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Blockade of invariant TCR-CD1d interaction specifically inhibits antibody production against blood group A carbohydrates

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Key Points

- Administrating anti-mouse CD1d blocking mAb prior to the A-RBC immunization abolished IL-5 production and anti-A Ab production in mice.
- In human PBMC-NOD/SCID mice, administrating anti-human CD1d mAb prior to A-RBC immunization completely inhibited anti-A Ab production.

Abstract

Previously, we detected B cells expressing receptors for blood group A carbohydrates in the $CD11b^{+}CD5^{+}$ B-1a subpopulation in mice, similar to that in blood group O or B in humans. In the present study, we demonstrated that CD1d-restricted natural killer T (NKT) cells are required to produce anti-A antibodies (Abs), probably through collaboration with B-1a cells. After immunization of wild-type (WT) mice with human blood group A red blood cells (A-RBCs), interleukin (IL)-5 exclusively and transiently increased, and the anti-A Abs were elevated in sera; however, those were not observed at all in *CD1d*$^{-/-}$ mice, which lack NKT cells. Administration of anti-mouse CD1d blocking mAb prior to the immunization abolished IL-5 production by NKT cells and anti-A Ab production in WT mice. Administration of anti-IL-5 neutralizing mAb also diminished anti-A Ab production in WT mice, suggesting that IL-5 secreted from NKT cells critically regulates anti-A Ab production by B-1a cells. In NOD/SCID/γ_c^{null} mice, into which peripheral blood mononuclear cells from type O human volunteers were

engrafted, administration of anti-human CD1d mAb prior to the A-RBC immunization completely inhibited anti-A Ab production. Thus, anti-CD1d treatment might constitute a novel approach that could help in evading Ab-mediated rejection in ABO-incompatible transplants.

Introduction

Invariant natural killer T (iNKT) cells are CD1d (non-MHC-encoded class I-like molecule)-restricted, lipid antigen (Ag)-reactive T cells that express invariant Vα14-Jα18 T cell Ag receptors (iTCRs) in mice and Vα24-Jα28 iTCRs in humans.[1,2] Upon activation by glycolipid Ags, including α-galactosylceramide (αGalCer),[3] these cells transactivate a variety of other cells, including NK, T, B, and dendritic cells.[4-7] Recent studies have reported that activated iNKT cells enhance antibody (Ab) responses against T-dependent and T-independent Ags and pathogens.[8-12] These observations prompted us to investigate the possible role of iNKT cells in the Ab production against transplant-related Ags such as ABO blood group carbohydrates, xenogeneic carbohydrates, and histocompatibility complex allopeptides.

Previously, we carried out surface staining of B cells using fluorescein-labeled synthetic human blood group A carbohydrates and demonstrated that B cells with surface IgM (sIgM) receptors for group A carbohydrate determinants are present exclusively in a small, significant B cell subpopulation—sIgM$^+$ CD11b$^+$ CD5$^+$ B-1a cells—in mice, similar to the case of humans with blood group O or B.[13,14] The serum anti-group A Ab levels in the mice significantly increased through the activation of these B-1a cells, which bear receptors for A determinants following their immunization with human

group A red blood cells (RBCs). Further, we used a similar technique and demonstrated that $sIgM^+$ $CD11b^+$ $CD5^-$ B-1b cells with receptors for Galα1-3Galβ1-4GlcNAc (Gal) epitopes, which are major xenogeneic Ags, were present in α1,3-galactosyltransferase deficient (*GalT*$^{-/-}$) mice, similar to the case in humans deficient in this enzyme.[15] When these mice were immunized with Gal-bearing rat thymocytes, the serum anti-Gal Ab levels significantly increased following the activation of the above mentioned B-1b cells bearing receptors for Gal determinants. We have also shown that cytidine monophospho-*N*-acetylneuraminic acid hydroxylase-deficient (*CMAH*$^{-/-}$) mice, which are completely deficient in *N*-glycolylneuraminic acid (NeuGc), non-Gal antigenic epitopes, produce anti-NeuGc Abs.[16,17] In the present study, using *GalT*$^{-/-}$, *CMAH*$^{-/-}$, *Jα18*$^{-/-}$ and *CD1d*$^{-/-}$ mice, we investigated whether iNKT cells function to produce anti-A, anti-Gal, anti-NeuGc, or anti-allopeptide Abs.

Methods

Mice

C57BL/6J (B6) (H-2b), BALB/c (H-2d), and nude mice (Balb/c) and F344 rats were purchased from CLEA Japan (Tokyo, Japan). *Jα18*$^{-/-}$ mice on a B6 genetic background and *CD1d*$^{-/-}$ mice on a B6 and Balb/c background, which are established by specific deletion of the Jα18 and CD1d gene segments, respectively, were kindly provided by Dr. K. Seino, Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan.[18] MHC class II-deficient *C2ta*^{tm1Ccum} (C2D) mice on the B6 background were purchased from Jackson Laboratory. *GalT*$^{-/-}$ mice on the B6 background were kindly provided by Dr. M. Sykes, Massachusetts General Hospital, MA, USA, and completely lacked Gal expression.[19] *CMAH*$^{-/-}$ mice on the B6 background, which are completely deficient in NeuGc, were kindly provided by Dr. Y. Kozutsumi, Kyoto University, Japan, and completely lacked NeuGc expression.[17] Both *GalT*$^{-/-}$ and *CMAH*$^{-/-}$ mice were crossed with *CD1d*$^{-/-}$ mice to produce double-knockout mice. To generate double-knockout mice, F2 mice (produced by intercrossing F1 mice) were typed for each gene, and the appropriate mice were intercrossed and typed until double-gene knockouts were established (typically 4 generations). Finally, the genotypes were confirmed by fluorescence-activated cell sorting analysis (FACS), genomic Southern blotting, and PCR. All the mice were housed in the animal facility of

Hiroshima University, Japan, in a specif c pathogen-free, micro-isolated environment and used when they were 8–16 weeks of age.

Anti-NeuGc and anti-Gal Ab production was elicited by intraperitoneal immunization of *CMAH*$^{-/-}$ and *GalT*$^{-/-}$ mice with NeuGc- and Gal-expressing thymocytes obtained from F344 rats 2 times with a 1-week interval (10×10^6 cells/mouse at each immunization). As indicated, anti-A Ab production was similarly elicited by intraperitoneal immunization of mice with human A-RBCs from blood group A volunteers 2 times with a 1-week interval (5×10^8 cells/mouse at each immunization). Informed consent was obtained in accordance with the Declaration of Helsinki from all human volunteers.

All the experiments were approved by the Institutional Review Board of Hiroshima University and conducted according to the guidelines of the National Institutes of Health (National Institutes of Health publication no. 86–23, revised 1996).

Conditioning regimen for experimental mice

As indicated, each mouse was intraperitoneally injected with 500 μg anti-mouse CD1d mAb (1B1) or with 100 μg anti-mouse interleukin (IL)-5 mAb (TRFK5) (BD PharMingen, San Diego, CA) diluted in PBS two times at one week interval. Mice that

received injections of isotype-matched Abs served as the controls.

To determine whether iNKT cells enhance Ab responses to specific Antigen, we immunized mice with human A-RBCs together with intraperitoneal injection of either αGalCer (KRN7000) (4 μg/mouse) or PBS (control).

Human PBMC-chimeric mouse study

Non-obese diabetic/severe combined immunodeficient (NOD/SCID)/γ_c^{null} mice were purchased from the Central Institute of Experimental Animals (Kawasaki, Japan). Human peripheral blood mononuclear cells (PBMCs) (20×10^6 cells/mouse) from Type O volunteers were engrafted in NOD/SCID/γ_c^{null} mice by intraperitoneal injection after 1 Gy of whole body irradiation. The human PBMC-chimeric mice received intraperitoneal injection of anti-human CD1d mAb (CD1d42) diluted in PBS at a dose of 500 μg/mouse at days 7 and 10 following the engrafting. Mice that received injections of isotype-matched Ab served as the controls. The CD1d42 clone cell line was kindly provided by Dr. S. Porcelli, Albert Einstein College of Medicine (Bronx, NY).[20,21]

Cell preparation and flow cytometry (FCM) analyses

Anti-NeuGc and anti-Gal Abs were detected by indirect immunof uorescence staining of rat thymocytes. A total of 10^6 thymocytes were incubated with 100 μL of serially diluted mouse serum, washed, and then incubated with biotin-conjugated rat anti-mouse IgM mAb (R6-60.2: BD PharMingen) or rat anti-mouse IgG Ab (eBioscience, San Diego). The biotinylated mAbs were visualized using allophycocyanin-streptavidin (BD PharMingen). Median fluorescence intensity (MFI) values were used to follow Ab levels.

