on the basis of their markers or the ligands. In our study, it was discovered that IL-17 producing NKT cells upon α-GalCer stimulation differed from the IL-4- and IFN-γ-producing NKT cell populations. To investigate whether this novel population possessed the properties of NKT cells, we tested the anergic properties of NKT cells in IL-17-producing cells and found that α-GalCer stimulation results in the unresponsiveness of IL-17-producing NKT cells. IL-17 induced by α-GalCer mediated the recruitment of neutrophils in the airway but had no effect on AHR development.

IL-17 is a proinflammatory cytokine that regulates various inflammatory cytokines, chemokines, and metalloproteinases [13, 24, 25]. Originally IL-17 was thought to be expressed in activated $CD4^+$ T cells. These cells are now defined as Th17 and are thought to be critical in chronic inflammation, especially autoimmunity [13]. Other types of cells, including γδ T and NKT-like cells, produce IL-17 and are regulated by IL-23, participating in granulopoiesis by inducing the production of

G-CSF [17]. Recently, others showed that NKT cells can produce IL-17 after stimulation with a specific ligand which is not dependent of IL-23 [18].

It is possible that NKT cells regulate granulopoiesis by secreting IL-17 upon TCR stimulation. Our investigation revealed an increased percentage of splenic granulocytes after α-GalCer stimulation. This increase did not depend on IL-17 produced by NKT cells, as a blockade of IL-17 showed no difference in the increase (data not shown). IL-17 from NKT cells under these conditions might be too subtle because NKT cells produce far less IL-17 than they do such granulopoietic cytokines as GM-CSF [26]. Based upon these findings, we postulate that IL-17-producing NKT cells might play an important role in local infection or inflammation.

NKT cells include several functionally distinct populations. For example, in mice, a double-negative subset of NKT cells is more effective in rejecting tumor cells than a $CD4^+$ subset [10], suggesting that double-negative NKT cells are functionally distinct from $CD4^+$ NKT cells. Although the $CD4^-$ population was previously reported to be primarily responsible for IL-17 production [17], we found that α-GalCer-stimulated IL-17 production was not biased by the CD4 marker. NK1.1 which was used to define NKT cell originally, also can be used as a marker to divide NKT cells into subsets. It has been demonstrated that recent thymic

emigrant NKT cells do not express NK1.1 and obtain NK1.1 within days of export [27]. Thus, NK1.1 phenotype has been regarded as a marker of maturity rather than that of a subset. It has not been defined clearly how each population of peripheral NKT cells functions under physiological conditions. Our findings and those recently reported by Michel et al. [18] suggest that NKT cells with or without the NK1.1 marker function in different ways by producing different cytokines after stimulation, showing IL-17 produced by $NK1.1^-$ NKT cells mediates the recruitment of neutrophils into the lung. Another recent report also suggests that periphery $NK1.1^-$ NKT cells are a stable and mature population that has different properties from immature thymic $NK1.1^-$ NKT cells [28]. These studies suggest that $NK1.1^-$ NKT cells are not only immature form of NKT cells but could be functionally distinct subset. On the other hand, we evaluated that there are cells producing IL-4 or IFN-γ among $NK1.1^-$ NKT cells that are distinguished from IL-17-producing $NK1.1^-$ NKT cells. It seems that $NK1.1^-$ NKT cells are heterogeneous population because there are considerable IL-4- and IFN-γ-producing NKT cells as well as IL-17 producing cells. Therefore, NK1.1 is inappropriate to define IL-17 producing NKT cells and the surface expressing specific marker of IL-17 producing NKT cells remains to be defined.

Several studies have shown that NKT cells are required for the development of allergen-induced AHR and airway inflammation [29-31]. In addition, the direct activation of pulmonary NKT cells upon administration of glycolipids intranasally could develop AHR and both IL-4 and IL-13 are critical to the development of AHR [23]. Although intranasal administration of α-GalCer induces eosinophilia in the airway, infiltration of eosinophils is not responsible for the development of AHR [23]. On the other hand, the presence of IL-17 in the sputum of asthma patients suggests that IL-17 may play an important role in development of AHR [32]. IL-17 is known to mediate the infiltration of neutrophils into tissues, but its function during the development of AHR has yet to be defined. One report suggests that administration of exogenous IL-17 into lung exerts a negative effect on allergic asthma [33]. In our study, blockade of IL-17 had no effect on the induction of AHR despite the reduced numbers of neutrophils in lungs during the airway inflammation induced by NKT-specific activation. It means that IL-17 seems to participate in leukocyte infiltration but be dispensable to the development of AHR. A recent study that describes the role of a costimulatory molecule of NKT cells observed that the treatment with α-GalCer induces infiltration of neutrophils into the airway, consistent with the above data [34].

In conclusion, we found that a small population of NKT cells has the ability to secrete IL-17 upon TCR stimulation and that IL-17-producing NKT cells can function *in vivo*. Although the amount of IL-17 produced by NKT cells was relatively small, the function of IL-17-producing NKT cells may be important under certain conditions. Further studies are needed to determine the roles played by IL-17-producing NKT cells in other infectious or inflammatory diseases.

CHAPTER II

Retinoic acid alleviates Con A-induced hepatitis: Differential regulation of effector production in NKT cells

II.1. Summary

Retinoic acid (RA) is a diverse regulator of immune responses. Although RA promotes natural killer T (NKT) cell activation in vitro by increasing CD1d expression on antigen-presenting cells (APCs), the direct effects of RA on NKT-cell responses in vivo are not known. In the present study, we demonstrated the effect of RA on the severity of Con A-induced hepatitis and molecular changes of NKT cells. First, we demonstrated that Con A-induced liver damage was ameliorated by RA. In correlation with cytokine levels in serum, RA regulated the production of IFN-γ and IL-4, but not TNF-α by NKT cells without influencing the NKT-cell activation status. However, RA did not alleviate α-GalCer-induced liver injury, even though it reduced IFN-γ and IL-4 but not TNF-α levels in serum. This regulation was also detected when liver mononuclear cells (MNCs) or NKT hybridoma cells were treated with RA in vitro. The regulatory effect of RA on NKT cells was mediated by RAR-α and RA reduced the phosphorylation of MAPK. These results suggest that RA differentially modulates the production of effector cytokines by NKT cells in hepatitis, and the suppressive effect of RA on hepatitis varies with the pathogenic mechanism of liver injury.

II.2. Introduction

Liver damage induced by various agents, such as viral infection, results in serious problems accompanied by an excessive immune response [35]. Uncontrolled severe responses in the liver by immune cells are observed in diverse animal models, including Con A-induced hepatitis. Following the administration of Con A, T cells, granulocytes, and Kupffer cells infiltrate into the liver, resulting in the death of hepatocytes [36-38]. NKT cells are responsible for liver injury in this model [39-44]. NKT cells are a distinct T cell subset with an invariant T cell receptor (TCR) that recognizes glycolipids loaded on the cell-surface protein CD1d, and they rapidly secrete cytokines upon stimulation. [3, 45-47]. In Con A-induced liver injury, inflammatory cytokines, such as IFN-γ, TNF-α, and IL-4, from NKT cells are pathogenic [39, 41, 43, 44]. In addition, a specific ligand of NKT cells, α-GalCer, can induce liver injury mediated by FasL and TNF-α rather than IFN-γ [48-50]. Although NKT cells are critical to induce both Con A- and α-GalCer-induced liver injury, their pathologic mechanisms are different from each other.

