[Frontiers in Bioscience 13, 6214-6228, May 1, 2008]

Alpha-galactosylceramide-driven immunotherapy for allergy

Yasuyuki Ishii[1], Risa Nozawa[1], Yukiko Takamoto-Matsui[2], Annabelle Teng[2], Haruka Katagiri-Matsumura,[2] Hiroshi Nishikawa[2], Hiroyuki Fujita[1,2], Yuki Tamura[1], Masaru Taniguchi[3]

[1]Laboratory for Vaccine Design, RIKEN Research Center for Allergy and Immunology, 1-7-22, Suehiro, Tsurumi, Yokohama, Kanagawa, Japan 230-0045, [2]Department of Environmental Immuno-Dermatology, Yokohama City University, Graduate School of Medicine, 3-9, Fukuura, Kanazawa, Yokohama, Kanagawa, Japan 236-0004, [3]Laboratory for Immune regulation, RIKEN Research Center for Allergy and Immunology, 1-7-22, Suehiro, Tsurumi, Yokohama, Kanagawa, Japan 230-0045

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
- *3.1. Mice*
- *3.2. Reagents*
- *3.3. Antibodies*
- *3.4. Preparation of liposomes*
- *3.5. Ab measurement by ELISA*
- *3.6. Cell culture and culture medium*
- *3.7. Preparation of splenic DCs*
- *3.8. Flow cytometry*
- *3.9. RT-PCR*
- *3.10. Statistical analysis*

4. Results
- *4.1. Suppression of T cell priming by pretreatment of mice with aGalCer-liposomes*
- *4.2. Antigen presenting cells involved in the regulation of IL-10 production by pretreatment of mice with aGalCer liposomes*
- *4.3. Expansion of CD11c^{low}CD45RB^{high} cell population in vivo by the treatment of aGalCer-liposomes*
- *4.4. Differentiation of Treg cells in vitro by antigen-pulsed CD11c^{low}CD45RB^{high} cells*
- *4.5. Differentiation of Treg cells in vivo by administration of aGalCer-liposomes encapsulated an antigen*
- *4.6. Antigen-specific suppression by the treatment with aGalCer-OVA-liposomes*

5. Discussion
6. Acknowledgement
7. References

1. ABSTRACT

We report here that the delivery of both α-galactosylceramide (αGalCer), a representative ligand for invariant natural killer T (iNKT) cells, and an antigenic polypeptide to marginal zone B cells induces the differentiation of regulatory cells *in vivo*, and suppresses the secondary antibody responses in mice. Splenic $CD21^+CD23^-$ B cells of mice treated with αGalCer-liposomes produce IL-10 when co-cultured with iNKT cells, whereas the cells treated with aqueous αGalCer fail to do so. Adoptive transfer of the B cells into syngenic mice leads to the expansion of splenic $CD11c^{low}CD45RB^{high}$ cells, which convert naïve $CD4^+$ T cells from RAG2-deficient DO11.10 mice to $CD4^+CD25^{high}$ $Foxp3^+$ T cells in the presence of $OVA_{323-339}$ peptide. Administration of αGalCer-OVA-liposomes into OVA-primed mice causes the development of $CD4^+CD25^{high}$ $Foxp3^+$ T cells that produce both IL-10 and IFN-γ, and induced the antigen-specific suppression of the secondary antibody responses when boosted with OVA alone. These results indicate that antigen-containing αGalCer-liposomes can facilitate the development of tolerogenic antigen-presenting cells and inducible regulatory T cells that are involved in the suppression of immune responses to antigens.

2. INTRODUCTION

It is generally accepted that polarization of an immune response towards the Th2 type is an important prerequisite for the generation of antigen-specific IgE formation involved with atopic disorders (1). Several approaches have been developed for the deviation of the T cell response to the Th1 type. Among these include chemical conjugation of CpG oligonucleotides (ODN) to antigen, which is a device to deliver CpG-ODN and antigen to the same antigen presenting cells. This proved to be a potent immunogen for priming Th1-biased immune response to the antigen (2). Repeated injections of a CpG-ragweed antigen conjugate to antigen-primed mice prevented the secondary IgE antibody response to this allergen (3). Clinical trials of this conjugate are in progress (4).

An alternative approach for the suppression of the IgE antibody response is to induce immune tolerance via the development of antigen-specific regulatory T (Treg)[1] cells. It is well known that intravenous injection of denatured or modified antigen, which has lost the major B cell epitopes while maintaining the T cell epitope of the native antigen, into antigen-primed mice facilitates the differentiation of antigen-specific suppressor (regulatory) T

cells (5, 6). This effectively suppresses the secondary IgE antibody response to the native antigen. More recently it was shown that administration of antigen along with heat-killed listeria monocyotogenes (HKL) as an adjuvant reversed an established Th2 response and IgE antibody formation, and induced a Th1 type immune response to the antigen (7, 8).

Further studies on the cellular mechanisms of the immune deviation revealed that immunization with HKL and antigen, included in incomplete Freund adjuvant, induced not only a conventional Th1 response, but also the differentiation of antigen-specific Treg cells that produced IL-10 and IFN-γ (7). The Treg cells were responsible for inhibition of allergen-induced airway hyper-reactivity and for suppression of the IgE antibody response (7). A possible role of IL-10-producing Treg cells for the suppression of an IgE response is supported by the fact that transfer of OVA-specific Tr1 T cell clones concomitant with OVA immunization inhibited the primary IgE antibody response, whereas transfer of the same number of the antigen-specific Th1 clone failed to do so (8).