B cells with receptors for human blood group A trisaccharide were detected using FITC –conjugated GalNAcα1–3Fucα1–2Gal–BSA (A-BSA: Dextra, Reading, United Kingdom) and control FITC-conjugated BSA (Roche, Indianapolis, IN). FITC conjugation of A-BSA and BSA was performed using a SureLINK Fluorescein Labeling Kit (KPL, Gaithersburg, MD, USA). We incubated 10^6 spleen cells/100 μL from human PBMC-chimeric mice with 0.5 μg/100 μL FITC-A-BSA or control FITC-BSA in medium for 1 hr at 4 °C. Non-specific Fcγ receptor binding of labeled Abs was blocked by anti-mouse CD16/32 (2.4G2: BD PharMingen). The cells were further stained with PE–conjugated anti-human CD19 mAb (HIB19: BD PharMingen). Isotype-matched irrelevant mAb was used as the control. Dead cells detected using light scatter and

staining with propidium iodide were excluded from the analysis.

All FCM analyses were performed on a FACSCalibur® flow cytometer (Becton Dickinson, Mountain View, CA).

Cell sorting

Liver mononuclear cells (LMNCs) were stained with APC-conjugated anti-mouse CD1d-tetramer (Proimmune, Bradenton, FL, USA) and PE-Cy7 conjugated anti-mouse TCRβ (H57-597) (eBioscience, San Diego). NKT cells (CD1d-tetramer^{+}, TCRβ^{+}), T cells (CD1d-tetramer^{-}, TCRβ^{+}), and the others (CD1d-tetramer^{-}, TCRβ^{-}) were isolated by sorting with FACS Aria II (BD Biosciences).

ELISA

Total mouse immunoglobulin and the serum anti-A- and anti-Gal-specific Ab levels were determined by enzyme-linked immunosorbent assay (ELISA) as described previously.[22,23] Briefly, ELISA plates were coated with 5 μg/mL of goat anti-mouse Ig (IgG + IgM + IgA, heavy chain + light chain; Southern Biotechnology, Birmingham, AL), 5 μg/mL synthetic A-BSA (Dextra), Gal-BSA (Dextra) or control BSA (Roche). The diluted serum samples were added to the plates and incubated for 2 hrs, and the

bound Abs were detected using horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoReserch) / IgM-specif c Abs (KPL, Guilford, United Kingdom). Color development was achieved using 0.1 mg/mL *O*-phenylenediamine (Sigma, St. Louis, MO) in a substrate buffer. The reaction was discontinued by adding 3 M H_2SO_4, and absorbance was measured at 492 nm. Anti-A- and anti-Gal-specif c Ab levels were determined by subtracting the absorbance of the wells coated with control BSA from that of the wells coated with A-BSA. Similarly, the serum anti-A IgM and IgG levels in the humanized mice were determined. The diluted serum samples were added to the ELISA plates precoated with either A-BSA or BSA and incubated, and the bound Abs were detected using horseradish peroxidase-conjugated goat anti-human IgG (Jackson ImmunoReserch) / IgM-specif c Abs (KPL, Guilford, United Kingdom).

Cytometric bead array

Cytokine levels in sera were analyzed by BD™ Cytometric Bead Array using a mouse Flex Set, (BD Bioscience), according to the manufacturer's instructions, for the production of IL-4, IL-5, IL-9, IL-17, IL-21 and INF-γ.

ELISPOT

Enzyme-linked immunospot (ELISPOT) assay to detect IL-5 producing cells was

performed using ELISPOT kits (R & D Systems, Minneapolis, Minn). A mAb specific for mouse IL-5 was pre-coated onto a PVDF (polyvinylidene defluoride)-backed microplate. Serial dilutions of cell suspension were prepared in RPMI 1640 medium, supplemented with 2mM L-glutamine, 10mM Hepes, 0.2% sodium carbonate, 100U/ml penicillin, and 10% fetal bovine serum. The cell suspension was incubated in PVDF-backed microplates at 37°C for 24 hours. After the membranes were dried, the wells of 96-well filtration plates were observed using a Leica MZ6 microscope (Leica, Wetsler, Germany; magnification 10x/0.63), and the spots in each well were counted.

Statistical analysis

Data are presented as mean ± SEM. The results were statistically analyzed using the unpaired Student *t* test of means or analysis of variance (ANOVA). A P value of less than .05 was considered statistically signif cant.

Results

Ab production against blood group A-determinants is dependent on iNKT cells but independent of T cells

Previously, we had detected naturally occurring Abs against anti-human blood group A carbohydrate determinants in the sera obtained from mice.[13,14] Extensive anti-A IgM and IgG production occurred when the mice were immunized with human group A-RBCs. To determine whether or not anti-A Ab responses were T cell-dependent, we immunized Balb/c nude mice and B6 C2D mice, both of which lack $CD4^+$ T cells, using human group A-RBCs 2 times per week. Even after the immunization, there was no increase in the anti-A Ab titer in nude mice, whereas the serum anti-A Ab titer in C2D mice had significantly increased (Figure 1A, B). Since unlike C2D mice, nude mice completely lacked NKT Cells,[24] we could not rule out the possibility that NKT cells play a role in the production of anti-A Abs. The predominant NKT subset is represented by type I NKT cells, which express Vα14-Jα18 iTCRs in mice. Type II NKT cells have variable Vα usage, and while they are CD1d restricted, they are thought to be stimulated by many glycolipids but not αGalCer.[1,2] In *CD1d*$^{-/-}$ mice deficient in types I and II NKT cells, the anti-A Ab response was completely impaired even in the presence of αGalCer (Figure 1C). Further, the anti-A Ab response was impaired in *Jα18*$^{-/-}$ mice expressing

CD1d but lacking type I αGalCer-reactive NKT cells (Figure 1D). We also observed that αGalCer significantly enhanced blood group A-specific Ab titers in Balb/c wild type (WT) mice, but this was not observed at all in *CD1d*$^{-/-}$ mice (Figure 2A-D). Therefore, these results suggest that induction of anti-A Ab production requires iNKT cells, which are not required for naturally produced anti-A Abs.

Ab production against Gal and NeuGc epitopes is independent of iNKT cells

We then investigated the possible role of iNKT cells in Ab production against xenogeneic carbohydrates such as Gal and NeuGc epitopes, to which B-1b cells respond.[15,19] To this end, we generated *CD1d*$^{-/-}$*GalT*$^{-/-}$ mice and *CD1d*$^{-/-}$*CMAH*$^{-/-}$ mice, both of which lacked iNKT cells. These mice were immunized with Gal-bearing and NeuGc-bearing rat thymocytes, and anti-Gal and anti-NeuGc Abs were determined in their sera, respectively. The serum anti-Gal Ab titers (both IgM and IgG subclasses) of *CD1d*$^{-/-}$*GalT*$^{-/-}$ mice were elevated to levels similar to those in *CD1d*$^{+/+}$ *GalT*$^{-/-}$ mice (Figure 3A-B). Likewise, *CD1d*$^{-/-}$*CMAH*$^{-/-}$ mice also exhibited increased anti-Gal Ab levels similar to that in *CD1d*$^{+/+}$*CMAH*$^{-/-}$ mice (Figure 3C-D). In addition, αGalCer administration at the immunization with xenogeneic cells did not accelerate the anti-Gal Ab and anti-NeuGc Ab production in the *CD1d*$^{+/+}$*GalT*$^{-/-}$ and *CD1d*$^{+/+}$*CMAH*$^{-/-}$ mice,

respectively (Figure S1). Thus, unlike anti-A Ab production, the production of anti-Gal and anti-NeuGc Abs does not require iNKT cells.

Ab production against allopeptides does not require iNKT cells

To investigate the possible role of iNKT cells in the Ab production against allopeptides, to which it is believed that conventional B cells (B-2 cells) respond, Balb/c WT (*CD1d*$^{+/+}$) mice and Balb/c *CD1d*$^{-/-}$ mice were immunized with thymocytes obtained from B6 mice. In the sera of the *CD1d*$^{-/-}$ mice, the levels of anti-B6 IgM and IgG1 subclass Abs were rather lower, but the levels of IgG2 and IgG3 subclass Abs were higher, when compared with those in *CD1d*$^{+/+}$ mice; the difference however was not statistically significant. Thus, Abs against histocompatibility complex allopeptides were produced in response to allostimulation in *CD1d*$^{-/-}$ mice, although the class-switching of the Abs might be somewhat influenced (Figure S2).

CD1d$^{-/-}$ mice display a slightly reduced proportion of B-1a cells

We investigated the possibility of difference in the proportion of B cell subclasses between *CD1d*$^{+/+}$ and *CD1d*$^{-/-}$ mice. The proportion of B-1a cells in the peritoneal cavity and the liver was slightly lower, but the proportion of B-1b cells was relatively higher in Balb/c *CD1d*$^{-/-}$ mice, as compared to that in Balb/c *CD1d*$^{+/+}$ mice (Figure S3).

In contrast, the proportion of B-2 cells in those anatomical sites did not differ between *CD1d*$^{-/-}$ and *CD1d*$^{+/+}$ mice. This suggests that CD1d-restricted NKT cells somewhat play a role in the differentiation of B-1a cells but not of B-1b cells and B-2 cells.