RA, an active metabolite of vitamin A, regulates various diseases through anti-tumor and anti-inflammatory effects [51, 52]. RA is associated with anti-inflammatory effects in diverse diseases [53]. RA also enhances T cell effector

responses and is critical in vaccine responses [54-58]. These contradictory findings imply that the exact physiological function of RA remains to be discovered. RA promotes the proliferation and activation of NKT cells indirectly *in vitro* by increasing CD1d expression in APCs [59-61]. However, the direct effects of RA on NKT cells and NKT cell-dependent diseases *in vivo* have not been examined. In the current study, we observed that RA differentially regulates NKT cell-mediated hepatitis and cytokine production by NKT cells. Our results suggest the selective regulatory effects and the therapeutic potential of RA in NKT cell-dependent diseases.

II.3. Materials and methods

Mice

Six- to 8-wk-old female C57BL/6 mice were purchased from Orient Bio. All mice were bred and maintained in specific pathogen-free conditions. All studies conformed to the principles for laboratory animal research outlined by Seoul National University (Seoul, Korea).

Reagents

α-GalCer, kindly provided by Dr. S. Kim (Seoul National University, Seoul, Korea), was dissolved in 0.5% Tween 20 in saline [19]. ATRA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO, further diluted in olive oil for injection, and 35 mg/kg of RA was intraperitoneally (i.p.) injected into the mice 16 hours before injecting Con A or α-GalCer. Disulfiram was dissolved in DMSO, further diluted in olive oil, and injected i.p. at a concentration of 10 mg/kg. The antagonist of RAR-α (Ro 41-5253) was purchased from Enzo Life Science (NY, USA), and the antagonists against RAR-γ (MM11253) and RXR (UVI3003) were purchased from Tocris Bioscience (Bristol, UK). They were dissolved in DMSO.

Flow cytometry and intracellular cytokine staining

Intracellular staining was performed with BD Cytofix/Cytoperm Plus (BD Biosciences) according to the manufacturer's instructions without additional stimulation ex vivo. The antibodies were purchased from BioLegend (San Diego, CA, USA). The stained cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and CellQuest Pro software (BD Biosciences).

Con A- or α-GalCer-induced hepatitis

Con A (Sigma-Aldrich) was dissolved in PBS and intravenously (i.v.) injected into the mice at a concentration of 20 mg/kg. For the survival study, the Con A dosage was increased to 30 mg/kg. The mice were euthanized after becoming moribund. For the disulfiram treatment study, the Con A dosage used for alanine aminotransferase (ALT) detection was 15 mg/kg and for survival monitoring was 17 mg/kg. The level of ALT was measured using Fuji-Dri Chem (Fuji Film, Tokyo, Japan) in accordance with the manufacturer's instructions. Five micrograms of α-GalCer was further diluted in PBS and i.v. injected into the mice. For histology analysis, livers were fixed in 10% formalin and embedded in paraffin. Sections were stained with H&E at Reference Biolabs (Seoul, South Korea). Anti-

asialoGM1 (200 μg) was administered i.p. to mice, followed by ATRA treatment (35 mg/kg) 16 hours before Con A i.v. injection. To deplete Kupffer cells, 200 μg of gadolinium chloride ($GdCl_3$) was injected i.v. 24 and 36 hours before administration of Con A. To deplete Tregs, 300 μg of anti-CD25 (PC61) was injected i.p. 16 and 40 hours before Con A injection.

Isolation of liver mononuclear cells

The liver MNCs were isolated as described previously [62]. Briefly, cells in supernatants were resuspended in 40% Percoll (GE healthcare), overlaid on 70% Percoll and centrifuged for 30 min at 750×*g*. Cells in interphase were collected and washed. Adhesive cells in liver were isolated with collagenase solution as described previously [63].

Cell culture

The liver MNCs (3.5×10^5 cells) and the DN32.D3 hybridoma cells (5×10^4 cells, provided by Dr. Park, Korea University, Seoul, Korea) were incubated with Con A (5 μg/ml) or α-GalCer (200 ng/ml) for 24 h in the presence of 100 nM ATRA. The supernatants were collected for ELISA. For the antagonist assay, chemicals were used at a concentration of 4 μM, and ATRA was used at a concentration of 10 nM.

Cytokine ELISA

The levels of IFN-γ, IL-4 and TNF-α in serum or supernatants were evaluated with ELISA kits in accordance with the manufacturer's instructions (BD Biosciences).

Western blot analysis

Con A-stimulated DN32.D3 hybridoma cells in the presence of vehicle (DMSO) or ATRA were lysed with Triton lysis buffer. SDS-PAGE was performed on 8% polyacrylamide gels, and then proteins were transferred to PVDF membranes. Following blocking using 5% BSA buffer, the blots were incubated in the presence of primary Abs specific for pERK, ERK, pJNK, JNK, phospho-p38 MAPK, p38 MAPK, IκB (all from Cell Signaling Technology, MA, USA), and GAPDH (Abcam, Cambridge, UK), followed by HRP-conjugated goat anti-rabbit IgG. The membrane was developed using WEST-one reagent (iNtRON Biotechnology, Gyeonggi-do, Korea) and detected on an X-ray film. The membrane was stripped and re-blotted.

Quantitative real-time PCR

Total RNA was extracted from cells using RNeasy kit (Qiagen) and reverse-transcribed into cDNA using oligo-dT primers and MMLV reverse transcriptase

(Roche). Quantitative real-time PCR was performed using an ABI 7500 (Applied Biosystems) and SYBR green PCR MasterMix (Fermentas). Primer sequences were as follows: for Hprt, 5′-AAGACTTGCTCGAGATGTCATGAA-3′ (forward) and 5′-ATCCAGCAGGTCAGCAAAGAA-3′ (reverse); for IFN-□, 5′-AACCCACAGGTCCAGCGCCA-3′ (forward) and 5′-CACCCCGAATCAGCAGCGACT-3′ (reverse); for IL-4, 5′-GGGCTTCCAAGGTGCTTCGC-3′ (forward) and 5′-TCCAGGCATCGAAAAGCCCGA-3′ (reverse); for TNF-□, 5′-GCCAGCCGATGGGTTGTACC-3′ (forward) and 5′-CTTGGGGCAGGGGCTCTTGA-3′ (reverse). The reaction conditions were 10 min at 95°C, followed by 15 s at 95°C, 30 s at 57°C and 30 s at 72°C for 45 cycles, and 30 min at 72°C. The comparative Ct method for relative quantification was used, and all of the expression levels of the target genes were normalized to the expression of Hprt.

Statistics

The results are expressed as the mean values ± SD. To compare the differences between two groups, Student's *t* test was used. The Kaplan-Meier method was

used to analyze the statistical significance of differences in survival time. The log-rank test (Mantel-Cox) was applied using SPSS 16.0 for Windows.