In an experimental model of anterior chamber-associated immune deviation (ACAID), Sonoda *et al* (9, 10) have shown that NKT cells are required for the development of systemic immune tolerance. They provided evidence that the NKT cells expressed an invariant Vα14-Jα281 chain, and that the NKT cell-derived IL-10 played a critical role for the development of antigen-specific Treg cells and immune tolerance. These findings suggested the possibility that stimulation of NKT cells with a specific epitope, i.e., α-galactosylceramide (αGalCer), at the time of (or prior to) antigen-presentation to naïve T cells may facilitate the differentiation of Treg, rather than Th, cells specific for the antigen.

In the present studies, we prepared liposomes that contained αGalCer in a lipid bilayer with encapsulated OVA and examined their effect on antigen-induced immune responses. The results show that administration of the αGalCer-OVA-liposomes induces the development of antigen-specific Treg cells *in vivo*, and that the treatment of mice with these liposomes is effective for suppressing secondary antibody responses.

3. MATERIALS AND METHODS

3.1. Mice

BALB/c and (C57BL/6 X DBA/2) BDF1 mice were purchased from Charles River Japan, Inc. Vα14 NKT-deficient (Jα281-/-) mice were generated at Chiba University (11) and backcrossed for ten generations onto BALB/c background. OVA-specific TCR transgenic DO11.10 mice were kindly provided by M. Kubo (RIKEN Research Center for Allergy and Immunology). All mice were maintained under pathogen-free conditions in our animal facility. The institutional committee for animal care and use in RIKEN approved all experimental procedures.

3.2. Reagents

α-galactosylceramide (αGalCer), KRN7000, was purchased from Kirin Brewery Co., Ltd., Japan. Dioleoylphosphatidylcholine (DOPC) (Wako Pure Chemical Industries, Japan), cholesteryl 3β-N- ((dimethylamino)ethyl)carbamate (DC-Chol) (Sigma-Aldrich), and 1,2-Distearoyl-sn-Glycero-3-Phosethanolamine-N- (Methoxy (Polyethyleneglycol)-2000) (Ammonium Salt) (PEG-PE) (Avanti Polar Lipids, Inc.) were used for the formation of liposomes. Phorbol 12-Myristate 13-Acetate (PMA), ionomycin and $OVA_{323-339}$ peptide were purchased from Sigma-Aldrich. Crystalline ovalbumin (OVA) was purchased from Seikagaku Co., Japan. 2,4-dinitrophenyl derivatives of OVA (DNP-OVA) and keyhole limpet hemocyanin (DNP-KLH) were purchased from Cosmo Bio, Japan.

3.3. Antibodies

For depletion of lineage-specific cells from spleens, biotinylated mAbs to CD3 (145-2C11), CD19 (1D3), CD49b (DX5), Ly-6G (Gr-1), TER-119 (BD Bioscience Pharmingen) and streptavidin-conjugated microbeads (Miltenyi Biotech) were used.

For flow cytometry, FITC-conjugated mAbs to IFN-γ (XMG1.2), and CD4 (RM4-5), PE-conjugated mAbs to CD25 (PC61) and CD45RB (C363.16A), APC-conjugated mAbs to IL-10 (JES5-16E3) and CD11c (HL3), biotinylated anti-CD4 (RM4-5) mAb and streptavidin-conjugated PerCP Cy5.5 were purchased from BD Bioscience Pharmingen. Anti-CD16/32 (2.4G2; BD Bioscience Pharmingen) mAb was used for blocking the Fcγ receptor. PE-conjugated anti-Foxp3 mAb (FJK-16s) and staining kit were purchased from eBioscience.

For ELISA, biotinylated anti-mouse IgE (6HD5) was purchased from Seikagaku Co. Japan. Mouse IgE anti-DNP mAb H1 DNP-ε-26 and IgG1 anti-DNP mAb H1 DNP-γ-109 (12) was a gift from Dr. Fu-Tong Liu (University of California, Davis, CA). IgG2a anti-DNP mAb (MA-DNP-2) was purchased from Cosmo Bio, Japan. Horse radish peroxidase (HRP)-conjugated anti-IgG1 and IgG2a were purchased from Zymed.

3.4. Preparation of liposomes

Firstly, DC-Chol (0.83 mg), DOPC (0.77 mg), PEG-PE (0.029 mg), and α-GalCer (0.16 mg) were dissolved in chloroform and methanol (1:1). The dried films were suspended in 200 μl of PBS containing 400 μg/ml OVA, homogenized by sonication and mixed with 600 μl of dH_2O. Uni-lamellar liposomes were prepared from multi-lamellar liposomes suspensions using a LiposoFast-Basic extruder (Avestin Inc.) by 25 cycles of manual extrusion through a 100 nm pore size polycarbonate membrane. Loss of α-GalCer and lipids trapped in the extruder was less than 10% of the starting material as estimated by the weight of lipids recovered. OVA-encapsulated αGalCer-liposomes were concentrated in an Amicon Ultra-4 centrifugal filter PL-100 (Millipore) and washed with PBS until no OVA protein was detectable in the flow through fraction. The concentrations of α-GalCer and OVA in the final liposome preparations were 200μg/ml and 50μg/ml, respectively. Liposome formulations were stored at 4 °C until use.