Administration of anti-mouse CD1d mAb abolishes anti-A Ab production in mice

We tested the hypothesis that the collaboration between iNKT and B-1a cells that proceeds via iTCR-CD1d interactions for anti-A Ab production is inhibited by anti-mouse CD1d-blocking mAb. A single injection of anti-mouse CD1d mAb (500 μg/mouse) adequately blocks CD1d molecules on B cells for at least 7 days (data not shown). When Balb/c mice were treated with anti-CD1d mAb 1 day before and after they were immunized with human blood group A-RBCs, they completely lost the ability to produce anti-A Abs, although their total immunoglobulin levels remained normal (Figure 4A–F). In contrast, when Balb/c mice were injected with isotype-matched irrelevant control Abs, there was a significant elevation in the anti-A IgM and IgG class switching in the serum after the immunization.

IL-5 critically regulates anti-A Ab production via iTCR-CD1d interactions

To investigate the mechanism through which anti-CD1d mAb abolishes anti-A Ab

production in mice, we have analyzed the kinetics of serum levels of various cytokines (IL-4, IL-5, IL-9, IL-17, IL-21 and IFN-γ) after immunization with human blood group A-RBCs in *CD1d*$^{+/+}$ and *CD1d*$^{-/-}$ Balb/c mice. The stimulation with blood group A-RBCs rapidly and transiently induced IL-5 production in *CD1d*$^{+/+}$ mice (Figure 5A), while other cytokines were not detectable during the observation period (data not shown). In contrast, such IL-5 production was completely abolished in *CD1d*$^{-/-}$ mice. In addition, administration of anti-CD1d mAb also abrogated IL-5 production in *CD1d*$^{+/+}$ mice even after immunization with blood group A-RBCs (Figure 5B). To evaluate the impact of IL-5 on anti-A Ab production, anti-IL-5 neutralizing mAb was administered prior to each immunization with human blood group A-RBCs in *CD1d*$^{+/+}$ mice, which lead to constantly undetectable level of IL-5 in those mice (data not shown). The mice treated with anti-IL-5 mAb displayed a significantly lower level of ant-A Ab than the mice treated with the isotype-matched control Ab (Figure 5C-E). Consistent with an increase in the serum levels of IL-5 in WT *CD1d*$^{+/+}$ mice immunized with A-RBCs, IL-5-producing cells were detectable in the LMNCs by ELISPOT assay, whereas those were not in the spleen cells (Figure 6A-B). The IL-5producing cells were not observed in the LMNCs after administration of anti-CD1d mAb. Among the LMNCs, NKT cells (CD1d-tetramer^{+} TCRβ^{+}), T cells (CD1d-tetramer^{-} TCRβ^{+}), and others (CD1d-tetramer^{-} TCRβ^{-}) were isolated by multiparameter FCM sorting, and were

subjected to ELISPOT assay for the frequency of total IL-5-producing cells in each sorted cell fraction. NKT cell fraction was greatly enriched for IL-5-producing cells, whereas the other fractions were markedly depleted for those cells (Figure 6C-E), indicating that NKT cells were the predominant sources of IL-5 secreted after immunization with group A-RBCs. Thus, IL-5 critically regulates anti-A Ab production via iTCR-CD1d interactions.

Administration of anti-human CD1d mAb significantly inhibited anti-A Ab production in humanized mice

We further hypothesized that blocking the iTCR-CD1d interactions using anti-CD1d mAb could prevent Ab-mediated rejection in ABO-incompatible transplants. To address this possibility, we examined the inhibitory effects of anti-human CD1d mAb on anti-group A Ab production in a humanized mouse model where PBMCs from type O human volunteers had been engrafted into NOD/SCID/γ_c^{null} mice. The same dose of PBMCs from each human volunteer was then injected into 2 mice (20×10^6 cells/mouse) of which one subsequently received anti-human CD1d mAb and the other received the isotype-matched irrelevant control Ab 7 and 10 days after the engrafting. These PBMC-chimeric mice were then immunized with human blood group A-RBCs 8 days after the

PBMC injection. Anti-CD1d mAb completely inhibited anti-A IgM/IgG production in the humanized mice, whereas the mice treated with control Abs showed significant increase in the serum anti-A IgM/IgG levels (Figure 7A). Three weeks after the human PBMC engrafting, the recipients were sacrificed and the proportion of B cells with receptors for group A carbohydrates was assayed. We then used synthetic A carbohydrate determinants conjugated with FITC-labeled A-BSA and found $CD19^+$ B cells receptors for A carbohydrates in the spleen of humanized mice treated with the control Abs. In contrast, there were significantly fewer B cells receptors for A carbohydrates in the spleen of humanized mice treated with anti-CD1d mAb (Figure 7B-C). Thus, blocking the iTCR-CD1d interactions by CD1d mAb could be a novel approach for preventing Ab-mediated rejection in ABO-incompatible transplants.

Discussion

Unlike allopeptide Ags, which are presented to conventional T cells via MHC molecules, glycolipid Ags are presented to T cells by the MHC-like molecule CD1. Humans express several nonpolymorphic CD1 molecules, including CD1d, which presents lipids to NKT cells. NKT cells are innate-like lymphocytes defined by their characteristic semi-invariant T-cell receptor that recognizes the potent glycolipid Ag αGalCer.[3,25] In addition to this nonphysiological Ag, NKT cells have been shown to respond to exogenous bacterial lipid Ags as well as endogenous glycolipids presented by APCs responding to innate stimuli.[26-32] Glycosphingolipid isoglobotrihexosylceramide (iGb3) has been identified as an endogenous glycolipid Ag species recognized by healthy, non-infected NKT cells.[33] In addition, it has been recently demonstrated that a ubiquitous endogenous lipid, β-D-glucopyranosylceramide (β-GlcCer), accumulates during infection and in response to toll-like receptor agonists, and potently activates iNKT cells in both mice and humans through a cognate TCR interaction.[32,34] Despite the similarity in the molecular structure of histo-blood group Ags, Gal and NeuGc epitopes, with the either iGb3 or β-GlcCer glycolipid, it is not yet known whether these glycolipids are also recognized by NKT cells. The use of *Jα18*$^{-/-}$, *CD1d*$^{-/-}$, *CD1d*$^{-/-}$*GalT*$^{-/-}$, and *CD1d*$^{-/-}$*CMAH*$^{-/-}$ mice in this study allowed us to focus on the specific role of CD1d

molecules in the Ab response to those glycolipids. We demonstrated that Ab production against blood group A carbohydrates, but not against Gal and NeuGc epitopes, was dependent on CD1d and NKT cells. Recently, another study used a similar mouse model to demonstrate that Ab responses to Gal do not require CD1 molecules or NKT cells.[35] Taking these findings into consideration, our results indicate that anti-A Ab production could be specifically inhibited by blocking iTCR-CD1d interactions using anti-CD1d mAb, while maintaining Ab responses to Gal and NeuGc epitopes. Consistently, our results demonstrated that anti-CD1d mAb specifically inhibited the production of Abs against blood group A Ags in both mice and humans. Since Gal and NeuGc epitopes are expressed in environmental bacteria and neoplastic cells, and Abs against those determinants have been implicated in antibacterial and antitumor immunity,[36-40] this novel concept of using CD1d mAb is a preferable strategy for preventing Ab-mediated rejection in ABO-incompatible transplant recipients while preserving their immunity to infection and cancer.

B-1a and B-1b cells are essentially identical in their phenotype, and are distinguishable by the presence or absence of the CD5 marker alone. Thus far, no functional differences have been clearly identified between the 2 cell populations. However, differing activities of IL-5 and IL-9 have been reported. IL-5 transgenic mice have an expanded

B-1a population associated with high levels of auto-Abs, whereas IL-9 transgenic mice have an expanded B-1b population without the production of auto-Abs; however, both mice exhibit enhanced IgM production.[41] It has also been shown that IL-5 receptor α-chain-deficient (IL-5Rα$^{-/-}$) mice show decreased numbers of CD5^{+} B-1a cells and sustained numbers of CD5^{-} B-1b cells.[42] Those mice showed low serum IgG3 and IgM and no IL-5-induced enhancement of B-cell proliferation. These results suggest that IL-5 contributes to early development of B-1a cells, but not of B-1b cells. In addition, it has been reported that injection of IL-5 or IL-10, but not IL-4, increases serum anti-RBC auto-Ab and induction of hemolytic anemia in transgenic mice bearing Ig heavy and light chain genes encoding an antibody against the mouse RBCs, speculating that IL-5 or IL-10 may play an important role in the terminal differentiation of B-1a cells into Ab-producing cells *in vivo*.[43] Taking together with the difference in B-1a/B-1b proportion between *CD1d*$^{-/-}$ and *CD1d*$^{+/+}$ mice in our study (Figure 4), the cytokines derived from NKT cells may have some impact on the differentiation of B-1a cells responding blood group carbohydrates, but may not affect that of B-1b cells responding to Gal or NeuGc. As a more striking clue, we found that IL-5 exclusively increased after the immunization with A-RBCs in WT *CD1d*$^{+/+}$ mice, but remained undetectable in *CD1d*$^{-/-}$ mice. The combined FCM sorting and ELISPOT assay revealed that NKT cells predominantly secreted IL-5. Anti-mouse CD1d blocking mAb completely abolished

such IL-5 production in the WT mice. In addition, anti-IL-5 neutralizing mAb significantly diminished anti-A Ab production in the WT mice, indicating that IL-5 secreted from NKT cells critically regulates anti-A Ab production by B-1a cells. In addition, a recent demonstration that iNKT cells direct B cell responses to cognate lipid antigen in an IL-21-dependent manner would also pave the way for addressing how NKT cells stimulate B-1a cell responses to blood group carbohydrates.[44] The particular experiment, in which IL-21R-deficient mice were used, revealed that IL-21 derived from iNKT cells was required for Ab class switching, not merely for Ab production in responses to lipid-antigens. Further studies are needed to determine whether similar mechanism is responsible for Ab production/class switching from B-1a cells in response to blood group carbohydrate antigens, although IL-21 remained undetectable in sera of mice immunized with A-RBCs in this study.

