

Natural killer T cells suppress zymosan A-mediated granuloma formation in the liver by modulating interferon- γ and interleukin-10

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Introduction

The liver is a unique immune organ, in terms of both the unconventional leucocyte populations that reside there and the immunosuppressive responses often induced. This is related to the fact that the liver has to process many foreign antigens transported via the portal vein. Another possible reason may be that the liver originates phylogenetically from the intestine, which is central to mucosal immunity. For example, the liver contains many unique leucocytes, including Kupffer cells, dendritic cells, natural killer T (NKT) cells,^{1–5} and intermediate T-cell receptor cells of extrathymic origin.^{6,7} These leucocyte populations seem to mediate liver-specific immune responses. Of these, hepatic dendritic cells are unique in terms of morphology and function because they are immunosuppressive (e.g. they are associated with immune tolerance in the liver).^{8–13}

The NKT cell population is also unique, being most abundant in the liver, and recognizing some glycolipids in conjunction with the MHC class I-like molecule, CD1d.^{1–5} It is conceivable that NKT cells in the liver mediate some

Summary

Wild-type (WT) and CD1d^{-/-} [without natural killer (NK) T cells] mice were treated with zymosan A to induce granuloma formation in the liver. Increased granuloma formation was seen in NKT-less mice on days 7 and 14 after administration. WT mice showed limited granuloma formation, and zymosan A eventually induced NKT cell accumulation as identified by their surface marker (e.g. CD1d-tetramer). Zymosan A augmented the expression of Toll-like receptor 2 on the cell surface of both macrophages and NKT cells. One possible reason for accelerated granuloma formation in NKT-less mice was increased production of interferon- γ (IFN- γ); a theory that was confirmed using IFN- γ ^{-/-} mice. Also, zymosan A increased interleukin-10 production in WT mice, which suppresses IFN- γ production. Taken together, these results suggest that NKT cells in the liver have the potential to suppress zymosan A-mediated granuloma formation.

Keywords: granuloma; interferon- γ ; liver; natural killer T cells; zymosan A

unknown function to maintain liver-specific immune responses. In recent studies, we noticed that CD1d^{-/-} (NKT-less) mice are sensitive to zymosan A-induced granuloma formation in the liver. Therefore, in the present study, we investigated the possibility that NKT cells play a major role in suppressing granuloma formation in the liver.

Granulomatous diseases occur at various sites within the body; however, granuloma formation is very common in the liver. Because it is likely that many microbial antigens reach the liver via the portal vein,^{14–17} we speculated that NKT cells within the liver play a key role in regulating the immune responses in this organ.

Materials and methods

Mice

C57BL/6 [B6, wild-type (WT)] and CD1d^{-/-}¹⁸ interferon- γ -deficient (IFN- γ ^{-/-}) mice¹⁹ were used at the age of 8–12 weeks. All these mice had a B6 background. The CD1d^{-/-} mice lacked the generation of invariant NKT

Abbreviations: NKT, natural killer T; TLR2, toll-like receptor 2; WT, wild-type.

cells and almost all NK1.1⁺ CD3^{int} cells (NKT cells including invariant NKT cells) are absent in these mice. We therefore refer to these CD1d^{-/-} mice as NKT-less mice in the text. These mice were maintained at the animal facility of Niigata University (Niigata, Japan) under specific pathogen-free conditions.

Granuloma formation

Zymosan A (mainly β -1,3-glucan), which reacts with toll-like receptor 2 (TLR2)¹⁷ and dectin-1,^{20,21} was used to induce granulomas in the liver. Zymosan A (Sigma, St Louis, MO) was suspended in PBS, placed in a boiling water bath for 1 hr, then centrifuged for 30 min at 1300 g; the residue was resuspended in PBS. Zymosan A was injected intravenously at a dose of 1 mg/mouse in 200 μ l PBS.

Histology of organs

Tissues for histology were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections, 4 μ m in thickness, were stained with haematoxylin & eosin.

Cell preparation

Hepatic mononuclear cells and splenic lymphocytes were isolated by a previously described method.⁶ Briefly, the liver (or the spleen) was removed, pressed through 200-gauge stainless steel mesh, and suspended in Eagle's minimal essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5 mM HEPES and 2% heat-inactivated newborn calf serum. After being washed once with medium, the cells were resuspended in 15 ml of 35% Percoll solution (Amersham Pharmacia Biotech, Piscataway, NJ) containing 100 U/ml heparin and centrifuged at 300 g for 15 min. The pellet was resuspended in erythrocyte lysing solution (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM Na-EDTA, and 17 mM Tris-HCl; pH 7.3). Concanavalin A blasts and lipopolysaccharide blasts were prepared by using splenic lymphocytes as previously described.⁷

Immunofluorescence tests by a cell analyser

The surface phenotype was identified by using monoclonal antibodies (mAbs) in conjunction with two-colour or three-colour immunofluorescence tests.⁷ The mAbs used here included FITC-, phycoerythrin- (PE) or biotin-conjugated reagents of anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-Mac-1 (M1/70), anti-Gr-1 (RB6-8C5), anti-IFN- γ (XMG1.2, rat IgG1), isotype control (R3-34, rat IgG1) mAbs (BD Biosciences, San Diego, CA); anti-TLR2 (6C2) mAbs (eBioscience, San Diego, CA); and CD1d tetramer (ProImmune Ltd., Oxford, UK). Biotin-conjugated reagents were developed with Tri-Color-conjugated streptavidin (Caltag Labora-

tory, San Francisco, CA). To prevent non-specific binding of mAbs, CD32/16 (BD Biosciences) was added before staining with labelled mAbs. For the intracellular staining, Cytofix/CytoPerm Kit (BD Biosciences) was used. The fluorescence-positive cells were analysed by flow cytometry (FACScan; BD Biosciences). Dead cells were excluded by forward scatter, side scatter and propidium iodide gating.

Lymphocyte culture

Both WT and NKT-less mouse liver cells (1×10^6 /ml) were cultured in complete RPMI-1640 medium containing 10% fetal calf serum in the presence of 100 μ g/ml zymosan A in a 96-well microculture plate for 1, 3 and 5 days at 37°.

ELISA for the detection of IFN- γ , TNF- α and IL-10

Pooled sera and cultured supernatant fluid were used for measurement of the concentrations of IFN- γ , tumour necrosis factor- α (TNF- α) and interleukin-10 (IL-10) by ELISA using OptEIA mouse IFN- γ and IL-10 sets (BD Biosciences) and mouse TNF- α ELISA Ready-SET-Go! (eBioscience).