II.4. Results

RA regulates Con A-induced hepatitis and differentially regulates cytokine levels in serum

To determine the effect of RA itself, we injected RA directly into normal mice, and liver injury was induced by injecting Con A. The RA-treated group had a 100% survival rate, whereas the entire control group succumbed to the lethal dose (30mg/kg) several hours after the Con A injection (Figure II-1A). In addition, when the ALT activity was measured in animals with nonlethal (20 mg/kg) Con A-induced hepatitis, significantly less ALT activity was observed in the RA-treated group (Figure II-1B). And also, liver histology showed massive necrosis in vehicle treated mice, but in RA-treated mice, the liver tissue maintained the structure (Figure II-1C). Treatment with disulfiram, a blocking agent of RALDH which synthesizes RA, aggravated the survival rate and serum ALT activity, indicating the protective effect of endogenous RA against Con A-induced hepatitis (Figure II-1D and E). The pathogenesis and maintenance of Con A-induced liver injury is mediated by inflammatory cytokines, such as IFN-γ, IL-4, and TNF-α [39, 41, 43, 44]. Interestingly, treatment with RA reduced the levels of IFN-γ and IL-4 in serum significantly but failed to affect the level of TNF-α (Figure II-1F). These

data show that RA regulates Con A-induced hepatitis and that this effect is correlated with IFN-γ and IL-4 levels in serum.

Differential regulation of cytokine production from hepatic NKT cells by RA

Since NKT cells are responsible for early cytokine production in Con A-induced hepatitis [41, 42], the production of each effector cytokine in NKT cells was analyzed. As with the cytokine levels in serum, RA reduced the percentage of IFN-γ- or IL-4-producing NKT cells but not TNF-α-producing NKT cells (Figure II-2B and C). Conventional T cells did not seem to be critically involved in the reduced cytokine level (Figure II-2A, D and Figure II-3). In the RA-treated group, NK cells included a considerably reduced percentage of IFN-γ-producing cells 6 hours post-injection compared to the control, but they were not required for the regulation or pathogenicity of liver injury (Figure II-2E and II-4A). The percentage of IL-4- or TNF-α-producing T or NK cells was below 1% (data not shown). Furthermore, we found that Tregs, which can be induced by RA, was not altered by treatment of RA and they were dispensable in the protective effect of RA on hepatitis (Figure II-4B-E). Our observations indicate that NKT cells can play a

predominant role in the regulation of cytokine production and the modulation of liver injury by RA.

Treatment with RA does not modulate the activation of NKT cells or the expression of FasL

The suppression of cytokine-producing NKT cells by RA could be caused by an impaired activation of NKT cells. We therefore sought to determine if the observed effects of RA resulted from the inhibition of NKT cell activation. The population of NKT cells in the liver rapidly decreases in Con A-induced hepatitis [42], which may be considered a parameter of NKT cell activation. NKT cell populations decreased shortly after Con A injection, with no significant difference between the RA and control groups (Figure II-5A and B). In addition, the expression of CD69 and CD25 showed no difference before or after Con A injection between the two groups (Figure II-5C and D).

Some studies have suggested that FasL, which is upregulated upon stimulation in NKT cells, may act as an effector molecule during liver injury, even though such a role is controversial in Con A-induced hepatitis [63, 64]. We observed that the expression of FasL on the surface of NKT cells after injection of Con A was

similar between the two groups (Figure II-5C and D). Collectively, these data indicate that RA does not modulate the activation of NKT cells.

Next, we examined the effects of RA on other cells, such as Kupffer cells and other APCs that might participate in the regulatory effects of RA on NKT cells. As illustrated in Figure II-5E, the percentages of Kupffer cells before and after Con A injection were comparable in each group (Figure II-6A). In addition, RA tended to reduce ALT activity in Kupffer cell-depleted mice (Figure II-6B). Moreover, the expression of costimulatory molecules or CD1d was not modulated by RA (Figure II-5F and Figure II-6C). Overall, these data indicate that treatment with RA reduces IFN-γ and IL-4 but not TNF-α production in NKT cells without affecting Kupffer cells or other APCs.

Treatment of RA reduces cytokine levels in serum but does not regulate liver injury in α-GalCer-induced hepatitis

We next examined whether RA could also regulate α-GalCer-induced hepatitis. Consistent with Con A-induced hepatitis, RA reduced the levels of IFN-γ and IL-4 but not TNF-α in α-GalCer-induced hepatitis (Figure II-7A). Although α-GalCer-induced hepatitis is mediated by activated NKT cells, its pathogenic mechanism is not consistent with Con A-induced liver injury. For example, whereas TNF-α is

important in both liver injury models, IFN-γ is critical in Con A-induced hepatitis but not in α-GalCer-induced hepatitis [50, 63]. We found that treatment with RA failed to regulate α-GalCer-mediated liver injury, with comparable ALT levels to the control (Figure II-7B), correlating with unaltered level of TNF-α (Figure II-7A). These results indicate that RA can alleviate Con A- but not α-GalCer-induced hepatitis. The differential regulation of RA on cytokine production can explain the contrary effects of RA in two hepatitis models.

RA directly regulates the secretion of IFN-γ and IL-4 from NKT cells through RAR-α.

The observations described above led us to hypothesize that RA acts on NKT cells directly. Therefore, we examined the effects of RA on liver MNC cultures in vitro to exclude the environmental factors present in the liver. Consistent with the in vivo results, in the presence of RA, the secretion of IFN-γ and IL-4 but not TNF-α was reduced compared to vehicle in the presence of Con A or α-GalCer stimulation (Figure II-8A and B). RA has been suggested to act upon various cell types via its specific receptors. In NKT cells, the mRNA expression of RAR-α, RAR-γ, and RXR-α was detected (data not shown), and the addition of Ro 41-5253, a specific RAR-α antagonist, recovered the suppression of IFN-γ and IL-4 secretion induced

by RA (Figure II-8C). Taken together, these results indicate that RAR-α mediates the regulation of cytokine production by RA.

Next, to determine if RA directly affects NKT cells, the CD1d-expressing NKT cell line DN32.D3 was stimulated with Con A or α-GalCer in the presence of RA (Figure II-9A). As shown in Figure II-8D and E, the secretion of IFN-γ and IL-4 but not TNF-α was reduced by RA. The mRNA expression was consistent with the quantitation data of the secreted cytokines (Figure II-9B). Because TNF-α production□ which is regulated by NFAT, was not reduced by RA, we examined the changes in other signaling molecules that are activated upon TCR stimulation. As a result, the phosphorylation of MAPK, especially JNK, was reduced by RA (Figure II-8F). We measured the amount of IκB, as an indicator of NFκB signaling, by western blot, and it was not influenced by RA. Therefore, these data suggest that RA regulates cytokine production in NKT cells directly, the mechanism of which might include a modification of MAPK signaling pathway.