To demonstrate a different pattern of V_H family usage in B-1b cells as compared to B-1a or conventional B cells in mice, a previous study used FCM sorting and single-cell PCR. They found that the V_H1 (J558) and V_H2 (Q52) families were underutilized and the V_H10 (DNA4) and V_H3 (3660) families were over-represented among B-1b cells, suggesting differences in the repertoires between the B-1a and B-1b populations.[45] Currently, the question of which B cell subset can recognize glycolipids to which iNKT

cells respond can be answered only speculatively. Since Gal and NeuGc epitopes are independent of CD1d in response to B-1b cells, this B cell subset might not share the recognition of the same Ag with NKT cells. In contrast, B-1a cells, some of which recognize blood group A epitopes in a CD1d-dependent manner, might share recognition of the corresponding Ag with NKT cells.

In conclusion, we found that iTCR-CD1d interactions were required for the production of anti-A Abs, whereas these interactions were not required for the production of anti-Gal and anti-NeuGc Abs. Anti-CD1d mAb significantly inhibited the development of B cells with receptors for blood group A carbohydrates, and completely inhibited anti-A Ab production. This suggests that they could be used in a novel approach to prevent Ab-mediated rejection in ABO-incompatible transplantation.

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Authorship

Contribution: H. Ohdan, H. Tazawa, and T. Irei designed the research; H. Tazawa, T Irei, Y. Tanaka, Y. Igarashi, M. Yamashita, and H. Sakai performed the research; H. Ohdan, H. Tazawa, T. Irei, and H. Tashiro analyzed the data; H. Ohdan and H. Tazawa wrote the paper.

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Figure legends

Figure 1. Ab production against blood group A determinants was dependent on iNKT cells but independent of T cells. (A-B) Balb/c nude mice and B6 MHC class II-deficient *C2ta*^{tm1Ccum} (C2D) mice were immunized with human group A-RBCs 2 times per week. Balb/c and B6 wild-type (WT) mice were used as the respective controls. The serum anti-A Ab (IgM) concentrations at 2 weeks after the second immunization were measured by ELISA assay. Even after the immunization, the serum anti-A Ab titers were not elevated in nude mice, but these Ab concentrations had significantly increased in C2D mice. Balb/c nude mice, n = 3; Balb/c WT mice, n = 3; B6 C2D mice, n = 4; B6 WT mice, n = 3. (C-D) Balb/c *CD1d*$^{-/-}$ mice and B6 *Jα18*$^{-/-}$ mice were immunized with human group A-RBCs 2 times per week. Balb/c and B6 WT mice served as the respective controls. The serum anti-A Ab levels were measured 2 weeks after the second immunization. The response of anti-A Abs was completely impaired in *CD1d*$^{-/-}$ mice and partially impaired in *Jα18*$^{-/-}$ mice. Average values ± SEM for the individual groups are shown. *p* values were shown to compare pre- and post-levels at the bottom of each figure. Balb/c *CD1d*$^{-/-}$ mice, n = 4; Balb/c WT mice, n = 3; B6 *Jα18*$^{-/-}$ mice, n = 5; B6 WT mice, n = 4. *$P < 0.05$ compared to the respective control mice.

Figure 2. Effect of αGalCer administration on anti- blood group A responses in *CD1d*$^{-/-}$ mice. Balb/c *CD1d*$^{-/-}$ mice and Balb/c wild type (*CD1d*$^{+/+}$) mice were immunized using blood group A-RBCs together with intraperitoneal injection of either αGalCer (4 μg/mouse) or PBS (control) 2 times per week. (A-B) The serum anti-A-specif c IgM and IgG levels were determined using ELISA at 2 and 10 weeks after the last immunization, respectively. αGalCer significantly increased the blood group A-specific Ab levels in *CD1d*$^{+/+}$ mice, but the Ab levels did not increase at all in *CD1d*$^{-/-}$ mice. The average values ± SEM for the individual groups are shown. **P* < 0.05 compared to the data from *CD1d*$^{+/+}$ mice without αGalCer. (C-D) The kinetics of the serum IgM and IgG titers against blood group A determinants in Balb/c *CD1d*$^{+/+}$ mice and Balb/c *CD1d*$^{-/-}$ mice (25× diluted serum was used). Anti-A Ab production was elicited by intraperitoneal immunization of mice with A-RBC 2 times per week. The average values ± SEM for the individual groups are shown. Balb/c *CD1d*$^{-/-}$ mice, n = 4; WT Balb/c mice, n = 5. **P* < 0.05 compared to the respective *CD1d*$^{+/+}$ mice.

Figure 3. Correlation of iNKT cells with Ab production against Gal and NeuGc- epitopes. B6 *CD1d*$^{-/-}$*GalT*$^{-/-}$ and *CD1d*$^{-/-}$*CMAH*$^{-/-}$ mice were immunized with Gal- and NeuGc-bearing rat thymocytes, and the levels of anti-Gal and anti-NeuGc Abs were then determined in their respective sera using FCM. (A-B) *CD1d*$^{-/-}$*GalT*$^{-/-}$

mice showed increased anti-Gal Ab titer (both IgM and IgG subclasses) similar to that in $CD1d^{+/+}GalT^{-/-}$ mice (n = 4 per group). (C-D) $CD1d^{-/-}CMAH^{-/-}$ mice also showed increased anti-NeuGc Ab titer similar to that in $CD1d^{+/+}CMAH^{-/-}$ mice (n = 4 per group). Median fluorescence intensity (MFI) values were used to follow Ab levels. The average values ± SEM for the individual groups are shown.

Figure 4. Effect of administration of anti-mouse CD1d mAb on anti-blood group A titers in mice. Balb/c WT mice received intraperitoneal injection of anti-mouse CD1d mAb (n = 6). Mice that received injections of isotype-matched Ab served as the controls (n = 5). The mice were immunized with human blood group A-RBCs (5×10^8/mouse) on days 1 and 8 after the mAb administration. After immunization, blood samples were obtained and the total IgM/IgG and anti-A IgM/IgG concentrations were measured using ELISA. (A-B) Treatment with anti-CD1d mAb significantly inhibited Ab production against blood group A epitopes in the mice. Anti-A IgM levels were detected 6 weeks after the mAb administration, and anti-A IgG levels were detected at 8 weeks. (C-D) The kinetics of anti-A Abs in Balb/c WT mice that were injected with either anti-mouse CD1d mAb or isotype-matched Ab is shown (10× diluted serum was used). (E-F) The kinetics of the total serum immunoglobulin (IgM and IgG) levels of the Balb/c WT mice treated with anti-CD1d mAb is presented. The average values ±

SEM for the individual groups are shown. *$P < 0.05$ compared to the data from WT mice treated with isotype-matched Ab.

Figure 5. Impact of IL-5 on anti-A Ab production after stimulation with blood group A-RBCs. (A) Balb/c *CD1d*$^{-/-}$ mice and WT *CD1d*$^{+/+}$ mice (n = 5 in each group) were immunized with human blood group A-RBCs (5×10^8/mouse). The levels of cytokines in serum were analyzed at the indicated time points using Cytometric Bead Array Flex Sets (CBA). Blood group A determinants significantly increased the level of IL-5 in *CD1d*$^{+/+}$ mice, but this cytokine level did not increase at all in *CD1d*$^{-/-}$ mice. In contrast, Blood group A determinants did not increase the levels of IL-4, IL-9, IL-17, IL-21, and IFN-γ in either *CD1d*$^{-/-}$ or *CD1d*$^{+/+}$ mice. (B) Balb/c WT mice received intraperitoneal injection of anti-mouse CD1d mAb (n = 5). Mice that received injections of isotype-matched Ab served as controls (n = 5). The mice were immunized with human blood group A-RBCs (5×10^8/mouse) on day 1 after the mAb administration. The level of IL-5 in serum was analyzed at the indicated time points using CBA. Treatment with anti-CD1d mAb significantly inhibited IL-5 production against blood group A epitopes in the mice. (C-E) Balb/c WT mice received intraperitoneal injection of anti-mouse IL-5 mAb (n = 5) 30 minutes prior to immunization with human blood group A-RBCs (5×10^8/mouse). Mice that received

injections of isotype-matched Ab served as controls (n = 5). The mice were immunized with human blood group A-RBCs two times at one week interval after the mAb administration. (C) Anti-A IgM concentrations were measured using ELISA before the immunization. (D) Anti-A IgM concentrations were measured at 2 weeks after the first immunization. (E) Anti-A IgM concentrations were measured at 3 weeks after the first immunization. Treatment with anti-IL-5 mAb significantly inhibited Ab production against blood group A epitopes in the mice. The average values ± SEM for the individual groups are shown. $*P < 0.05$ compared to the data from *CD1d*$^{-/-}$ mice, and data from WT mice treated with isotype-matched Ab.