Reverse transcription-PCR and real-time PCR analysis

Total RNA was extracted from cells of WT and NKT-less mice. To detect mRNAs of cytokines, RNA was reverse transcribed using the primers of these genes and such cDNA was further amplified by PCR methods. Briefly, total RNA was prepared from cells with Isogen (Nippon Gene, Tokyo, Japan). The cDNA was synthesized using 1 μ g RNA with a SuperScript β First-Strand Synthesis System for reverse transcription-PCR (RT-PCR) (Invitrogen, Carlsbad, CA) and oligo-dT 15 Primer (Promega, Madison, WI). The PCR amplification of synthesized cDNA was then conducted. Forward primers for IL-4, IL-10, IL-12p40, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were paired with a reverse primer for a constant region sequence that is shared by all T-cell receptor c clusters. The PCR was carried out with an initial denaturation for 10 min at 95°, followed by 40 cycles of 1 min at 95°, 1 min at 57°, and 1 min at 72°. Their sequences are as follows: IL-4 sense, 5'-CCAGCTA GTTGTCATCCTGC-3'; IL-4 antisense, 5'-GTGATGTGGA CTTGGACTCA-3'; IL-10 sense, 5'-GGACAACATACTGCT AACCGG-3'; IL-10 antisense, 5'-ATATTTTCGGAGAGAG GTACA-3'; IL-12p40 sense, 5'-CGTGCTCATGGCTGGTG CAAAG-3'; IL-12p40 antisense, 5'-CTTCATCTGCAAGT TCTTGGGC-3'; GAPDH sense, 5'-ACCACAGTCCATGA AATCAC-3'; GAPDH antisense, 5'-TCCACCACCCTGTT GCTGTA-3'. PCR products were visualized on 2% agarose gel stained with ethidium bromide under UV illumination.

To quantify the amount of IL-10 RNA, a real-time PCR, based on SYBR green fluorescence, was performed using

SYBR Premix Ex *Taq* polymerase and Takara real-time Thermal Cycler Dice (Takara, Shiga, Japan). The following primers were used to specifically amplify respective genes: IL-10 sense, 5'-GCCAGAGCCACATGCTCCTA-3'; IL-10 antisense, 5'-GATAAGGCTTGGCAACCCCAAGTAA-3'; GAPDH gene used as a control, GAPDH sense, 5'-TGTGTCCGTCGTGGAT CTGA-3'; GAPDH antisense, 5'-TTGCTGTTGAAGTCGCAGGAG-3'.

Statistical analysis

Significance of differences was determined by unpaired *t*-test or two-way analysis of variance. A value of *P* < 0.05 was considered to be significant.

Results

Predominant formation of granulomas in CD1d^{-/-} mice

Mononuclear cells were isolated from the livers of WT and NKT-less mice and the number of mononuclear cells was counted (Fig. 1a). After the administration of zymosan A, the number of mononuclear cells increased up until

day 7 before decreasing again. The number of cells was comparable in both mouse strains, but was slightly higher in NKT-less mice. During the same time period, granulomas formed in both mouse strains. The number of granulomas observed in liver sections was compared on days 7 and 14 (Fig. 1b) and the results showed a significantly higher number in NKT-less mice than in WT mice (*P* < 0.01). Representative images are shown in Fig. 1(c). On day 14 after zymosan A administration, many granulomas were seen in the livers of NKT-less mice along with some areas of necrosis. The granulomas disappeared spontaneously by day 28 in both strains of mice.

Temporal kinetics regarding the number of NKT cells and macrophages in the liver of mice treated with zymosan A

We next examined how NKT cells were regulated in the liver of WT mice after zymosan A administration (Fig. 2a). NKT cells were identified by their expression of CD1d-tetramer and CD3^{int}. This cell population was completely absent from NKT-less mice (data not shown). On days 7 and 14, the proportion of NKT cells decreased markedly;

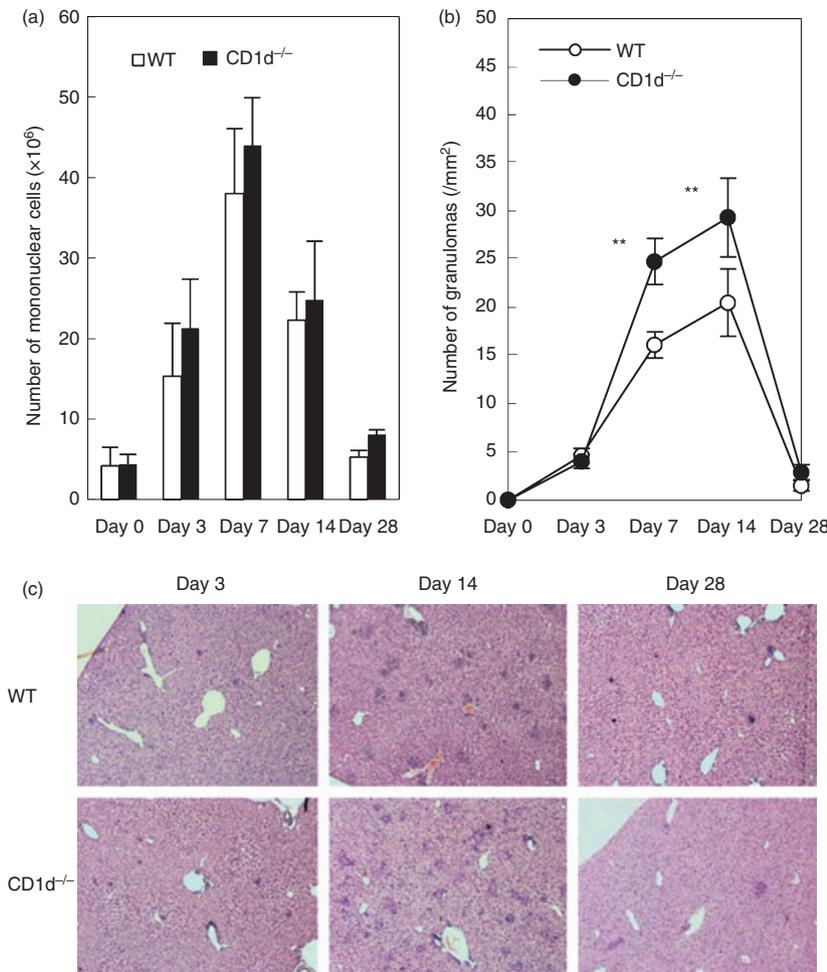


Figure 1. Granuloma formation in the livers of wild-type (WT) and CD1d^{-/-} [natural killer T (NKT) cell-less] mice. (a) The number of mononuclear cells in the liver. (b) The number of granulomas in the liver. (c) Histology. WT and NKT-less mice were treated with zymosan A and various parameters were examined at the indicated time-points. Data represent the mean ± SD from four mice. ***P* < 0.01.