3.5. Ab measurement by ELISA

Serum anti-DNP IgG1 and IgG2a antibodies were measured as described (13). Anti-DNP IgE Ab was measured with minor modifications. Briefly, microtiter plates (Corning) were coated overnight with 1 μg/ml rat anti-mouse IgE mAb (6HD5) at 4 °C. After blocking and washing, appropriate dilutions of serum samples or serial dilutions of anti-DNP IgE Ab as a standard were added. After incubation for 3 hr at 37 °C, plates were washed and 2.5 μg/ml biotinylated DNP-BSA (Cosmo Bio, Japan) in "Can Get Signal" immunoreaction enhancer solution (TOYOBO, Japan) was added to each well. The binding of biotinylated DNP-BSA was measured by HRP-conjugated streptavidin (Dako Cytomation) in "Can Get Signal" immunoreaction enhancer solution and TMB+ substrate system (Dako Cytomation).

3.6. Cell culture and culture medium

All cells were cultured in Click's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 10 mM Hepes (Sigma-Aldrich) and antibiotics. $CD4^+$ naïve T cells were isolated from spleen cells of OVA-specific TCR transgenic DO11.10 mice by using $CD4^+$ T cell isolation kit (Miltenyi Biotech). For the isolation of iNKT cells, dimeric rmCD1d:Ig was loaded with molar excess of αGalCer according to the instruction manuals of BD Biosciences for 16 h at 37 ° C. Spleen or lymph node cells were incubated with αGalCer-loaded rmCD1d:Ig dimer for 30 min at 4 °C. After washing, the cells were incubated with rat anti-mouse IgG1 microbeads for 30 min at 4 °C. iNKT cells were recovered by the binding to magnet.

3.7. Preparation of splenic DCs

DCs were prepared as previously described (14). Briefly, spleens were swollen by infusion of 1 mg/ml collagenase D (Roche Molecular Biochemicals) in HBSS containing 10 mM Hepes buffer (pH 7), and incubated for 45 min at 37 °C. After cutting the spleen into small pieces, the cells were filtered through a 70 μm cell strainer (BD Bioscience), and suspended in 3 ml of 14.1% HistoDenz (Sigma-Aldrich). After the addition of 5 ml of X-VIVO 15 medium (BioWhittaker), the cell suspension was centrifuged at 1700 x g for 15 min and the low-density cell fraction was recovered. After incubation with a cocktail of biotinylated anti-CD3, CD19, CD49b, Ly-6G and TER119 mAbs at 4°C for 30 min, the cells were bound to streptavidin-conjugated microbeads. The enriched DCs were recovered from the flow-through fraction of a magnetic column (Miltenyi Biotech).

3.8. Flow cytometry

Cells were pre-treated with anti-CD16/CD32 mAb to prevent non-specific binding of Abs to cell Fc receptors. After washing with PBS containing 2% FCS and 0.09% sodium azide, the cells were incubated for 30 min at 4 °C with fluorescently-labeled mAbs. For staining of intracellular cytokines, cells were incubated with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 h in the presence of 2 μM monensin (Protocol of BD Bioscience). Cells were fixed, and permeabilized by using Cytofix/Cytoperm in Perm/Wash buffer (BD Bioscience), and then incubated for 30 min at 4 °C with fluoro-labeled mAbs. Cytometric analysis and cell sorting were done using FACSCalibar, FACSVantage SE and FACSAria (BD Bioscience).

3.9. RT-PCR

Total RNA was isolated using RNeasy MinElute Cleanup kit (Qiagen). For the amplification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), Foxp3 and IL-21, specific primers were synthesized as follows: 5'- accacagtccatgccatcac -3' (forward for G3PDH); 5'- tccaccaccctgttgctgta -3' (reverse for G3PDH); 5'- gccagtctggaatgggtgtccagg -3' (forward for Foxp3); 5'- ggtcgcatattgtggtacttgaag -3' (reverse for Foxp3); 5'- cccttgtctgtctggtagtcatc-3' (forward for IL-21) and 5'- atcacaggaagggcatttagc-3' (reverse for IL-21). RT-PCR was performed for 30 cycles at 57 °C for annealing.

3.10. Statistical analysis

The statistical significance of differences between the experimental groups was determined by nonparameric Mann-Whitney *U* test.

4. RESULTS

4.1. Suppression of T cell priming by pretreatment of mice with αGalCer-liposomes

In order to test possible effects of the liposome-formulation of αGalCer (αGC-liposomes) on the antibody response, either αGC-liposomes containing 2 μg αGalCer or αGC-OVA-liposomes containing 2 μg αGalCer and 0.5 μg OVA was injected intraperitoneally into groups of BDF1 mice 3 days prior to immunization with alum-absorbed DNP-OVA. Control groups received aqueous αGalCer or liposomes alone prior to immunization. As shown in Figure 1A, primary anti-DNP IgE, IgG1 and IgG2a antibody responses were completely suppressed by pretreatment with αGC-liposomes or αGC-OVA-liposomes, but not by the pretreatment with aqueous αGalCer. Total serum immunoglobulin levels were not affected by either of the pre-treatments (data not shown). We wondered whether the lack of the antibody responses in the αGC-liposomes-treated mice was due to suppression of T cell priming. To test this possibility, we treated groups of BDF1 mice with an intraperitoneal injection of αGC-liposomes or aqueous αGalCer, and then immunized the mice with alum adsorbed DNP-OVA three days later. Splenic $CD4^+$ T cells were obtained from each group of the mice 7 days after the immunization, and the cells were cultured for 48 hr with irradiated syngenic splenocytes in the presence of DNP-OVA of various concentrations. As shown in Figure 1B, the pretreatment with αGC-liposomes prevented IL-2 production and cell proliferation, whereas pretreatment with aqueous αGalCer rather enhanced them. It was also noted that antigen-nonspecific proliferation of $CD4^+$ T cells induced by PMA/ionomycin was also substantially inhibited following αGC-liposome-treatment (Figure 1B). To elucidate the mechanism for the prevention of T cell priming, cytokine production of splenic $CD4^+CD25^+$ T cells from αGC-liposome-treated mice were analyzed 7, 10 and 14 days after the immunization with