Figure

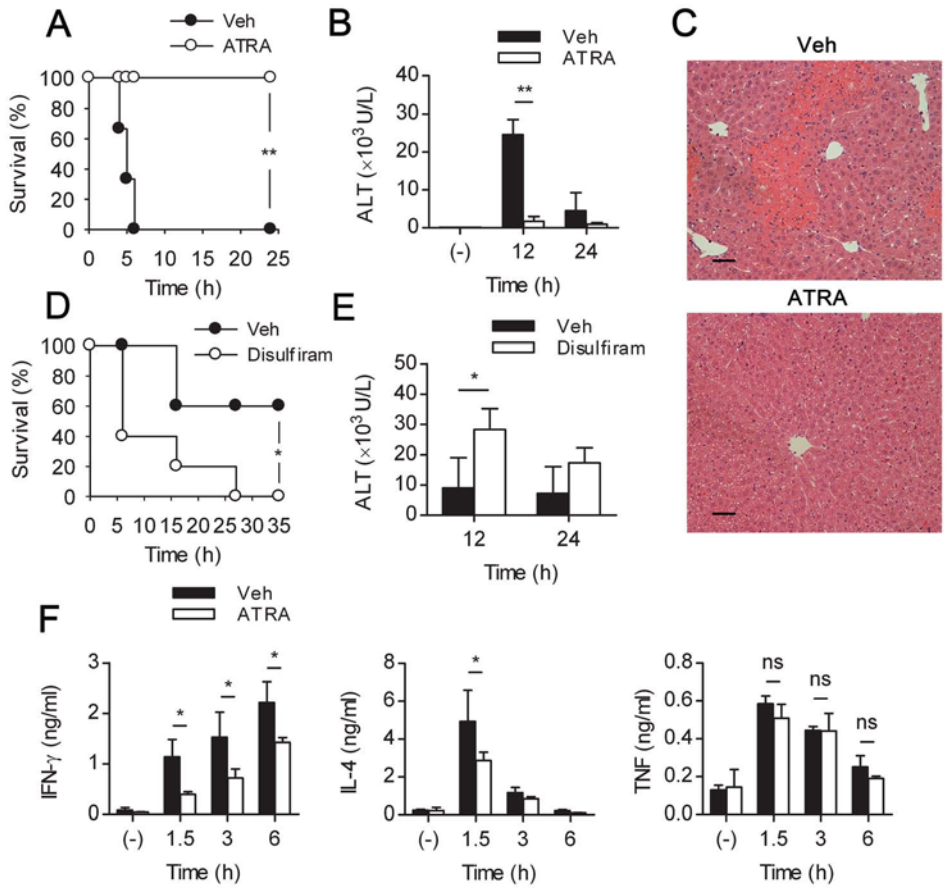

Figure II-1. RA ameliorates Con A-induced hepatitis

(A) ATRA was administered to C57BL/6 mice i.p. 16 h before Con A i.v. injection. Survival was monitored after the injection of a lethal dose of Con A (30 mg/kg). n=6 to 7; **p<0.005 by the Kaplan-Meier method and the log-rank test. (B) At 12 h and 24 h after the injection of a sublethal dose of Con A (20 mg/kg), the serum was collected, and ALT activity was measured. Data are presented as the mean+SD. n=3 to 4; **p<0.005 from each Veh-treated group by Student's *t* test.

Data are representative of three independent experiments (A, B). (C) Liver tissue was collected 12 h after Con A injection for H&E staining (original magnification, ×100). Each scale bar indicates 100 μm. (D) Disulfiram was injected i.p. for 3 days, followed by the injection of Con A (17 mg/kg), after which survival was monitored. n=5; *$p<0.05$ by the Kaplan-Meier method and the log-rank test. (E) At 12 h post-injection of Con A (15 mg/kg), ALT in the serum was measured. Data are presented as the mean+SD. n=4; **$p<0.005$, ns=non-significant from each Veh-treated group by Student's *t* test. Data are representative of two independent experiments (D, E). (F) Serum was collected at each time point after the injection of Con A (20 mg/kg), and IFN-γ, IL-4 and TNF-α levels were determined via ELISA. Data are presented as the mean+SD. n=3 to 4; **$p<0.005$ from each Veh-treated group by Student's *t* test. Data are representative of three independent experiments. ATRA and Veh (DMSO) were dissolved in olive oil.

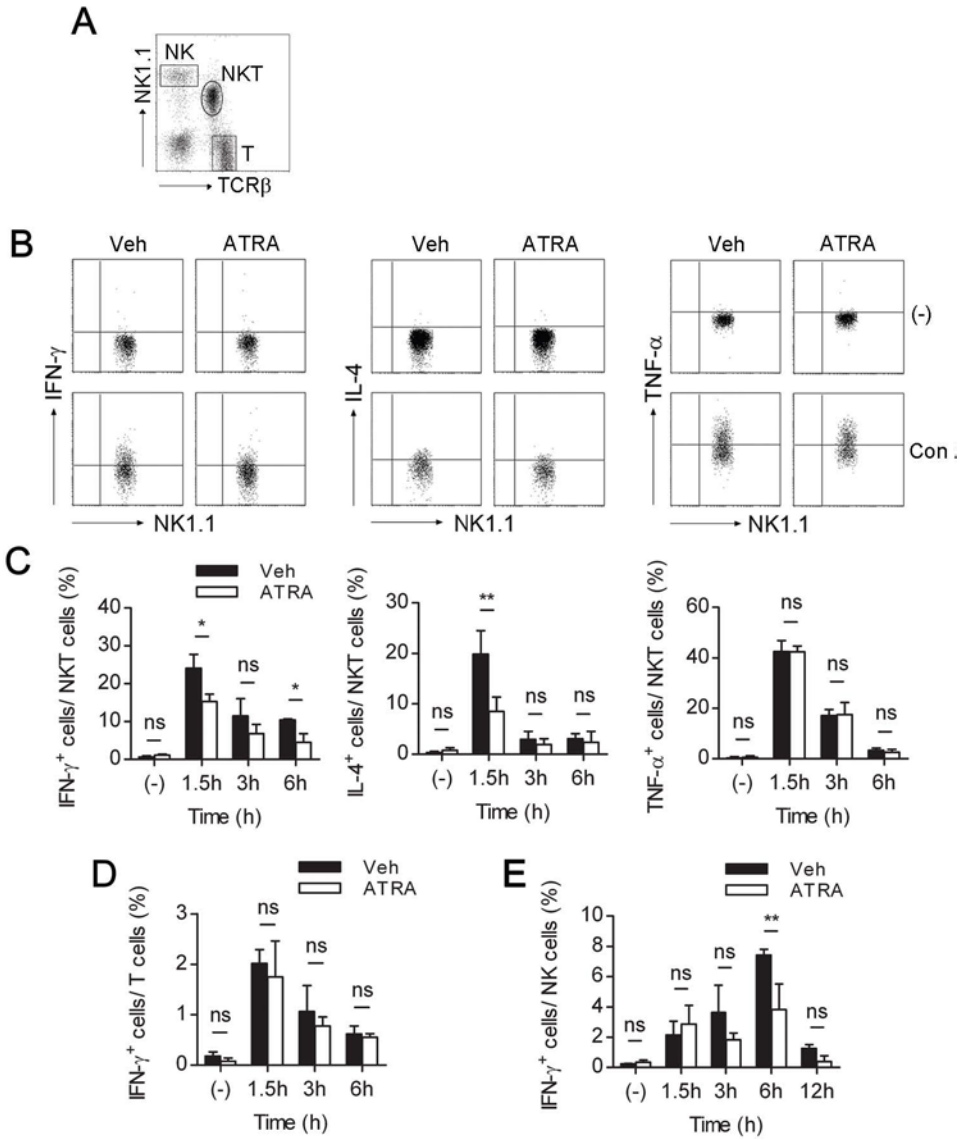

Figure. II-2. RA regulates the production of IFN-γ and IL-4 but not TNF-α by NKT cells in the liver during the induction of hepatitis

Mice were pretreated with ATRA, and liver MNCs were collected 1.5 h after the injection of Con A (20 mg/kg), followed by intracellular cytokine staining and flow cytometry analysis. Liver MNCs were stained with NK1.1 and TCRβ. (A) The gating strategy for NKT, T and NK cells. (B) NKT (TCRβintNK1.1+) cells were gated and shown. (C) The percentage of NKT cells positive for each cytokine at each time point. (D, E) The percentage of IFN-γ-positive cells in T cells (TCRβhiNK1.1-)(D) and NK (TCRβ-NK1.1+)(E) cells at each time point. All data are representative of three independent experiments. n=3; Veh, DMSO; *$p<0.05$, **$p<0.005$, ns=non-significant from each Veh-treated group by Student's *t* test.