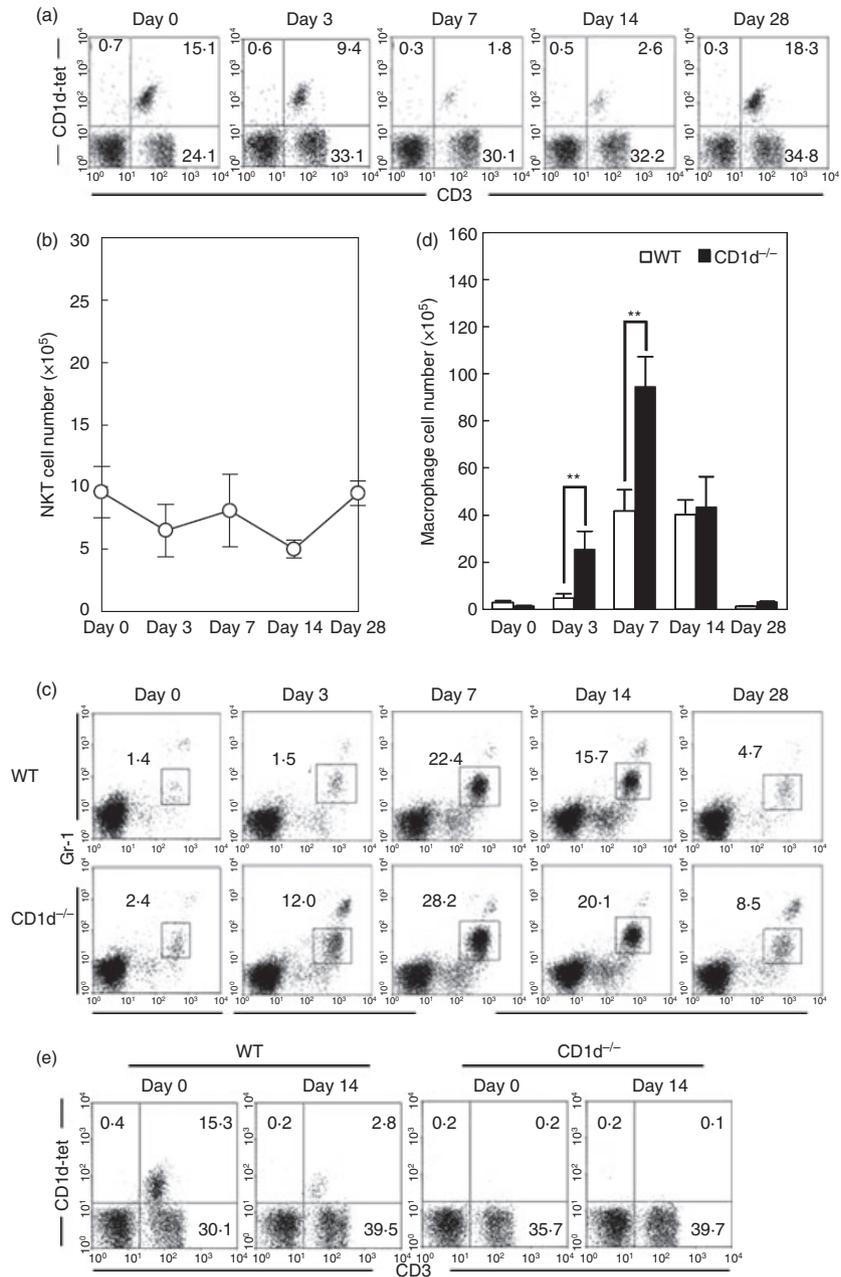


Figure 2. Identification of natural killer T (NKT) cells and macrophages. (a) Two-colour staining for CD3 and CD1d-tetramer. (b) Number of NKT cells. (c) Two-colour staining for Mac-1 and Gr-1. (d) Number of macrophages. (e) Identification of NKT cells in CD1d^{-/-} mice. Mononuclear cells were isolated from the livers of wild-type (WT) and NKT-less mice. Immunofluorescence studies were done to identify NKT cells (CD3^{int} CD1d-tetramer⁺) and macrophages (Gr-1^{int} Mac-1⁺). The data represent the mean ± SD from four mice. ***P* < 0.01.

however, the absolute number remained relatively constant (Fig. 2b). The reason for this was an increase in the total number of mononuclear cells within the liver.

Next, we determined the proportion of macrophages (Gr-1^{int} Mac-1⁺) in the livers of both strains of mice (Fig. 2c). Most of this population was F4/80 positive (data not shown). On days 7 and 14, the proportion of macrophages increased markedly in both strains; however, this increase was observed earlier (day 3), and to a greater extent, in NKT-less mice. The absolute number of macrophages was then calculated (Fig. 2d), and was found to be significantly higher in NKT-less mice than in WT mice, especially on days 3 and 7. In a final portion of this experiment (Fig. 2e), it was

confirmed that NKT cells were rarely found in CD1d^{-/-} mice irrespective of zymosan A treatment.

Augmented expression of TLR2 by NKT cells and macrophages

The expression of TLR2 (a receptor for β-1,3-glucan) by mononuclear cells in the livers of zymosan A-treated WT mice was examined using three-colour staining for NK1.1, CD3 and TLR2, or for Gr-1, Mac-1 and TLR2 (Fig. 3). The expression of TLR2 on NKT cells (NK1.1⁺ CD3^{int}), macrophages (Gr-1^{int} Mac-1⁺) and T cells (NK1.1⁻ CD3^{high}) was determined using gated analysis. On day 0,