Figure 6. NKT cells were predominant sources of IL-5 secreted after immunization with group A-RBCs. WT *CD1d*$^{+/+}$ Balb/c mice received intraperitoneal injection of anti-mouse CD1d mAb (n = 3). Mice that received injections of isotype-matched Ab served as controls (n = 3). The mice were immunized with human blood group A-RBCs (5×10^8/mouse) on day 1 after the mAb administration. The mice were sacrificed to determine the IL-5 producing cells 6 hours after the immunization. (**A-B**) The liver mononuclear cells (LMNC) and spleen cells were seeded . The representative pictures of ELISPOT wells are shown in **A** and the frequency of IL-5 producing cells is depicted in **B**. Number in each picture refers to the

total cells seeded per well ($\times 10^3$). (**C-E**) Six hours after immunization with A-RBCs, the LMNCs were isolated from *CD1d*$^{+/+}$ Balb/c mice (n=12). The pooled cells were used in ELISPOT assay to determine the frequency of IL-5-producing cells. The LMNC were stained with APC-conjugated anti-mouse CD1d-tetramer and PE-Cy7 conjugated anti-mouse TCRβ. NKT cells (CD1d-tetramer$^+$, TCRβ$^+$), T cells (CD1d-tetramer$^-$, TCRβ$^+$), and the others (CD1d-tetramer$^-$, TCRβ$^-$) were isolated by sorting with FACS Aria. After sorting, the purities of NKT, T and other cells were reanalyzed by FCM. (**D-E**) The representative pictures of ELISPOT wells are shown in **D** and the frequency of IL-5 producing cells is shown in **E**. Number in each picture refers to the total cells seeded per well ($\times 10^3$). The results shown are the average ± SEM calculated from red spot number in quadruplicate wells. The results are representative of two similar experiments. $*P < 0.05$.

Figure 7. Effect of administration of anti-human CD1d mAb on anti-A Ab production in humanized mice. The same dose of PBMCs from each type O human volunteer was intraperitoneally injected into 2 NOD/SCID/γ_c^{null} mice (20×10^6 cells/mouse). Of these mice, one subsequently received anti-human CD1d mAb and the other received isotype-matched irrelevant control Ab at days 7 and 10 after the PBMC engrafting. The humanized mice were immunized with human blood group A-RBCs 8

days after the PBMC injection. (A) The serum anti-A IgM and IgG levels in the humanized mice were determined using ELISA at 14 and 21 days after the engraftment. Each point represents an individual mouse. Each group contained 5 animals. (B) Three weeks after the human PBMC engrafting, the humanized mice were sacrificed to determine the proportion of B cells with receptors for group A carbohydrates. Spleen cells were prepared from the humanized mice (n = 4 in each group). The pooled cells were stained with FITC-labeled A-BSA or control FITC-labeled BSA together with PE–conjugated anti-human CD19 mAb. Representative FCM results of group A-BSA-binding spleen cells. We analyzed 50,000 cells per contour plot. The percentages in the figure represent percentages of total $CD19^+$ B cells. (C) The frequencies of A-BSA-binding B cells among the total B cell population in mice treated with either anti-human CD1d mAb or isotype-matched control Ab are shown. $*P < 0.05$ compared to the data from humanized mice treated with isotype-matched Ab.

Figure 1

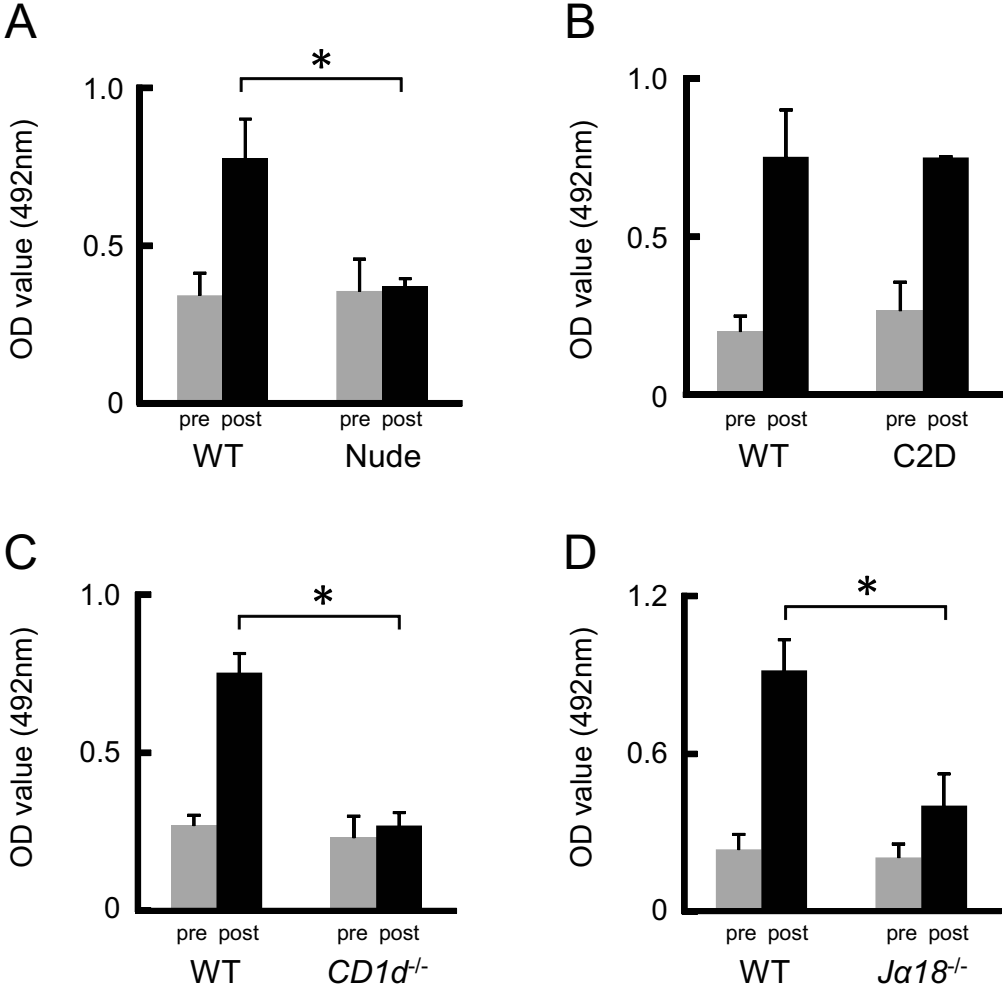
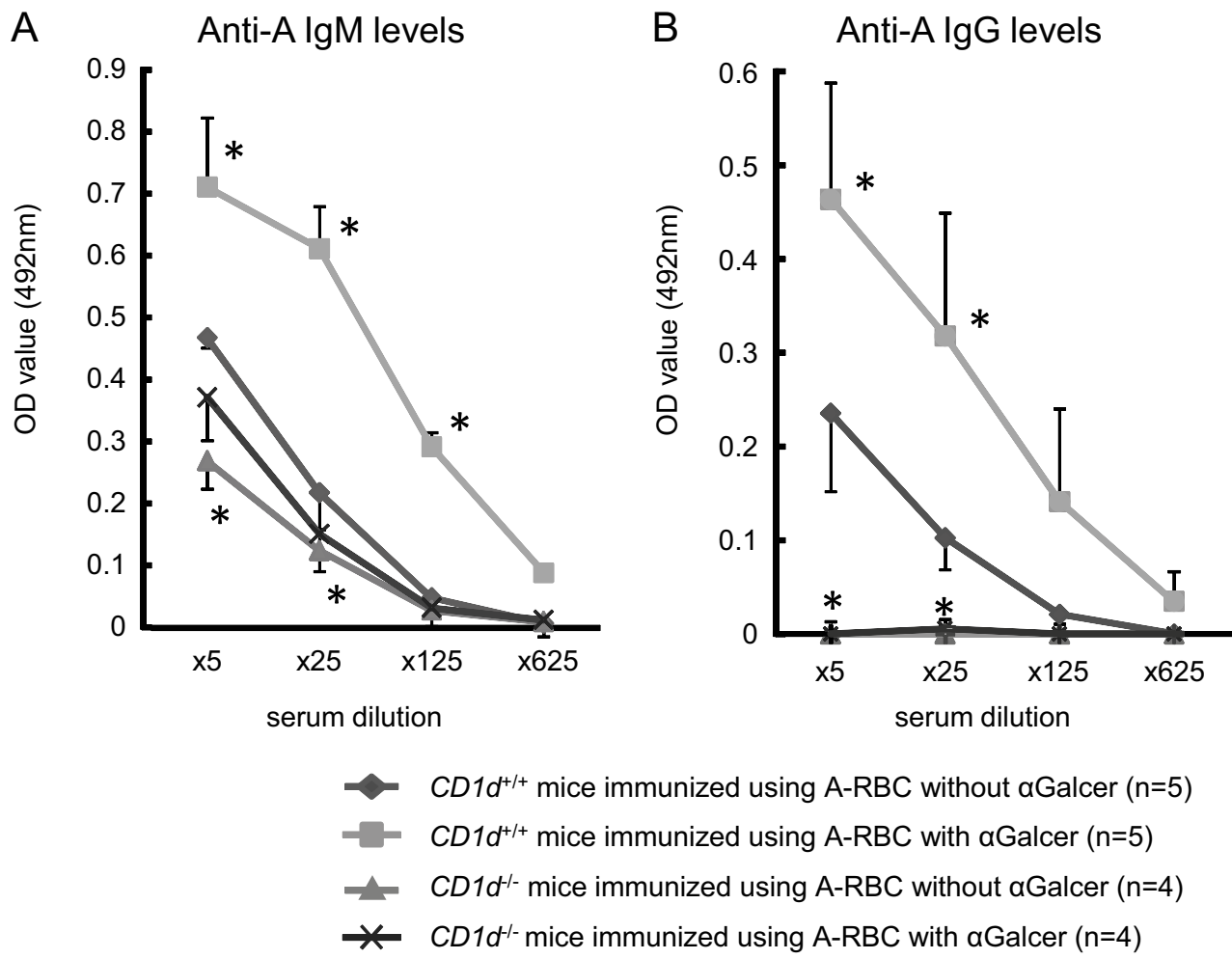