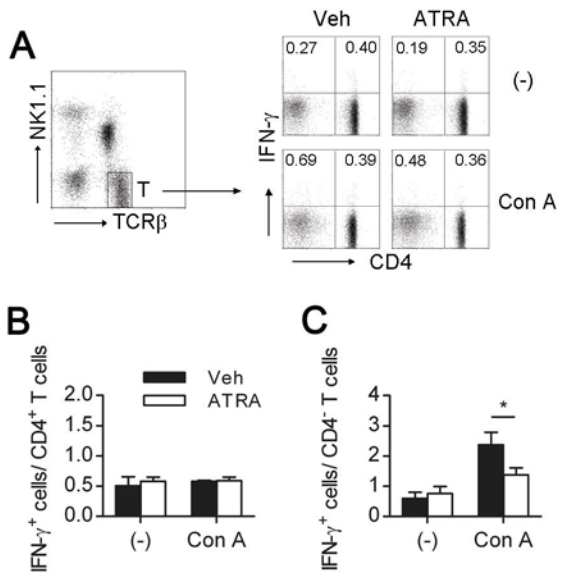

Figure. II-3. Conventional CD4$^+$ T cells are not responsible for production of IFN-γ

(A) Mice were pretreated with ATRA, and liver MNCs were collected 1.5 h after injection of Con A (20 mg/kg) followed by intracellular cytokine staining and flow cytometry analysis. Liver MNCs were stained with NK1.1, TCRβ and CD4. Conventional T cells were gated and shown. (B and C) The graphs show the percentage of IFN-γ-producing cells in CD4$^+$ conventional T cells (B) or CD4$^-$ conventional T cells (C). Data (mean+SD) are representative of two independent experiments. n=3; Veh, DMSO; *p<0.05 (Student's t-test).

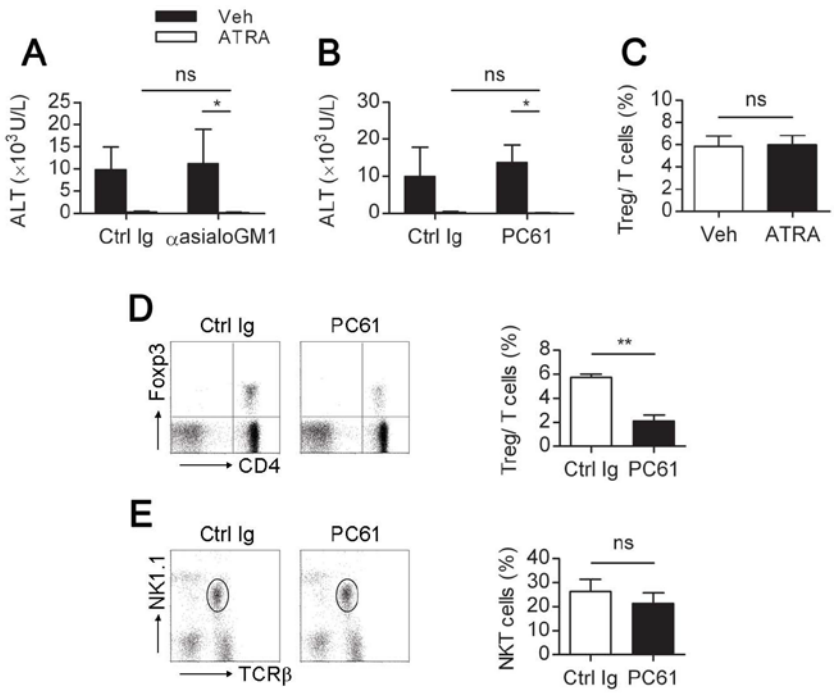

Figure. II-4. NK cells and Tregs do not participate in the regulation of Con A-induced hepatitis by RA

(A) Anti-asialoGM1 (200 μg) was administered to mice, followed by ATRA treatment (35 mg/kg) 16 h before Con A i.v. injection. (B) Anti-CD25 (PC61) (300 μg) was administered 40 and 16 h before Con A injection. ATRA was administered as in (A). Next, 12 h after Con A injection, serum was collected, and ALT activity was analyzed. (C) Liver MNCs were collected 16 h after ATRA treatment (35mg/kg), which was followed by intracellular staining of Foxp3 and flow cytometry analysis. The graph shows the percentage of Foxp3+ cells in

conventional T cells. (D and E) Anti-CD25 (PC61) (300 μg) was administered 40 and 16 h before Con A injection. Foxp3^{+} cell in T cells (D) and NKT cells (E) are shown. All data are presented as the mean+SD. n=3; *p<0.05, **p<0.005, ns=non-significant by Student's t test. Data are representative of two independent experiments.

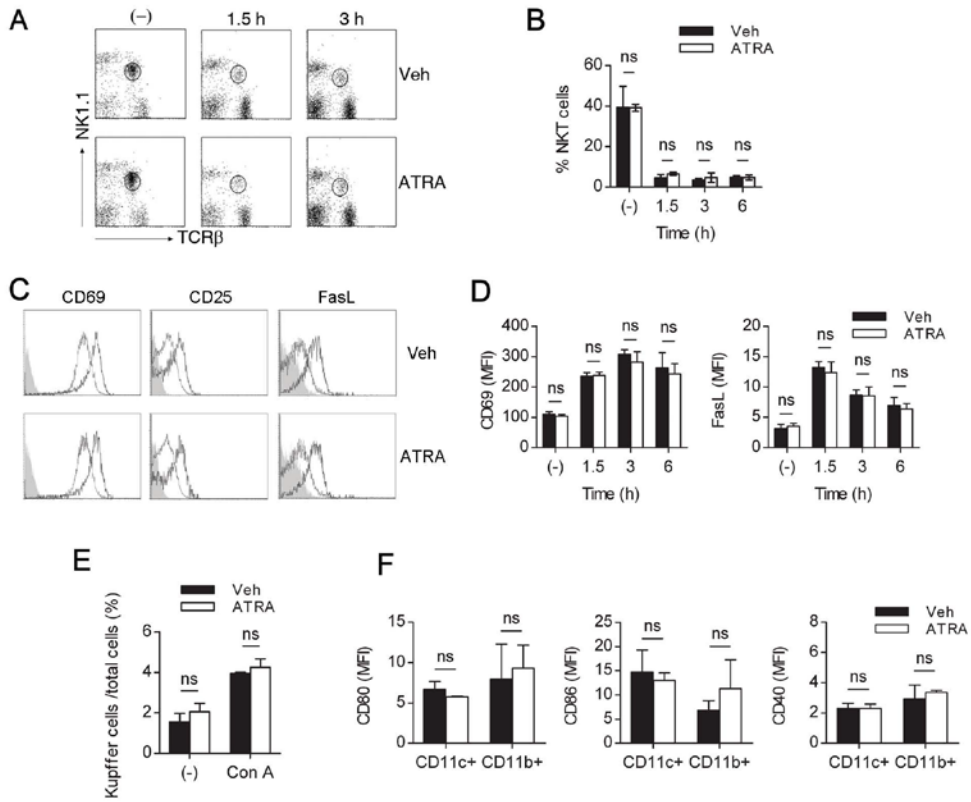

Figure II-5. Treatment with RA does not inhibit the activation of NKT cells or the expression of FasL