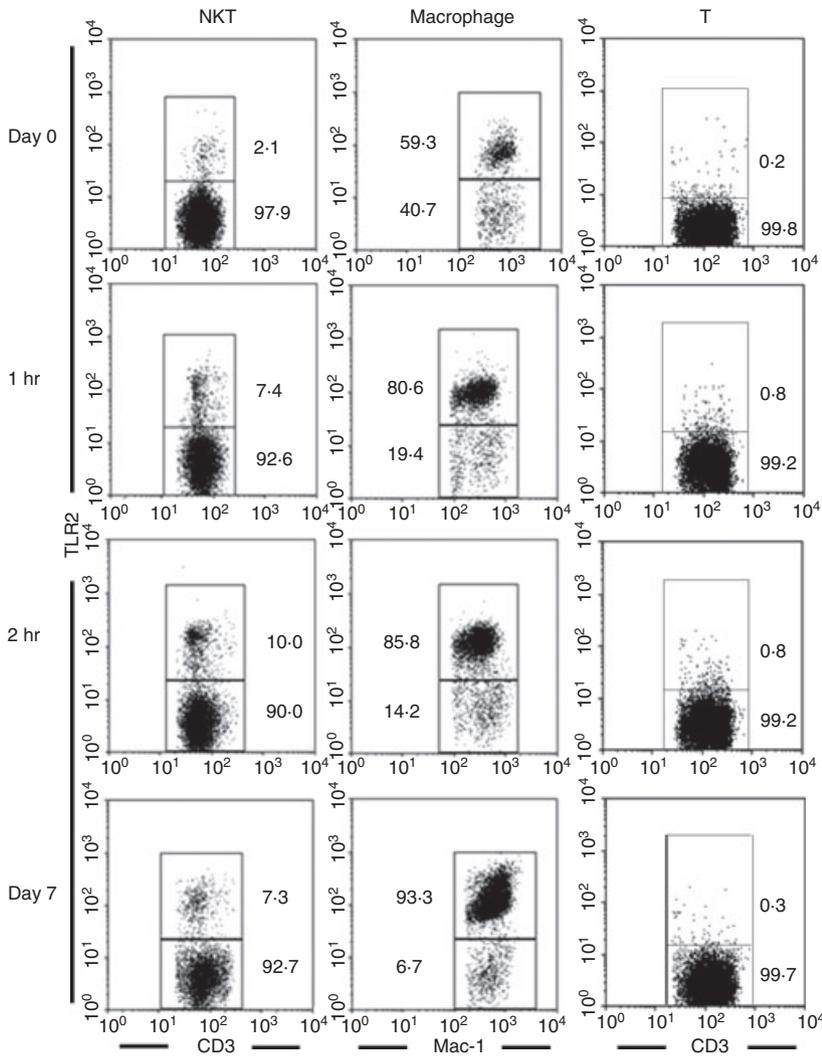


Figure 3. Expression of Toll-like receptor 2 (TLR2) on natural killer T (NKT) cells and macrophages. Wild-type (WT) mice were treated with zymosan A and mononuclear cells were isolated from the liver on the indicated days. Three-colour staining for TLR2, NK1.1 (or Gr-1) and CD3 (or Mac-1) was then performed. To show the expression of TLR2, the gated analysis was conducted for NKT cells (NK1.1⁺ CD3^{int}), macrophages (Gr-1^{int} Mac-1⁺), and T cells (NK1.1⁻ CD3^{high}). Representative results from three independent experiments are shown. The numbers in the figure represent the percentage of fluorescence-positive cells.

TLR2 was expressed at very low levels by NKT cells, but was positive in 59% of macrophages. After the administration of zymosan A TLR2 was expressed by 10% of NKT cells after 2 hr and on day 7. The majority of macrophages also showed increased levels of TLR2 expression after 2 hr and on day 7.

CD1d^{-/-} mice treated with zymosan A show increased IFN- γ production

The results of our experiments revealed that NKT-less mice showed increased granuloma formation, and that these granulomas contained a higher number of macrophages after treatment with zymosan A. As macrophages are activated by cytokines such as IFN- γ and TNF- α , the levels of these cytokines were examined both in sera and in *in vitro* culture (Fig. 4). High levels of IFN- γ were detected in the sera of NKT-less mice treated with zymosan A (Fig. 4a), reaching a peak by day 14. In contrast, the level of TNF- α was similar between WT mice and NKT-less mice.

Next, liver mononuclear cells were isolated from both WT mice and NKT-less mice and cultured with or without zymosan A (Fig. 4b). The levels of IFN- γ and TNF- α in the culture supernatants were then measured on the indicated days. Higher titres of IFN- γ were detected in NKT-less mice, whereas the levels of TNF- α were similar in both strains.

Next, the leucocyte subsets responsible for IFN- γ production in zymosan A-treated NKT-less mice were analysed (Fig. 4c). Liver mononuclear cells were isolated on day 14 and three-colour staining was performed (the third colour stained cytoplasmic IFN- γ). All the tested cell populations (except CD8⁺ T cells) contained IFN- γ -producing cells (between 4.9 and 7.1%). However, the difference between WT mice and NKT-less mice was insignificant.

IFN- γ ^{-/-} mice show decreased granuloma formation

It was hypothesized that IFN- γ was intimately involved in granuloma formation after zymosan A administration. To confirm this, tissue sections from IFN- γ ^{-/-} mice were pre-