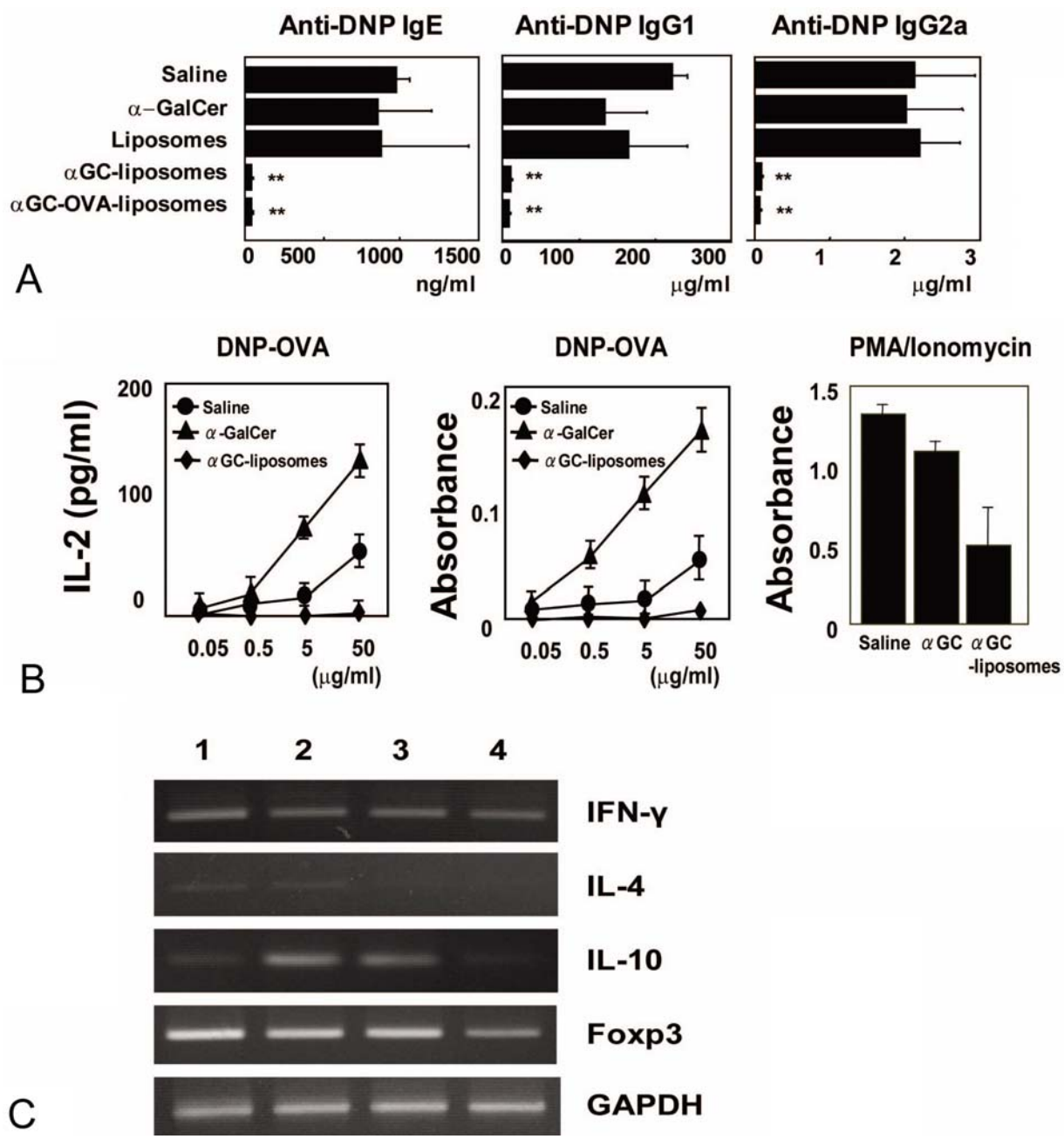

Figure 1. Suppression of primary antibody responses by administration of αGC-liposomes. **(**A) Groups of BDF1 mice (n=5) received an intraperitoneal injection of αGC-liposomes or αGC-OVA-liposomes containing 2 μg α-GalCer, the same dose of aqueous αGalCer, or liposome alone. Three days later, they were immunized with alum-absorbed DNP-OVA (0.1 μg), and the concentration of anti-DNP Abs in their sera was determined by ELISA at day 14. ** $p < 0.001$ for αGC-liposome and αGC-OVA-liposome groups versus saline group. (B) Groups of BDF1 mice were pretreated with the same dose of aqueous αGalCer, αGC-liposomes, or saline, and then immunized with alum-adsorbed DNP-OVA 3 days later. Their splenic $CD4^+$ T cells were obtained 7 days after the immunization, and aliquots (2×10^5 cells) of the cells were cultured with serial dilutions of DNP-OVA together with irradiated syngenic splenocytes (1×10^6 cells) or in the presence of 50 ng/ml PMA and 500 ng/ml ionomycin. After 48 h, IL-2 in the culture supernatants was assessed by ELISA. The cell proliferation was assessed by using the uptake of methylthiazole tetrazolium (MTT). Stimulation index represents the ratio of absorbance of samples versus that of a negative control. (C) Groups of BDF1 mice were pretreated with the same dose of αGC-liposomes or saline, and then immunized with alum-absorbed DNP-OVA three days later. Splenic $CD4^+CD25^+$ T cells of non-treated mice were obtained 7 days after the immunization (lane 1). The cells of αGC-liposome-treated mice were obtained 7 (lane 2), 10 (lane 3) and 14 days (lane 4) after the immunization. Total RNAs were extracted from each cell fraction. Expression of IFN-γ, IL-4, IL-10, Foxp3 or GAPDH mRNAs was analyzed by RT-PCR.

alum-absorbed DNP-OVA. Splenic $CD4^+CD25^+$ T cells were isolated by using anti-CD25-coupled microbeads after the depletion of cells bound to anti-CD8, anti-CD11b, anti-B220, anti-CD49b or anti-Ter-119 mAb. Almost comparable numbers of $CD4^+CD25^+$ T cells were recovered from all mice despite the treatment with αGC-liposomes with different time periods after immunization (data not shown). Analysis for mRNA expression showed that IL-10, but neither IL-4 nor IFN-γ, mRNA expression was augmented on 7 and 14 days after immunization, but the expression level of Foxp3 mRNA of αGC-liposome-treated mice was almost comparable with that of non-treated mice (Figure 1C). These results suggested that αGalCer in liposome formulation, but not in aqueous solution, could prevent both $CD4^+$ helper T cell priming and the primary antibody responses initiated by the priming with alum-adsorbed antigen and IL-10-producing $CD4^+CD25^+$ T cells might be involved in these suppressive phenomena.

4.2. Antigen presenting cells in the regulation of IL-10 production by pretreatment with αGalCer-liposomes

We suspected that the opposite effects of αGC-liposomes versus aqueous αGalCer on the antigen-priming of naïve T cells might be due to the uptake of αGC-liposomes by non-DCs. To test this possibility, either aqueous αGalCer or αGC-liposomes was intraperitoneally injected into separate groups of BDF1 mice, and their spleens were obtained 24 hr after the injection. DCs were purified from the splenocytes by using the microbeads conjugated with anti-CD11c mAb. B cells and macrophages were then recovered successively from the residual cells using microbeads conjugated with anti-B220 mAb and anti-CD11b mAb, respectively. Aliquots of normal spleen cells as a source of iNKT cells were then co-cultured with the purified DCs, B cells or macrophages from