(A) Mice were pretreated with ATRA, and liver MNCs were collected at the indicated time points after Con A injection (20 mg/kg) and stained with anti-NK1.1 and anti-TCRβ. (B) The percentage of NKT cells at each time point. Data are presented as the mean+SD. n=3 to 4; ns=non-significant from each Veh-treated group by Student's *t* test. (C) CD69, CD25, and FasL were detected using flow

cytometry analysis 1.5 h after Con A injection. Each histogram includes the isotype control (gray histogram), unstimulated NKT cells (dashed line) and Con A-treated NKT cells (solid line). (D) The median fluorescence intensity (MFI) of CD69 and FasL on the surface of NKT cells at each time point. Data are presented as the mean+SD. n=3 to 4; ns=non-significant from each Veh-treated group by Student's t test. (E) The percentage of Kupffer cells (CD11b+CD68+) with or without Con A injection (1.5 h). Data are presented as the mean+SD. n=3 to 4; ns=non-significant from each Veh-treated group by Student's *t* test. (F) The MFI of CD80, CD86, and CD40 on CD11c+ or CD11b+ cells before Con A injection. Data are presented as the mean+SD. n=3 to 4; ns=non-significant from each Veh-treated group by Student's t test. Data are representative of three independent experiments. Veh, DMSO.

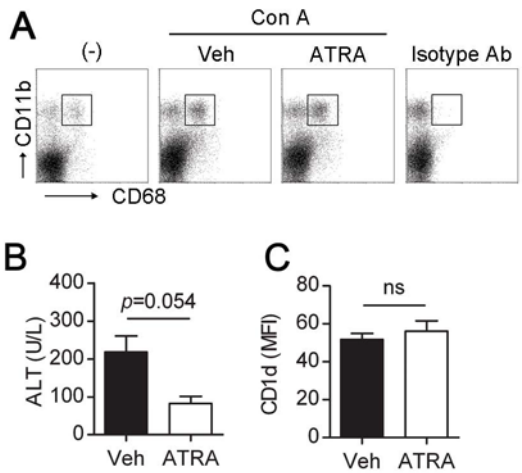

Figure. II-6. In Kupffer cell-depleted mice, RA tends to reduce ALT activity

(A) The gating strategy for Kupffer cells. (B) $GdCl_3$ (200 μg) was injected i.v. 36 and 24 h before Con A administration. ATRA was injected at 35 mg/kg 16 h before Con A injection. Next, 12 h after injection of Con A, serum was collected, and ALT activity was analyzed. Data are presented as the mean+SD. *n*=3; the comparison was made using Student's *t* test. (C) The MFI of CD1d on liver MNCs from the mice injected with ATRA or Veh without Con A administration. Data are presented as the mean+SD. *n*=3; ns=non-significant by Student's *t* test. Data are representative of two independent experiments. Veh, DMSO.

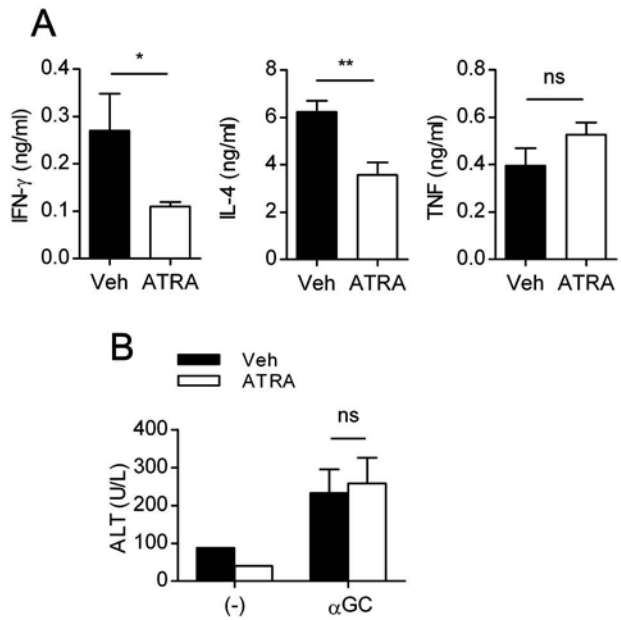

Figure II-7. Treatment of RA reduces cytokine levels in serum but does not regulate liver injury during α-GalCer-induced hepatitis

(A) ATRA (35 mg/kg) was administered to C57Bl/6 mice i.p., 16 h prior to the i.v. administration of 5 μg of α-GalCer. Serum was collected 1.5 h after the injection of α-GalCer, and IFN-γ, IL-4 and TNF-α levels were detected using ELISA. Data are presented as the mean+SD. n=3; *$p<0.05$, **$p<0.005$ from each Veh-treated group by Student's *t* test. (B) Serum was collected 12 h after the injection of α-GalCer, and ALT activity was measured. Data are presented as the mean+SD. n=5; ns=non-significant from each Veh-treated group by Student's *t* test. All data

are representative of two independent experiments. Veh, DMSO; αGC, α-galactosylceramide.