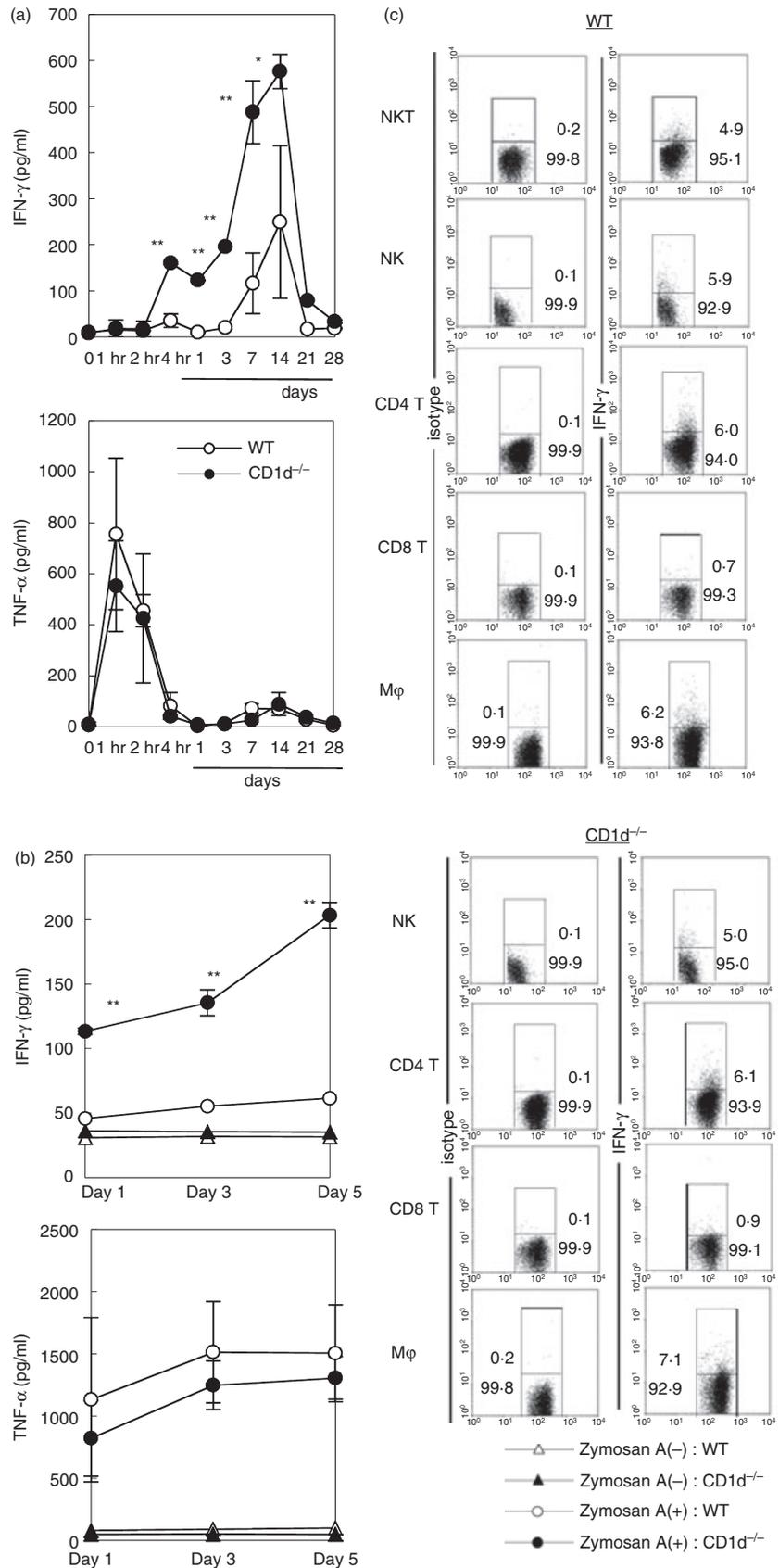


Figure 4. Interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) production in wild-type (WT) and CD1d^{-/-} mice. (a) IFN- γ and TNF- α levels in the sera. (b) IFN- γ and TNF- α levels in the culture supernatants. (c) Cytoplasmic detection of IFN- γ and TNF- α in different leucocyte populations. Cytoplasmic cytokines were detected using immunofluorescence. Data represent the mean \pm SD from four experiments.

pared on day 14 and the numbers of granulomas in WT, NKT-less and IFN- $\gamma^{-/-}$ mice were compared (Fig. 5a). The results showed that granuloma formation was most pronounced in NKT-less mice, and least in IFN- $\gamma^{-/-}$ mice. The actual number of granulomas was compared on days 7 and 14 (Fig. 5b), and a large decrease in the number of granulomas was observed in IFN- $\gamma^{-/-}$ mice at both time points.

WT mice show high levels of IL-10 after zymosan A treatment

Why did the deficiency of NKT cells result in increased production of IFN- γ and subsequent activation of macrophages? The answer to this question may involve IL-10, which is known to suppress IFN- γ production. Hence, IL-10 levels were measured in WT and NKT-less mice treated with zymosan A (Fig. 6a). High levels of IL-10 were detected in the sera of WT mice as early as 1 to 2 hr after zymosan A administration. However, only minimal levels were detected in NKT-less mice. Interleukin-10 was not detectable in the sera of either mouse strain between day 1 and day 28.

Increased IL-10 production was confirmed using RT-PCR (Fig. 6b). The signal for IL-10 mRNA increased between 1 hr and 1 day after zymosan A administration in WT mice, but not in NKT-less mice. Both IL-4 mRNA and IL-12 mRNA were examined as controls. The signal for IL-4 mRNA was only detected in concanavalin A blasts, whereas that for IL-12 mRNA was detected in both concanavalin A blasts and lipopolysaccharide blasts. Real-time PCR was also conducted (Fig. 6c). The data confirmed the result from Fig. 6(b).

Discussion

In the present study, we showed that CD1d $^{-/-}$ mice had an unusually high number of granulomas in the liver after zymosan A treatment. This animal model of zymosan A-induced granuloma formation is linked to activation of macrophages in WT mice.²²⁻²⁶ At this time, TLR2¹⁷ and dectin-1^{20, 21} might be associated with such activation. These are known to be binding sites for many bacterial products, including zymosan A. However, we did not analyse which molecules of TLR2 or dectin-1 played a major role for zymosan A-induced granuloma formation in this protocol. Compared with WT mice, NKT-less mice

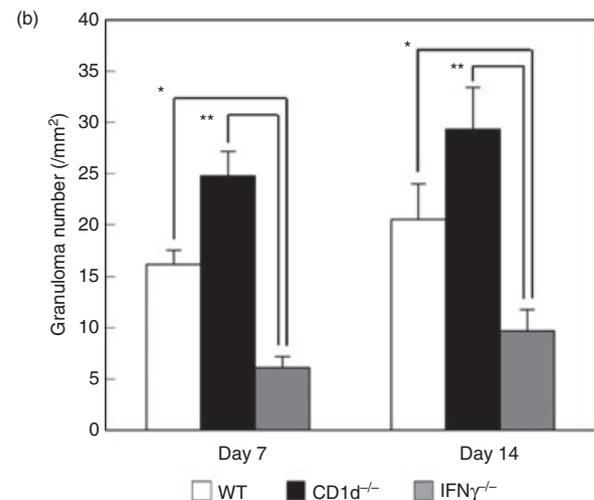
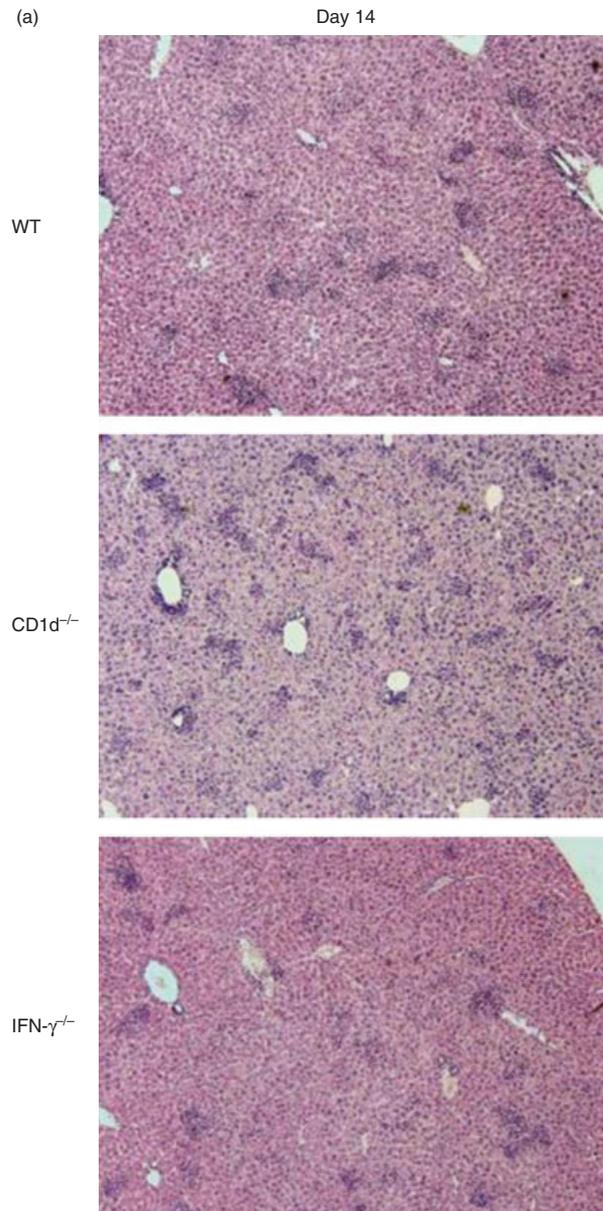