Figure 2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0.4
0.3
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0.1
0
x5
x25
x125
x625
serum dilution
B
Anti-A IgG levels
OD value (492nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
x5
x25
x125
x625
serum dilution
CD1d+/+ mice immunized using A-RBC without αGalcer (n=5)
CD1d+/+ mice immunized using A-RBC with αGalcer (n=5)
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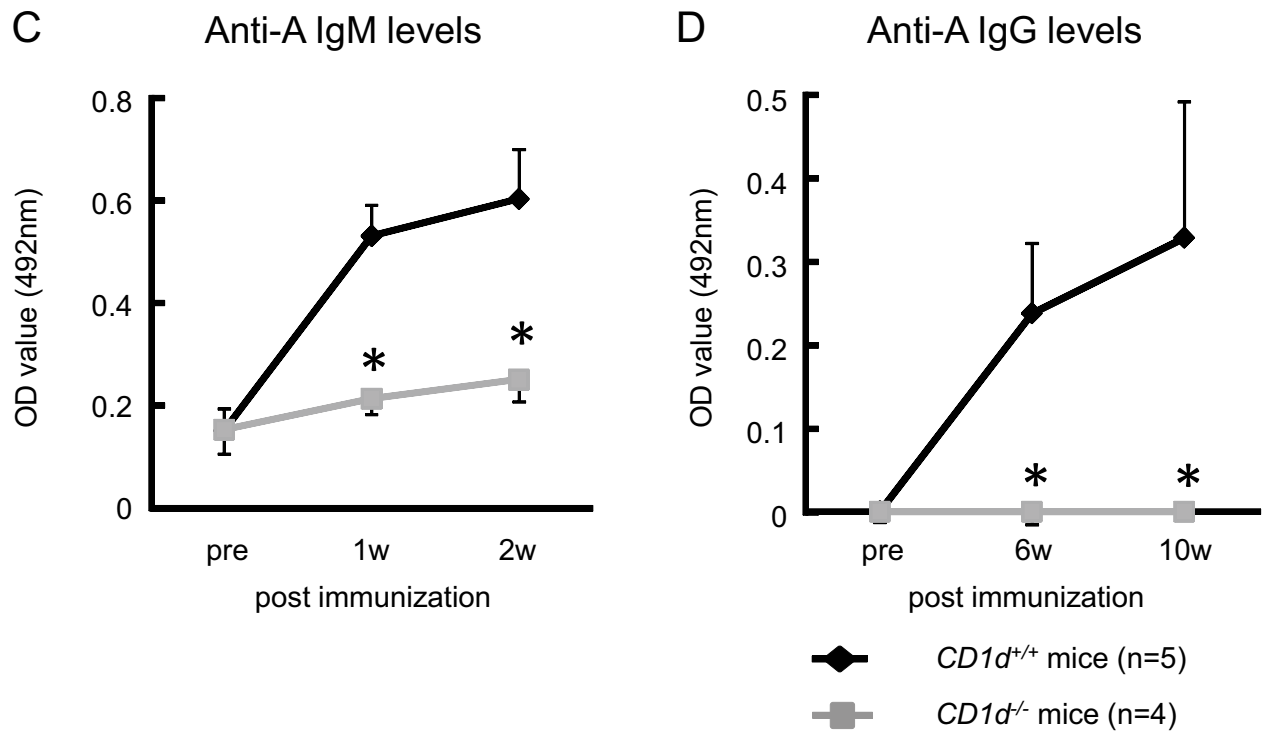
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OD value (492nm)
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0.6
0.4
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pre
1w
2w
post immunization
D
Anti-A IgG levels
OD value (492nm)
0.5
0.4
0.3
0.2
0.1
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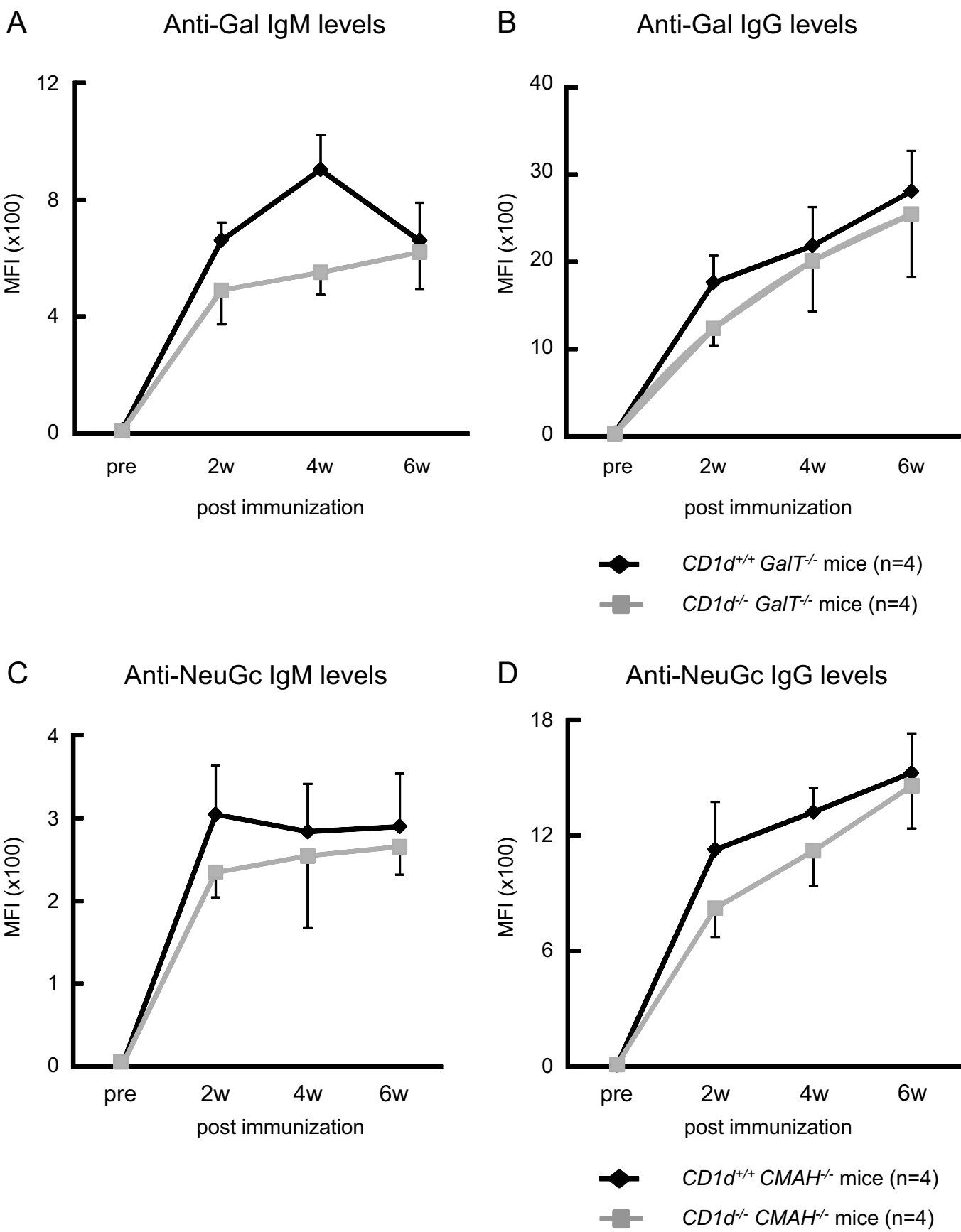
pre
6w
10w
post immunization
CD1d+/+ mice (n=5)
CD1d-/- mice (n=4)