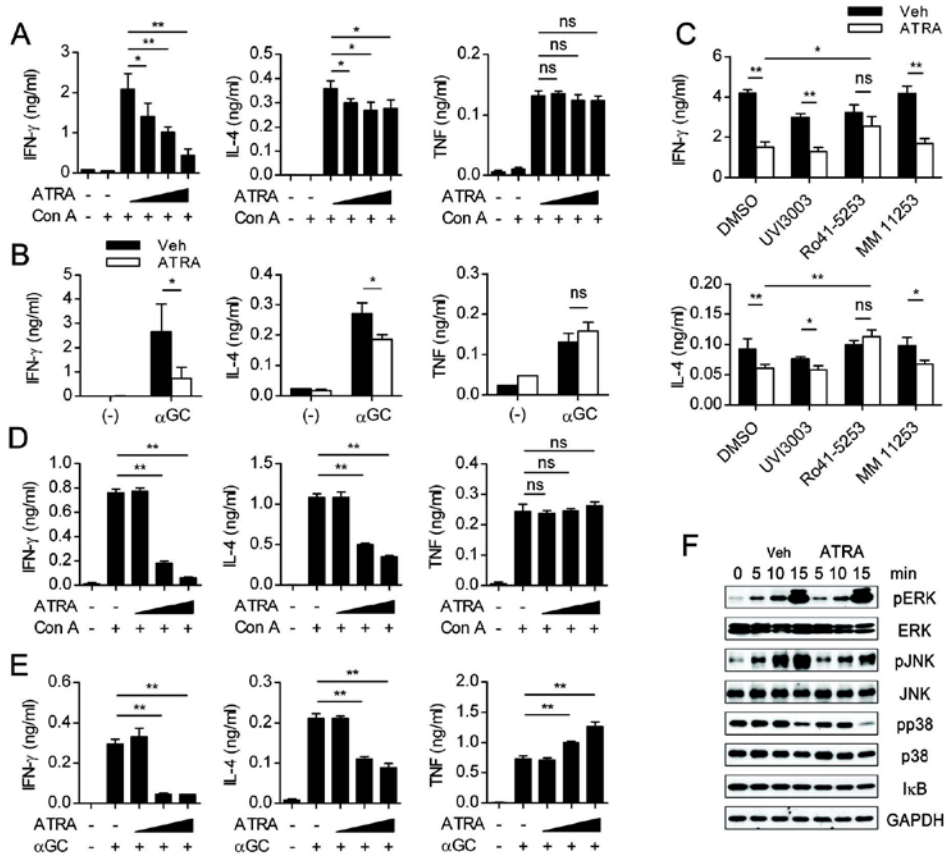

Figure II-8. RA regulates IFN-γ and IL-4 secretion via RAR-α and reduces MAPK activation *in vitro*

(A) Liver MNCs were stimulated with 5 μg/ml Con A in the presence of different concentrations of ATRA for 24 h, and the cytokine levels in the supernatants were measured using ELISA. ATRA was serially diluted by 1/500 starting with 100 nM. (B) Liver MNCs were stimulated with 200 ng/ml α-GalCer in the presence of 100

nM ATRA for 24 h, and the cytokine levels in the supernatants were measured using ELISA. Data are presented as the mean+SD. n=4 to 5; *p<0.05, **p<0.005, ns=non-significant from each Veh-treated group by Student's *t* test. (C) Cells were treated with 4 μM of an antagonist against RAR-α (Ro41-5253), RAR-γ (MM11253) or RXR (UVI3003), combined with RA (10 nM) and Con A (5 μg/ml) for 24 h. The culture supernatants were collected, and cytokines were measured using ELISA. Data are presented as the mean+SD. n=3 to 4; *p<0.05, **p<0.005, ns=non-significant by Student's *t* test. (D, E) DN32.D3 (NKT hybridoma) cells were stimulated with 5 μg/ml Con A (D) or 200 ng/ml α-GalCer (E) in the presence of different concentrations of ATRA for 24 h, and the cytokine levels in the supernatants were measured using ELISA. ATRA was serially diluted by 1/100 starting with 100 nM. Data are presented as the mean+SD. n=3 to 4; *p<0.05, **p<0.005, ns=non-significant from each Veh-treated group by Student's *t* test. (F) DN32.D3 cells were incubated with Con A in the presence of ATRA for the indicated time periods, and the cell extracts were subjected to western blot analysis with the indicated antibodies. GAPDH was used as a loading control. All data are representative of two independent experiments. Veh, DMSO; αGC, α-galactosylceramide.

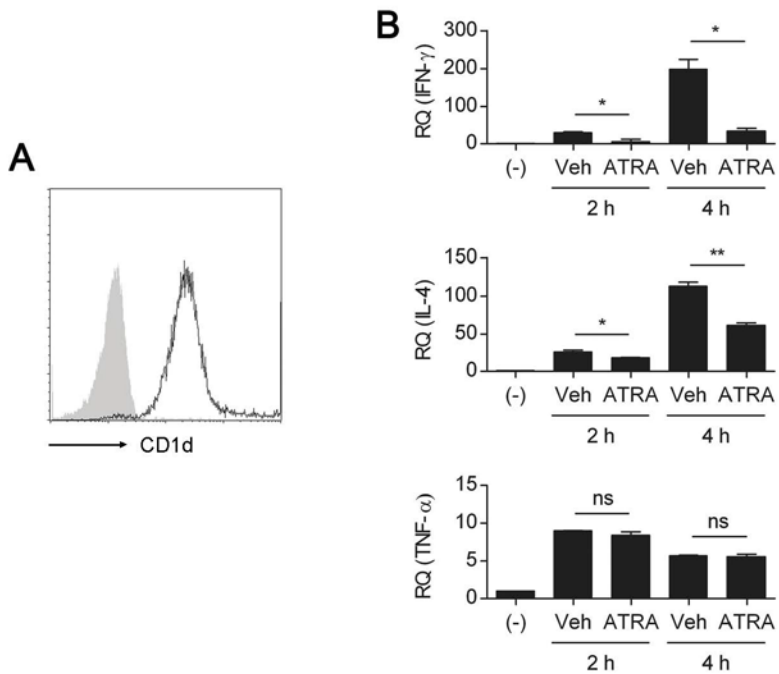

Figure. II-9. Treatment of RA reduces gene expression of IFN-γ and IL-4

(A) CD1d expression of DN32.D3 cells was analyzed by flow cytometry. Isotype control (gray histogram) and anti-CD1d (solid line) are shown. (B) DN32.D3 cells were stimulated with 5 μg/ml Con A in the presence of 100nM ATRA for 2 h or 4 h, and mRNA transcription of IFN-γ, IL-4 and TNF-α was measured using real-time PCR. After normalization against hprt, the RQ was calculated relative to levels in unstimulated cells. Data (mean+SD) are representative of two

independent experiments. n=3; Veh, DMSO; *p<0.05; **p<0.005; ns=non-significant (Student's t-test).

II.5. Discussion

In the current study, we demonstrated, for the first time, how RA regulates NKT cell-mediated diseases and NKT cell responses in vivo. We showed that RA ameliorated Con A- but not α-GalCer-induced hepatitis. This distinct role of RA can be explained by the finding that RA differentially regulated the secretion of various pathogenic cytokines from NKT cells, with unaltered NKT cell activation. Mechanistically, our observations indicate that RA affects NKT cells directly by modulating signaling molecules such as RAR-α and MAPK.

We first attempted to examine the influence of endogenous RA using vitamin A-deficient mice; however, the results did not correlate with the data obtained from RA-pre-treated animals (manuscripts in preparation). We found that RA deficiency affected the activation status of cells in naïve mice by an unknown mechanism. RA signaling is biphasic and has the potential to display opposite effects in various models [53-58]. These controversial findings have not been explained completely, and our observations and future studies may explain this discordance. In this study, to minimize the effect of vitamin A-deficiency on NKT cells, disulfiram was used to pre-treat the animals for 3 days to reduce the amount of endogenous RA. Aggravated liver injury was observed in disulfiram-treated

mice, demonstrating the regulatory role of endogenous RA in Con A-induced hepatitis (Figure I-1D and E). Disulfiram can induce liver injury by hitherto unknown mechanisms when it is administered to treat alcohol abuse [65, 66]. Our observations suggest that a defect in RA synthesis via disulfiram treatment might cause the liver to become susceptible to inflammation and increase liver injury in patients. In addition, we speculate that RA supplementation might be useful to protect against disulfiram-induced liver injury.