Figure 5. Association of interferon- γ (IFN- γ) with granuloma formation. (a) Liver histology. (b) Number of granulomas. Wild-type (WT), CD1d $^{-/-}$ and IFN- $\gamma^{-/-}$ mice were treated with zymosan A. On the indicated days, hepatic tissue was isolated. The data represent the mean \pm SD of the number of granulomas in four mice. * $P < 0.05$ and ** $P < 0.01$.

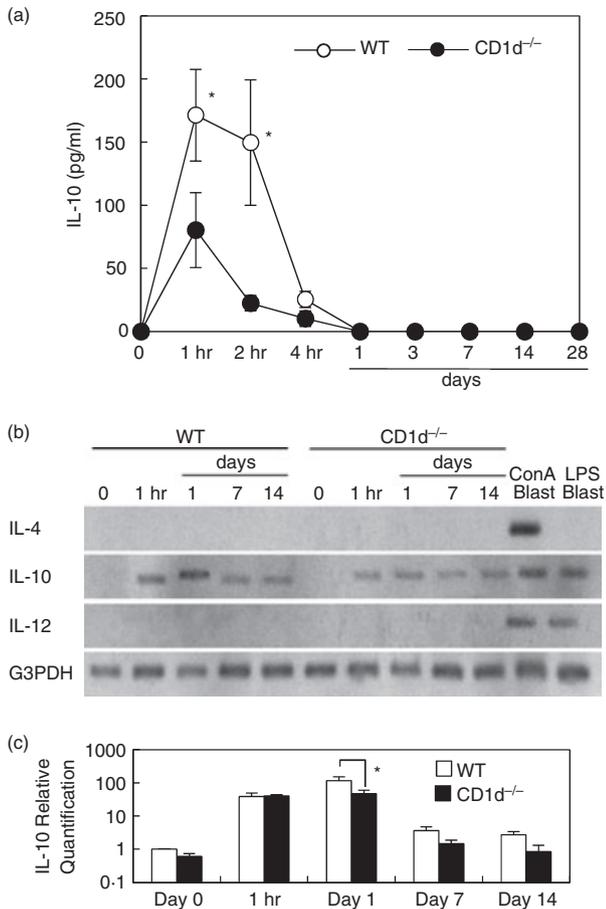


Figure 6. Increased interleukin-10 (IL-10) production in wild-type (WT) mice. (a) IL-10 levels in sera. (b) IL-10 mRNA expression. (c) Real-time PCR. RNA was isolated from concanavalin A (Con A) blasts and lipopolysaccharide (LPS) blasts was used as a positive control. Data represent the mean \pm SD IL-10 concentration in the sera of four mice. For experiment (b), representative results are shown. * $P < 0.05$.

treated with zymosan A showed an increase in the macrophage population in the liver (both in terms of number and proportion). As NKT cells in WT mice treated with zymosan A eventually down-regulated their expression of T-cell receptors on the cell surface, it is conceivable that activated NKT cells modulated the magnitude of zymosan A-mediated granuloma formation in the liver.

We first assessed the production of IFN- γ by mononuclear cells in the liver. This is because IFN- γ is the most important cytokine involved in macrophage activation^{27–31} and macrophages are the main leucocyte population found in granulomas.^{32–34} The results showed that unusually large amounts of IFN- γ (but not TNF- α) were produced by liver mononuclear cells in NKT-less mice treated with zymosan A. This was true in both the *in vivo* and *in vitro* experiments. We next examined the production of IL-10 – an immunosuppressive cytokine that is often produced by T cells or activated NKT cells.^{35–39} The

results showed that WT mice produced large amounts of IL-10, especially within 1–2 hr of zymosan A administration. This suggests that NKT cells play an important role in suppressing zymosan A-mediated granuloma formation in the liver by modulating the production of IFN- γ and IL-10.

There was also evidence that NKT cells were directly activated by zymosan A. In addition to down-regulated expression of the CD1-tetramer by NKT cells, the results showed elevated expression of TLR2 (from 2.1% to 10.0% of cells by 2 hr after zymosan A administration). In the case of macrophages, a large proportion (59.3%) of resting macrophages was TLR2⁺, and this was increased by treatment with zymosan A.

To determine the source of IFN- γ , the cytoplasmic expression of IFN- γ was examined in various leucocyte populations in both WT and NKT-less mice. The results showed that NKT cells, NK cells, CD4⁺ T cells and macrophages produced IFN- γ . Production of IFN- γ by CD8⁺ T cells was minimal. The importance of IFN- γ in granuloma formation was also examined in IFN- γ ^{-/-} mice. Few granulomas were seen in IFN- γ ^{-/-} mice treated with zymosan A.