Figure 3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MFI (x100)
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8
4
0
pre
2w
4w
6w
post immunization
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MFI (x100)
40
30
20
10
0
pre
2w
4w
6w
post immunization
CD1d+/+ GalT-/- mice (n=4)
CD1d-/- GalT-/- mice (n=4)
C
Anti-NeuGc IgM levels
MFI (x100)
4
3
2
1
0
pre
2w
4w
6w
post immunization
D
Anti-NeuGc IgG levels
MFI (x100)
18
12
6
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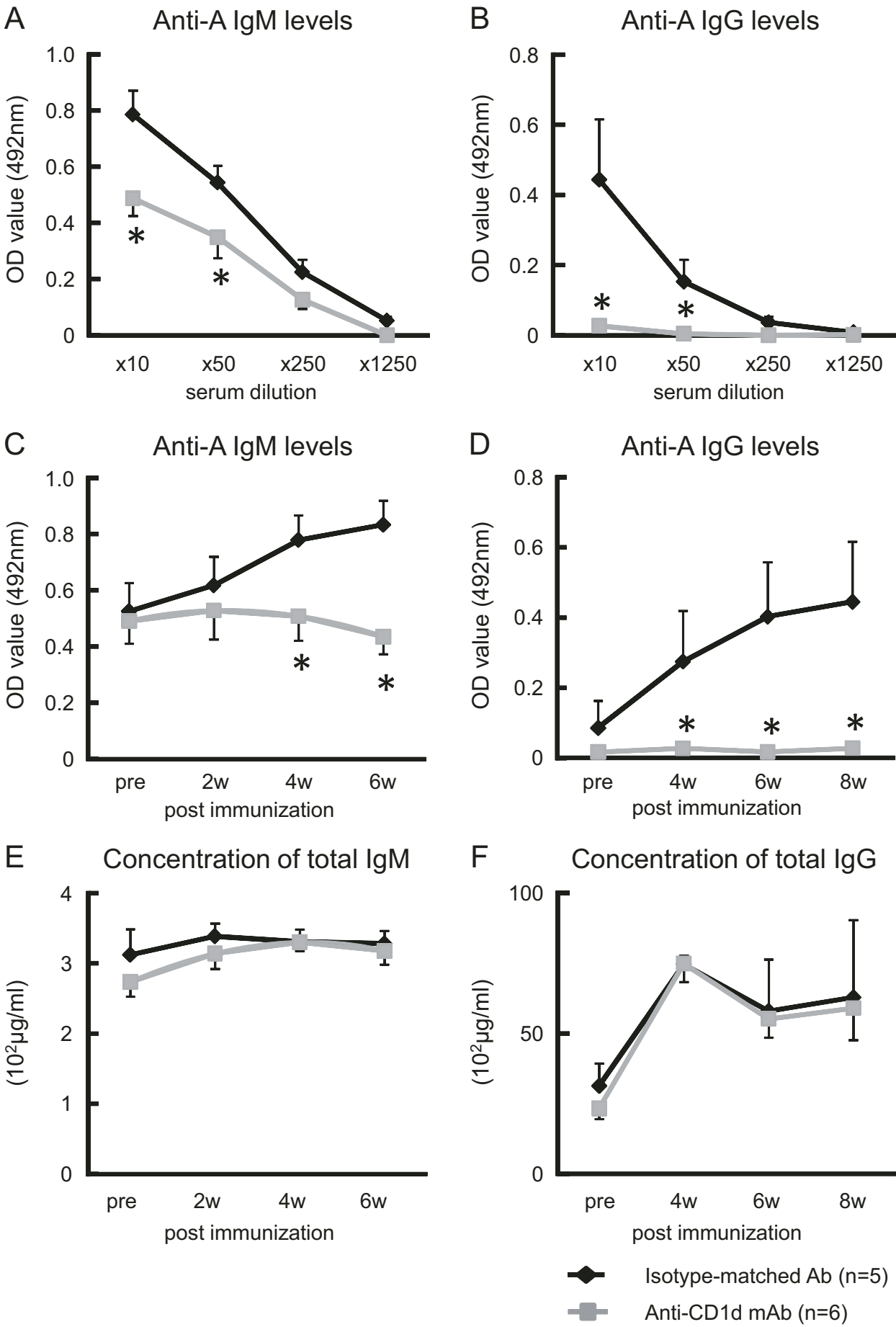
CD1d-/- CMAH-/- mice (n=4)

Figure 4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x1250
serum dilution
B
Anti-A IgG levels
OD value (492nm)
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x1250
serum dilution
C
Anti-A IgM levels
OD value (492nm)
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0.4
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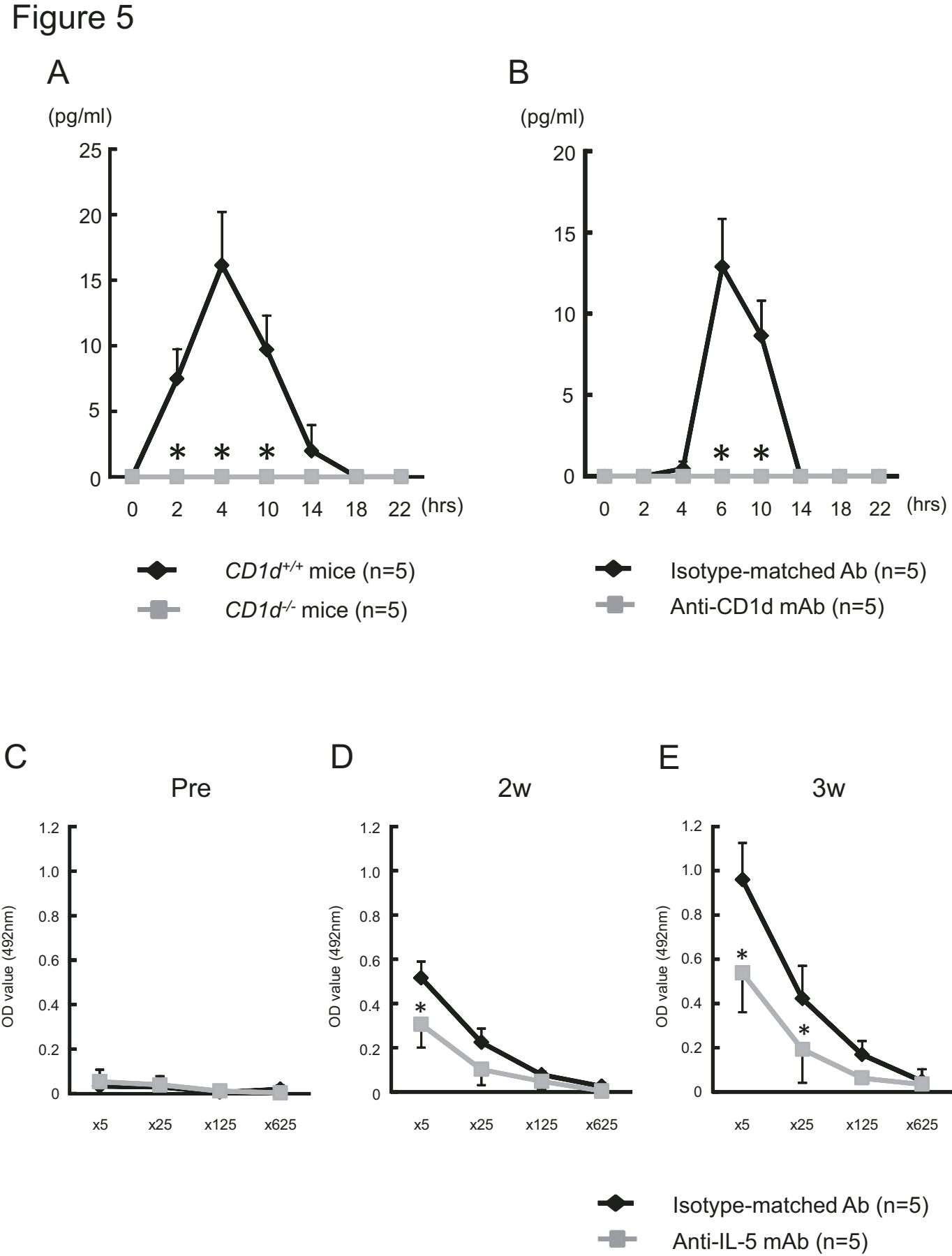
2w
4w
6w
post immunization
D
Anti-A IgG levels
OD value (492nm)
0.8
0.6
0.4
0.2
0
pre
4w
6w
8w
post immunization
E
Concentration of total IgM
(10²μg/ml)
4
3
2
1
0
pre
2w
4w
6w
post immunization
F
Concentration of total IgG
(10²μg/ml)
100
50
0
pre
4w
6w
8w
post immunization
Isotype-matched Ab (n=5)
Anti-CD1d mAb (n=6)