the αGalCer-treated mice or αGC-liposome-treated mice, and the concentrations of IFN-γ, IL-4 and IL-10 in the culture supernatants were determined. As shown in Figure 2A, DCs from the αGC-liposome-treated mice induced the higher production of cytokines than those from the aqueous αGalCer-treated mice. When B cells from the αGC-liposome-treated mice were co-cultured with normal spleen cells, IL-10 became detectable in the culture supernatant within 24 hr, and the concentration of the cytokine markedly increased by 48 hr. The concentration of IL-10 in the culture was much higher than that produced by the co-culture of the same spleen cells with DCs. Since the formation of IL-10 by the co-culture of the B cells with normal spleen cells was prevented by the addition of anti-CD1d antibody, and culture supernatants of the B cells alone did not contain a detectable amount of IL-10 (results not shown), it appeared that presentation of CD1d-associated αGalCer on B cells to iNKT cells was responsible for the production of IL-10. In contrast, B cells of aqueous αGalCer-treated mice barely induced the formation of IL-10 (Figure 2A). These results suggested that the difference between B cells from the αGC-liposome-treated mice and those from the aqueous αGalCer-treated mice, with respect to the ability to induce IL-10 formation, was probably due to an enhancement of uptake of αGalCer in liposome formulation by B cells.

Since the splenic B cells comprise follicular and marginal zone B cells, we determined which subset is involved in the IL-10 formation. BDF1 spleen cells were obtained 24 hr after an intraperitoneal injection of αGC-liposomes. DCs in the splenocytes were depleted by using anti-CD11c-coupled microbeads. The rest of the splenocytes were then fractionated by histodenz gradient, and B cells in both the high density (HD) and low density (LD) fractions were recovered by using anti-B220-coupled microbeads. The HD-B cells and LD-B cells were then cultured with normal spleen cells as the source of iNKT cells, and cytokines in the supernatants were determined. As shown in Figure 2B, only LD-B cells induced the formation of IL-10, whereas neither IL-4 nor IFN-γ was detected in the culture supernatant. Absence of both IL-4 and IFN-γ indicates that the formation of IL-10 in this system was not due to contamination of the B cell fraction with DCs. Moreover, LD-B cells were further fractionated into $CD21^{high}$ $CD23^-$ cell, $CD21^{middle}$ $CD23^{high}$ cell or $CD21^{low}$ $CD23^-$ cell fraction by flow cytometry. Each fraction was cultured with normal spleen cells, from which B cells had been depleted by using anti-B220-coupled microbeads. IL-10 production was detected in the culture supernatants of CD23-negative cell fractions at substantially higher amounts than in that of CD23-positive cell fraction (Figure 2C). To determine the cell source of IL-10 in this system, LD-B cells were obtained from the spleen of αGC-liposome-treated mice by the method described above, and the cells were cultured with normal spleen cells, from which B cells had been depleted. After 48 hr culture, supernatants were collected to confirm the presence of IL-10, and the cells were fractionated into $B220^+$ cell and αGC/CD1d-Ig^+ cell fraction. Each cell fraction was analyzed for the presence of IL-10 mRNA by RT-PCR. As shown in Figure 2D, IL-10 mRNA was detected in $B220^+$ B cell fraction more than in αGC/CD1d-Ig^+ cell fraction which contains iNKT cells. Taken together, the results suggested that presentation of αGalCer on CD23-negative LD-B cells to iNKT cells induced the formation of IL-10.