One of the functions of NKT cells was cytotoxicity against autologous thymocytes and hepatocytes. Cytotoxicity against thymocytes did not require specific activation,^{40,41} but cytotoxicity against hepatocytes required activation with α -GalCer.^{42–44} However, other immunosuppressive functions of NKT cells were also reported.^{45–48} For example, NKT cells suppress autoimmune uveitis by inhibiting T helper type 17 differentiation,⁴⁹ and play a protective role in acute and chronic arthritis by ameliorating antigen-specific T helper type 1 responses.⁵⁰

Granuloma formation in the liver and other organs is important for sealing microbes within the tissue and preventing their dissemination into the systemic circulation. It is thought that TLR2-mediated or other TLR-mediated granuloma formation is one such response.^{16,17} However, excessive granuloma formation is sometimes dangerous and can lead to tissue damage (e.g. hepatic failure). In this regard, positive and negative regulation of granuloma formation in the liver might be critical for the protection of the whole body. The present results suggest that NKT cells play an important role in regulating such responses. The key factor modulating macrophage activation appears to be IFN- γ , with IL-10 acting to provide negative feedback.

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Disclosures

None of the authors have any conflict of interest with any of the content of this manuscript.

References

- Bendelac A. Mouse NK1⁺ T cells. *Curr Opin Immunol* 1995; 7:367–74.
- MacDonald HR. NK1.1⁺ T cells receptor- α/β^+ cells: new clues to their origin, specificity, and function. *J Exp Med* 1995; 182:633–8.
- Bix M, Locksley RM. Natural T cells: cells that co-express NKR1 and TCR. *J Immunol* 1995; 155:1020–2.
- Vicari AP, Zlotnik A. Mouse NK1.1⁺ T cells: a new family of T cells. *Immunol Today* 1996; 17:71–6.
- Abo T, Kawamura T, Watanabe H. Physiological responses of extrathymic T cells in the liver. *Immunol Rev* 2000; 174:135–49.
- Sato K, Ohtsuka K, Hasegawa K, Yamagiwa S, Watanabe H, Asakura H, Abo T. Evidence for extrathymic generation of intermediate T cell receptor cells in the liver revealed in thymectomized, irradiated mice subjected to bone marrow transplantation. *J Exp Med* 1995; 182:759–67.
- Watanabe H, Miyaji C, Kawachi Y, Iiai T, Ohtsuka K, Iwanaga T, Takahashi-Iwanaga H, Abo T. Relationships between intermediate TCR cells and NK1.1⁺ T cells in various immune organs. NK1.1⁺ T cells are present within a population of intermediate TCR cells. *J Immunol* 1995; 155:2972–83.
- Jomantaité I, Dikopoulos N, Kröger A, Leithäuser F, Hauser H, Schirmbeck R, Reimann J. Hepatic dendritic cell subsets in the mouse. *Eur J Immunol* 2004; 34:355–65.
- Lau AH, Thomson AW. Dendritic cells and immune regulation in the liver. *Gut* 2003; 52:307–14.
- Kingham TP, Chaudhry UI, Plitas G, Katz SC, Raab J, DeMatteo RP. Murine liver plasmacytoid dendritic cells become potent immunostimulatory cells after Flt-3 ligand expansion. *Hepatology* 2007; 45:445–54.
- Pillarisetty VG, Shah AB, Miller G, Bleier JJ, DeMatteo RP. Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition. *J Immunol* 2004; 172:1009–17.
- Castellaneta A, Sumpter TL, Chen L, Tokita D, Thomson AW. NOD2 ligation subverts IFN- α production by liver plasmacytoid dendritic cells and inhibits their T cell allostimulatory activity via B7-H1 up-regulation. *J Immunol* 2009; 183:6922–32.
- Connolly MK, Bedrosian AS, Mallen-St Clair J et al. In liver fibrosis, dendritic cells govern hepatic inflammation in mice via TNF- α . *J Clin Invest* 2009; 119:3213–25.
- Bowdish DM, Sakamoto K, Kim MJ et al. MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and *Mycobacterium tuberculosis*. *PLoS Pathog* 2009; 5:e1000474.
- Rivas-Santiago B, Hernandez-Pando R, Carranza C, Juarez E, Contreras JL, Aguilar-Leon D, Torres M, Sada E. Expression of cathelicidin LL-37 during *Mycobacterium tuberculosis* infection in human alveolar macrophages, monocytes, neutrophils, and epithelial cells. *Infect Immun* 2008; 76:935–41.
- Fenhalls G, Squires GR, Stevens-Muller L, Bezuidenhout J, Amphlett G, Duncan K, Lukey PT. Associations between toll-like receptors and interleukin-4 in the lungs of patients with tuberculosis. *Am J Respir Cell Mol Biol* 2003; 29:28–38.
- Reiling N, Holscher C, Fehrenbach A, Kröger S, Kirschning CJ, Goyert S, Ehlers S. Cutting edge: toll-like receptor (TLR) 2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. *J Immunol* 2003; 169:3480–4.
- Mendiratta SK, Martin WD, Hong S, Boesteanu A, Joyce S, van Kaer L. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* 1997; 6:469–77.
- Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon- γ genes. *Science* 1993; 259:1739–42.
- Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. Collaborative induction of inflammatory responses by dectin-1 and toll-like receptor 2. *J Exp Med* 2003; 197:1107–17.
- Dillon S, Agrawal S, Banerjee K et al. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J Clin Invest* 2006; 116:916–28.
- Moriyama H, Yamamoto T, Takatsuka H, Umezu H, Tokunaga K, Nagano T, Arakawa M, Naito M. Expression of macrophage colony-stimulating factor and its receptor in hepatic granulomas of Kupffer-cell-depleted mice. *Am J Pathol* 1997; 150:2047–60.
- Tanaka K, Morimoto J, Kon S et al. Effect of osteopontin alleles on β -glucan-induced granuloma formation on the mouse liver. *Am J Pathol* 2004; 164:567–75.
- Ikedo Y, Adachi Y, Ishii T, Miura N, Tamura H, Ohno N. Dissociation of toll-like receptor 2-mediated innate immune response to zymosan by organic solvent-treatment without loss of dectin-1 reactivity. *Biol Pharm Bull* 2008; 31:13–8.