Figure 5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(pg/ml)
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15
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*
CD1d+/+ mice (n=5)
CD1d-/- mice (n=5)
B
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*
Isotype-matched Ab (n=5)
Anti-CD1d mAb (n=5)
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OD value (492nm)
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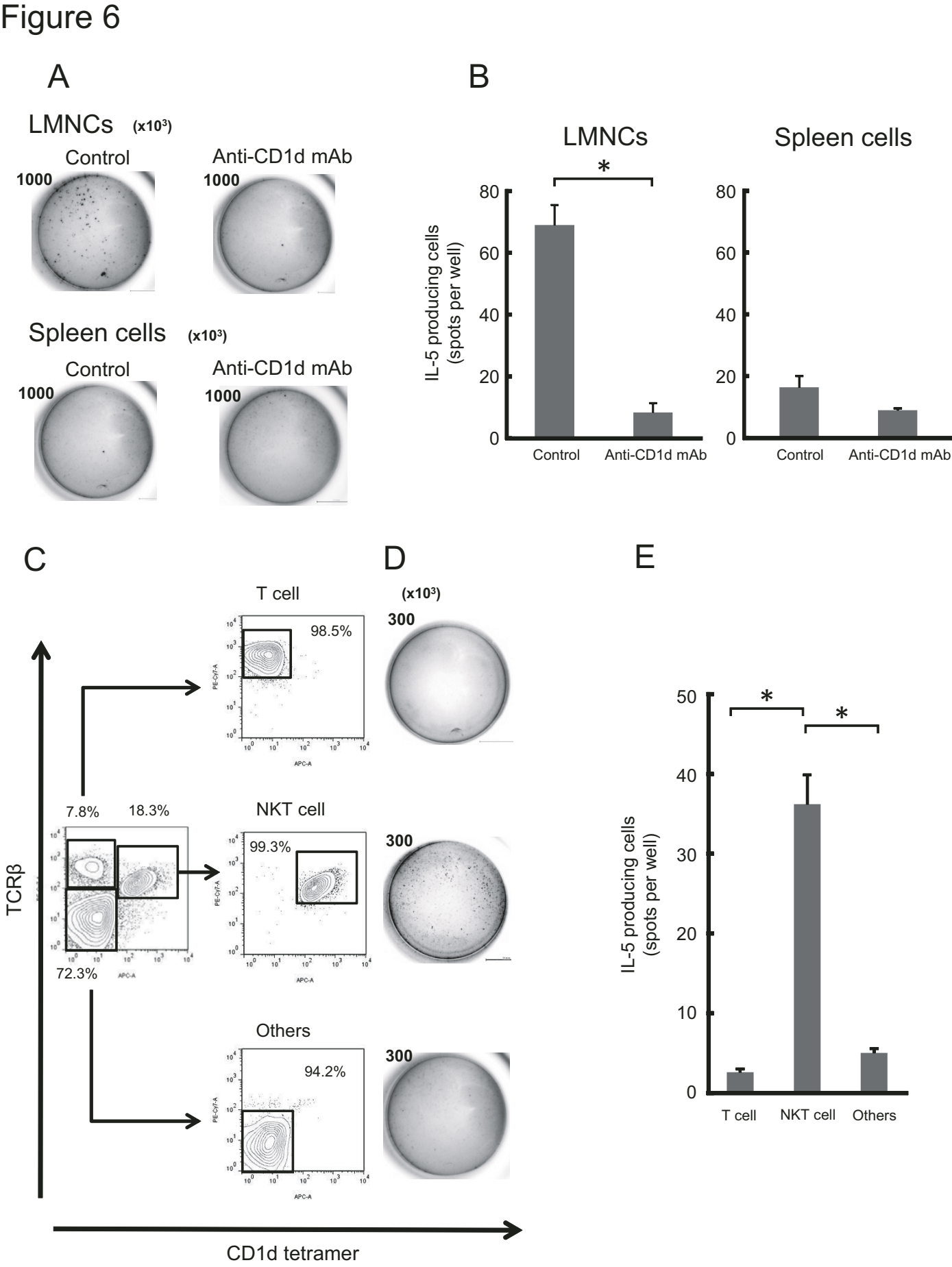
x5 x25 x125 x625
*
Isotype-matched Ab (n=5)
Anti-IL-5 mAb (n=5)

Figure 6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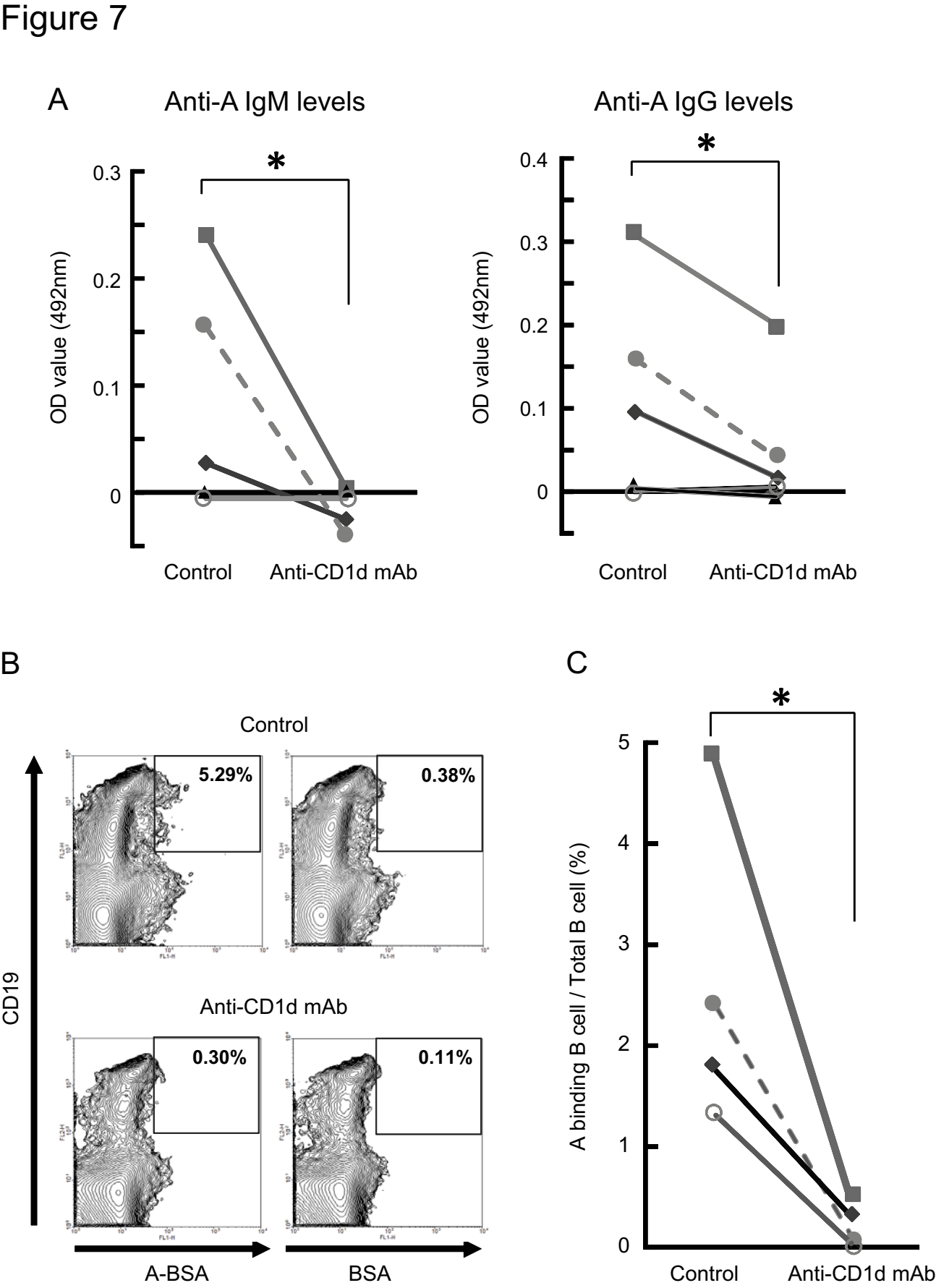
Control
Anti-CD1d mAb
1000
1000
Spleen cells (x10³)
Control
Anti-CD1d mAb
1000
1000
B
LMNCs
Spleen cells
*
IL-5 producing cells (spots per well)
80
60
40
20
0
Control
Anti-CD1d mAb
C
T cell
98.5%
7.8%
18.3%
72.3%
NKT cell
99.3%
Others
94.2%
TCRβ
CD1d tetramer
PE-Cy7-A
APC-A
D
(x10³)
300
300
300
E
*
*
IL-5 producing cells (spots per well)
50
40
30
20
10
0
T cell
NKT cell
Others

Figure 7


A
Anti-A IgM levels
OD value (492nm)
0.3
0.2
0.1
0
*
Control
Anti-CD1d mAb
Anti-A IgG levels
OD value (492nm)
0.4
0.3
0.2
0.1
0
*
Control
Anti-CD1d mAb
B
Control
5.29%
0.38%
Anti-CD1d mAb
0.30%
0.11%
CD19
A-BSA
BSA
C
A binding B cell / Total B cell (%)
5
4
3
2
1
0
*
Control
Anti-CD1d mAb