4.3. Expansion of $CD11c^{low}CD45RB^{high}$ cell population *in vivo* by the treatment of αGalCer-liposomes

Since αGC-liposomes are captured by LD-B cells in the spleen, when it was injected intraperitoneally (cf. Figure 2), we determined as to whether the transfer of the LD-B cells pulsed with αGC-liposomes prior to the priming might induce the suppression of antibody responses. LD-B cells were obtained from the spleen of normal BDF1 mice, and incubated for 18 hr with αGC-liposomes. After washing, these cells or un-pulsed LD-B cells were transferred intravenously into normal BDF1 mice and then immunized with DNP-OVA in alum. As shown in Figure 3A, primary anti-DNP IgE, IgG1 and IgG2a antibody responses were completely suppressed by the transfer of αGC-liposome-pulsed LD-B cells, but not by that of LD-B cells alone.

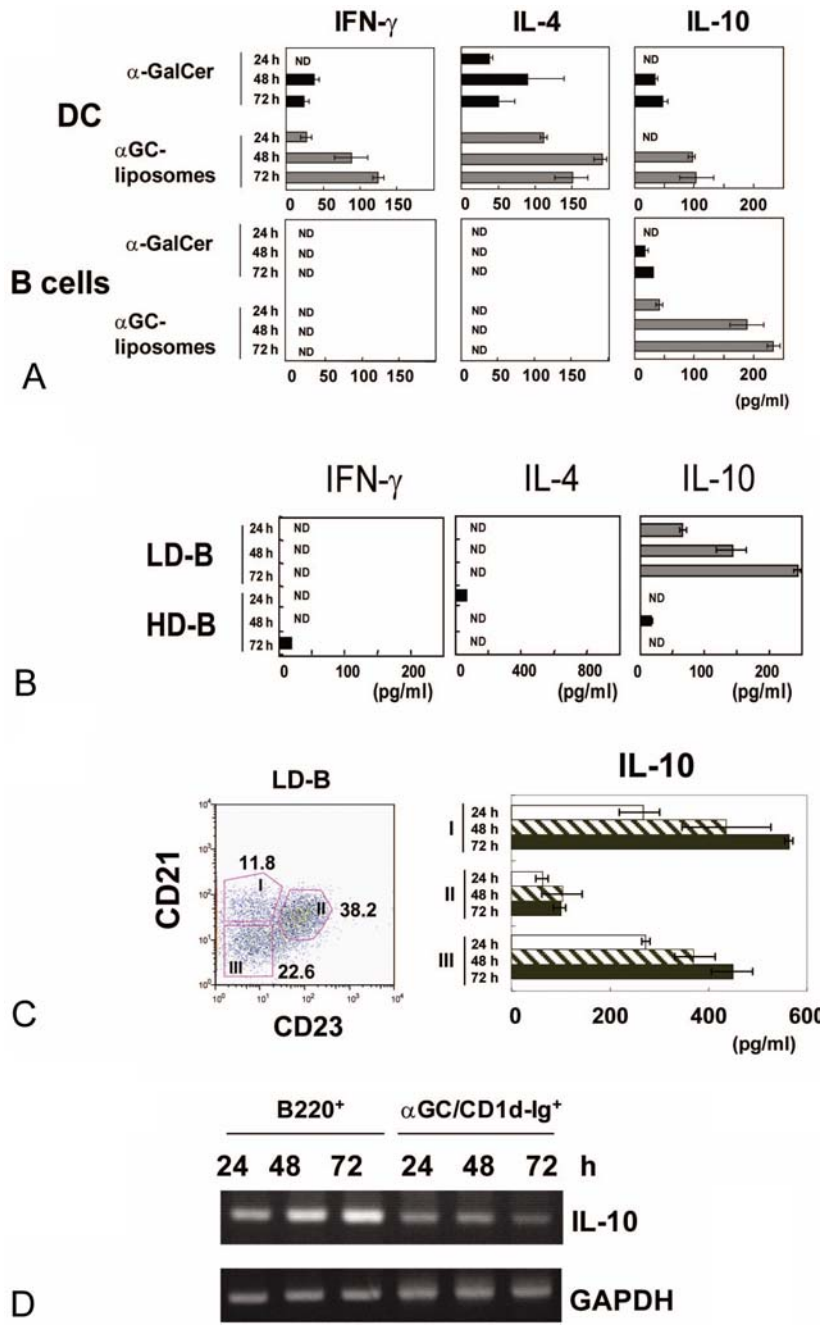

Figure 2. Uptake of αGC-liposomes by antigen-presentation cells. Splenocytes of BDF1 mice were obtained 24 h after an intraperitoneal injection of aqueous αGalCer (2 μg) or αGC-liposomes containing 2 μg α-GalCer. (A) Dendritic cells (DC) were purified from the splenocytes by using microbeads conjugated with anti-CD11c mAb. B cells were recovered from the residual cells using microbeads conjugated with anti-B220 mAb. Aliquots of normal spleen cells were then co-cultured with the purified DC or B cells. IFN-γ, IL-4 and IL-10 in the culture supernatants were determined by ELISA. ND; not detected. (B) Splenocytes of BDF1 mice were obtained 24 hr after an intraperitoneal injection of αGC-liposomes. After the depletion of $CD11c^+$ cells, the rest of the splenocytes were then fractionated by histodenz gradient, and B cells in the high density (HD) and low density (LD) fractions were recovered by using anti-B220-coupled microbeads. The HD-B cells and LD-B cells were then cultured with normal spleen cells depleted $B220^+$ cells for 24 hr. IFN-γ, IL-4 and IL-10 in the culture supernatants were determined by ELISA. (C) LD-B cells were stained with both FITC-labeled anti-CD21 and PE-labeled anti-CD23 mAbs, and sorted into $CD21^{high}$ $CD23^-$ cell (a), $CD21^{middle}$ $CD23^{high}$ cell (b) or $CD21^{low}$ $CD23^-$ cell (c) fraction by flow cytometry. Each fraction was co-cultured with normal spleen cells depleted $B220^+$ cells for 24, 48 and 72 hr. (D) The cultured cells in (C) were fractionated into B cell ($B220^+$) and iNKT cell (aGC/CD1d-Ig^+) fractions. Each cell fraction was analyzed for the presence of IL-10 mRNA by RT-PCR.