The present study shows that the regulatory effect of RA is restricted to liver injury induced by Con A but not α-GalCer. We also demonstrated that RA regulates IFN-γ and IL-4 but has no effects on TNF-α in Con A- or α-GalCer-induced hepatitis. NKT cells mediate the liver injury caused by Con A and by α-GalCer, but by different mechanisms. Several papers have demonstrated differences in the levels of effector cytokines between Con A- and α-GalCer-induced hepatitis [50, 63]. Although the papers could not demonstrate the cellular and molecular mechanism of how the same cytokine can function differently in two hepatitis models, they showed that IFN-γ was dispensable in α-GalCer-induced hepatitis but critical in Con A-induced hepatitis. Several possibilities might explain this difference between Con A- and α-GalCer-induced hepatitis. For example, CD1d-expressing antigen presenting cells could counteract tissue-destructive effect of

IFN-γ in α-GalCer-induced hepatitis via an unknown mechanism. In fact, the decrease of IFN-γ production does not ameliorate liver injury in α-GalCer-induced hepatitis. Moreover, the previous studies have established that α-GalCer-induced hepatitis is dependent on TNF-α [50, 63]. We observed that the treatment of RA did not alter liver injury induced by α-GalCer (Figure II-7B). This observation supports that RA dose not reduce TNF-α production of NKT cells and that RA does not inhibit activation of NKT cells. RA regulated effector cytokines in the same manner in both hepatitis models. That is, the production of IFN-γ and IL-4 was inhibited by RA but not TNF-α upon stimulation with Con A or α-GalCer. We speculate that the differential effect of RA treatment on the two hepatitis models is because of the difference of the pathologic effect of each cytokine in each model via an unknown mechanism. It is unclear how the pathogenic aspects of the same molecule in the liver have different effects. However, our observations expand the understandings on α-GalCer- and Con A-induced hepatitis. More important, the differential regulatory effects of RA could be important for the possible clinical application of RA to prevent potential liver damage.

RA skews conventional T cells toward a Th2 response in vitro [67-70]. In our study, RA reduces the production of IFN-γ and IL-4 both in NKT cells (Figure II-8). Moreover, MAPK was affected by RA, but other TCR signaling molecules

were not. The addition of RA during the initial stimulation suppresses Th1 and Th2 development, suggesting the involvement of AP-1 inhibition [67]. Although we did not show any inhibition of AP-1 by RA directly, AP-1 activity might be affected by RA via reduced MAPK activity in NKT cells. In addition, the genes regulated by NFAT differ depending on the cooperative recruitment of AP-1 [71-73]. These findings might explain the differential regulation of cytokine production by RA, as TNF-α expression can be activated by NFAT without AP-1 activation, in contrast to IFN-γ and IL-4. Thus, RA might be able to change the balance of AP-1 and NFAT activity during T cell activation, resulting in expression changes of specific genes.

In summary, RA ameliorated Con A- but not α-GalCer-induced liver injury. This protective effect of RA specific to Con A-induced hepatitis may be due to the different molecular mechanism of the liver injuries. According to our results, RA has therapeutic potential in protecting against liver damage by various agents, especially in the case of fulminant hepatitis. However, before administering therapy with RA, the pathogenic mechanism of specific hepatitis needs to be considered.

Conclusion

In the present study, we established that IL-17 producing NKT cells upon α-GalCer stimulation differed from the IL-4- and IFN-γ-producing NKT cell populations. To investigate whether this novel population possessed the properties of NKT cells, we tested the anergic properties of NKT cells in IL-17-producing cells and found that α-GalCer stimulation results in the unresponsiveness of IL-17-producing NKT cells. IL-17 induced by α-GalCer mediated the recruitment of neutrophils in the airway but had no effect on AHR development. We also demonstrated, for the first time, how RA regulates NKT cell-mediated diseases and NKT cell response *in vivo*. We showed that RA ameliorated Con A- but not α-GalCer-induced hepatitis. This distinct regulation of RA can be explained with the finding that RA regulated the expression of pathogenic cytokines from NKT cell differentially with unaltered NKT cell activation. Mechanistically, our observations indicate that RA affects NKT cells directly via modulation of signaling molecules such as RAR-α and MAPK. Collectively, our results demonstrated that NKT cells can function diversely in inflammatory diseases. Therefore, the results should be critically considered for clinical approaches in inflammation.

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국문 초록

NKT 세포는 특이적인 특성과 다양한 기능을 갖는 T 세포의 한 종류로서 다양한 질병 모델에서 조절 또는 주요 병인인자로서 작용하는 세포이다. NKT 세포는 α-GalCer 또는 Con A 와 같은 자극에 의해 cytokine 을 발현하여 면역기능을 조절하는 것으로 알려져 있다. 본 연구는 생쥐의 염증성 모델에서 기존의 밝혀지지 않은 NKT 세포의 역할과 조절에 관한 연구이다. 먼저, α-GalCer 자극에 의하여 IL-17 을 발현하며 NK1.1 을 발현하지 않는 NKT 세포를 규명하고 일반적인 IFN-γ, IL-4 를 발현하는 NKT 세포와는 다른 subset 임을 밝혔다. 또한 NKT 세포의 활성화에 의해 유도되는 기도과민성 모델에서 NKT 세포에서 발현하는 IL-17 는 기도저항성에는 직접적인 영향을 미치지 않았으나 조직을 손상할 수 있는 호중구의 폐 조직 이동을 증가시키는 역할을 하는 것으로 밝혀졌다. 다음으로 NKT 세포는 간에 가장 많이 분포하고 있는 특이적인 세포이며

간에서 NKT 세포의 기능 조절을 알아보았다. 특히 간은 레티노산의 전구체들이 많이 저장되어 있는 장소이다. 본 연구에서는 NKT 세포에 의해 유도되는 간염 모델에서 레티노산의 역할과 NKT 세포 조절에 관한 연구를 수행하였다. 레티노산의 선처리는 Con A 에 의해 유도되는 간손상을 경감시키는 것으로 나타났다. 레티노산은 간손상에 중요한 것으로 알려진 cytokine 중 IFN-γ, IL-4 의 감소를 유도하였으나 TNF 를 감소시키지는 않았다. 이러한 cytokine 의 조절은 α-GalCer 를 마우스에 투여한 경우에도 나타났으나 이 경우에 일어나는 간손상에서는 감소현상을 보이지 않았다. 이는 Con A 와는 달리 α-GalCer 에 의해 유도되는 간손상은 IFN-γ에 의해 유도되지 않고 TNF 에 의해 유도되는 것과 같은 병인 인자의 차이에 의한 것으로 생각된다. 이러한 cytokine 의 조절은 대부분 NKT 세포에서 나타났으며 이는 NKT 세포의 활성화 자체를 억제하여 나타나는 것이 아니었다. 또한 이러한 cytokine 의 감소는 RAR-α를 통한 것으로 관찰되었고 레티노산에 의해 MAPK 의 활성 저해가

일어나는 것으로 보아 TCR 신호 전달 자체의 부분적 신호 억제와 관련이 있을 것으로 생각된다. 본 연구는 염증성 모델에서 NKT 세포의 다양한 기능과 조절에 밝혔으며 임상적으로 적용 시에 중요하게 고려되어야 할 부분으로 생각된다.

주요어: 자연살해 T 세포, NKT 세포, 기도 염증 반응, 호중구, 레티노산, 간염, 사이토카인

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