- Kindler V, Sappini AP, Grau GE, Piguet PF, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell* 1989; 56:731–40.
- Senaldi G, Yin S, Shaklee CL, Piguet PF, Mak TW, Ulich TR. *Corynebacterium parvum*- and *Mycobacterium bovis* bacillus Calmette-Guérin-induced granuloma formation is inhibited in TNF receptor I (TNF-RI) knockout mice and by treatment with soluble TNF-RI. *J Immunol* 1996; 157:5022–6.
- Moreira AL, Tsenova L, Murray PJ, Freeman S, Bergtold A, Chiriboga L, Kaplan G. Aerosol infection of mice with recombinant BCG secreting murine IFN- γ partially reconstitutes local protective immunity. *Microb Pathog* 2000; 29:175–85.
- Boros DL, Whitfield JR. Endogenous IL-10 regulates IFN- γ and IL-5 cytokine production and the granulomatous response in *Schistosoma mansoni*-infected mice. *Immunology* 1998; 94:481–7.
- Weinstock JV, Elliott D. The substance P and somatostatin interferon- γ immunoregulatory circuit. *Ann NY Acad Sci* 1998; 840:532–9.
- Smith D, Hänisch H, Bancroft G, Ehlers S. T-cell-independent granuloma formation in response to *Mycobacterium avium*: role of tumour necrosis factor- α and interferon- γ . *Immunology* 1997; 92:413–21.
- Chensue SW, Warmington KS, Ruth JH, Lincoln P, Kunkel SL. Cytokine function during mycobacterial and schistosomal antigen-induced pulmonary granuloma formation. Local and regional participation of IFN- γ , IL-10, and TNF. *J Immunol* 1995; 154:5969–76.
- Joshi AD, Raymond T, Coelho AL, Kunkel SL, Hogaboam CM. A systemic granulomatous response to *Schistosoma mansoni* eggs alters responsiveness of bone-marrow-derived macrophages to Toll-like receptor agonists. *J Leukoc Biol* 2008; 83:314–24.
- Ordway D, Harton M, Heno-Tamayo M, Montoya R, Orme IM, Gonzalez-Juarrero M. Enhanced macrophage activity in granulomatous lesions of immune mice challenged with *Mycobacterium tuberculosis*. *J Immunol* 2006; 176:4931–9.
- Ma J, Chen T, Mandelin J, Ceponis A, Miller NE, Hukkanen M, Ma GF, Kontinen YT. Regulation of macrophage activation. *Cell Mol Life Sci* 2003; 60:2334–46.
- Imler TJ Jr, Petro TM. Decreased severity of experimental autoimmune encephalomyelitis during resveratrol administration is associated with increased IL-17⁺ IL-10⁺ T cells, CD4⁺ IFN- γ ⁺ cells, and decreased macrophage IL-6 expression. *Int Immunopharmacol* 2009; 9:134–43.
- Zhou L, Wang H, Zhong X et al. The IL-10 and IFN- γ pathways are essential to the potent immunosuppressive activity of cultured CD8⁺ NKT-like cells. *Genome Biol* 2008; 119:1–18.
- Singh UP, Singh S, Singh R, Cong Y, Taub DD, Lillard JW Jr. CXCL10-producing mucosal CD4⁺ T cells, NK cells, and NKT cells are associated with chronic colitis in IL-10^{-/-} mice, which can be abrogated by anti-CXCL10 antibody inhibition. *J Interferon Cytokine Res* 2008; 28:31–43.
- Miellot A, Zhu R, Diem S, Boissier MC, Herbelin A, Bessis N. Activation of invariant NK T cells protects against experimental rheumatoid arthritis by an IL-10-dependent pathway. *Eur J Immunol* 2005; 35:3704–13.
- Wei H, Zheng X, Lou D, Zhang L, Zhang R, Sun R, Tian Z. Tumor-induced suppression of interferon- γ production and enhancement of interleukin-10 production by natural killer (NK) cells: paralleled to CD4⁺ T cells. *Mol Immunol* 2005; 42:1023–31.
- Kawamura T, Takeda K, Mendiratta SK, Kawamura H, van Kaer L, Yagita H, Abo T, Okumura K. Critical role of NK1⁺ T cells in IL-12-induced immune responses *in vivo*. *J Immunol* 1998; 160:16–9.
- Bannai M, Oya H, Kawamura T et al. Disparate effect of beige mutation on cytotoxic function between natural killer and natural killer T cells. *Immunology* 2000; 100:165–9.
- Osman Y, Kawamura T, Naito T, Takeda K, van Kaer L, Okumura K, Abo T. Activation of hepatic NKT cells and subsequent liver injury following administration of α -galactosylceramide. *Eur J Immunol* 2000; 30:1919–28.
- Kaneko Y, Harada M, Kawano T, Yamashita M, Shibata Y, Gejyo F, Nakayama T, Taniguchi M. Augmentation of V α 14 NKT cell-mediated cytotoxicity by interleukin 4 in an autocrine mechanism resulting in the development of concanavalin A-induced hepatitis. *J Exp Med* 2000; 191:105–14.
- Takeda K, Hayakawa Y, van Kaer L, Matsuda H, Yagita H, Okumura K. Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc Natl Acad Sci U S A* 2000; 97:5498–503.
- Dieli F, Taniguchi M, Kronenberg M et al. An anti-inflammatory role for V α 14 NK T cells in *Mycobacterium bovis* bacillus Calmette-Guérin-infected mice. *J Immunol* 2003; 171:1961–8.
- Kuwata K, Watanabe H, Jiang S-Y, Yamamoto T, Miyaji C, Abo T, Miyazaki T, Naito M. AIM inhibits apoptosis of T cells and NKT cells in *Corynebacterium*-induced granuloma formation in mice. *Am J Pathol* 2003; 162:837–47.

- 47 Komocsi A, Lamprecht P, Csernok E *et al.* Peripheral blood and granuloma CD4⁺ CD28⁻ T cells are a major source of interferon- γ and tumor necrosis factor- α in Wegener's granulomatosis. *Am J Pathol* 2002; **160**:1717–24.
- 48 Tabata A, Kaneda K, Watanabe H, Abo T, Yano I. Kinetics of organ-associated natural killer cells and intermediate CD3 cells during pulmonary and hepatic granulomatous inflammation induced by mycobacterial cord factor. *Microbiol Immunol* 1996; **40**:651–8.
- 49 Oh K, Byoun OJ, Ham DI, Kim YS, Lee DS. Invariant NKT cells regulate experimental autoimmune uveitis through inhibition of Th17 differentiation. *Eur J Immunol* 2011; **41**:392–402.
- 50 Teige A, Bockermann R, Hasan M, Olofsson KE, Liu Y, Issazadeh-Navikas S. CD1d-dependent NKT cells play a protective role in acute and chronic arthritis models by ameliorating antigen-specific Th1 responses. *J Immunol* 2010; **185**:345–56.