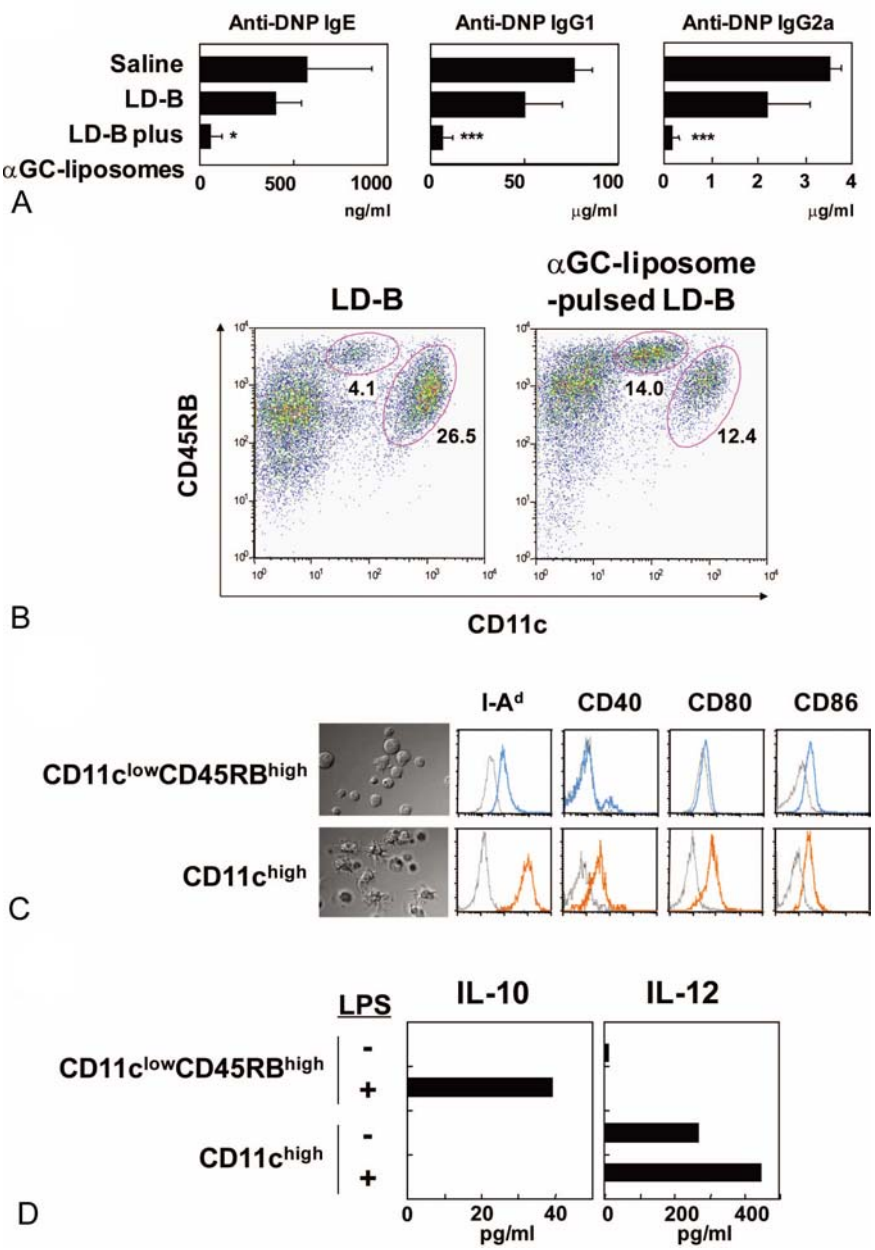

Figure 3. Preferential increase in $CD11c^{low}CD45RB^{high}$ cells in mouse spleen by the treatment with αGC-liposomes. (A) LD-B cells were obtained from the spleen of normal BDF1 mice, and incubated for 18 hr with 100 ng/ml of αGC-liposomes. After washing, these cells (6 X 10^5 cells), un-pulsed LD-B cells (6 X 10^5 cells) or saline were transferred intravenously into normal BDF1 mice (n=5) and immunized with alum-adsorbed DNP-OVA (0.1 μg). The concentration of anti-DNP Abs in their sera was determined by ELISA at day 14. * $p < 0.05$ and *** $p < 0.005$ for αGC-liposome-LD-B cell-transferred groups versus saline group. (B) αGC-liposome-pulsed LD-B cells (6 X 10^5 cells) or un-pulsed LD-B cells (6 X 10^5 cells) were transferred intravenously into normal BDF1 mice (n=5), of which splenocytes were obtained after three days. After depletion of erythrocytes, T cells, B cells, granulocytes, and macrophages, the reminder was stained with both APC-labeled anti-CD11c and PE-labeled anti-CD45RB mAbs, and the cells were analyzed by flow cytometry. Circles indicate the population of $CD11c^{low}CD45RB^{high}$ cells and $CD11c^{high}$ cells. The proportion (%) of the cells is shown in panel. (C) $CD11c^{low}CD45RB^{high}$ and $CD11c^{high}$ cells in the spleen of αGC-liposome-treated mice were sorted by flow cytometry, and examined by phase contrast microscopy. The sorted cells were stained with FITC-conjugated mAb to $I\text{-}A^d$, CD40, CD80 or CD86, and were analyzed by flow cytometry. (D) The sorted cells (2 x 10^5) were cultured in the Click's medium containing 10% FCS supplemented with 1 μg/ml LPS (+) or without LPS (-) in the presence of 10 ng/ml rmGM-CSF. After 24 hr, culture supernatants were recovered and assessed for IL-10 and IL-12 by ELISA.

Table 1. αGC-liposomes preferentially augment the absolute number of $CD11c^{low}CD45RB^{high}$ cells relative to $CD11c^{high}$ DCs in the spleens of BDF1 mice

Treatment	Total cells –Lineages[1]	$CD11c^{low}CD45RB^{high}$	$CD11c^{high}$
Transfer			
Saline	29.7	1.5 (4.9%)	5.6 (18.8 %)
LD-B[2]	43.1	1.8 (4.1 %)	11.4 (26.5 %)
LD-B pulsed with αGC-liposomes[3]	76.1	10.6 (14.0%)	9.4 (12.4 %)
Injection			
Saline	26.3	1.5 (5.5 %)	3.9 (14.9 %)
αGalCer	51.7	3.6 (6.9 %)	8.1 (15.7 %)
Liposomes	44.1	2.9 (6.5 %)	6.8 (15.3 %)
αGC-liposomes	121.6	30.8 (25.3 %)	4.5 (3.7 %)

Results represent the number of cells (x 10^{-5}) per mouse spleen. [1]Total cells-Lineages represent the numbers of enriched cells after depletion of CD3, CD19, CD49b, Gr-1, or TER-119-positive cells. [2]LD-B represents low-density B cells. [3]αGC-liposomes represent a liposome formulation α-galactocylceramide (αGalCer).

To clarify the suppression mechanism, we next analyzed splenic CD11c-positve cells three days after the transfer of αGC-liposome-pulsed LD-B cells. To enrich $CD11c^+$ DCs in the spleen cells, CD3, CD19, CD49b, Gr-1 or TER-119 marker positive cells were depleted by using the microbeads conjugated with specific mAbs. The results of flow cytometry of the DC-enriched fraction were shown in Figure 3B, and the average numbers of $CD11c^{low}CD45RB^{high}$ DCs and $CD11c^{high}CD45RB^{low}$ DCs recovered per spleen of the recipients were included in Table 1. The results clearly showed that the transfer of αGalCer-liposome-pulsed LD-B cells induced a selective increase in $CD11c^{low}CD45RB^{high}$ cells in the spleen of the recipients.

Since the $CD11c^{low}B220^+$ $Gr\text{-}1^+$ plasmacytoid DCs should have been depleted from the spleen cells in the process of the enrichment of $CD11c^+$ cells, we wondered whether the $CD11c^{low}$ cells might represent $CD45RB^{high}$ tolerogenic DCs. To confirm whether the expansion of $CD11c^{low}CD45RB^{high}$ cells depends on the treatment of αGC-liposomes, BDF1, BALB/c and Vα14NKT-deficient BALB/c-background mice received an intraperitoneal injection of αGC-liposomes, αGalCer-OVA-liposomes, or the same dose of aqueous αGalCer, and their splenocytes were recovered 3 days later. The proportion of $CD11c^{low}CD45RB^{high}$ cells in the total DCs in the BDF1 and BALB/c splenocytes increased by the treatment with αGC-liposomes or αGC-OVA-liposomes. In the Vα14 NKT cells-deficient mice, however, the proportion of the $CD11c^{low}$ cells did not change by the treatment, indicating that Vα14 NKT cells were indispensable for the expansion of the $CD11c^{low}CD45RB^{high}$ cell population (40). The numbers and the proportion of $CD11c^{low}CD45RB^{high}$ cells and $CD11c^{high}CD45RB^{low}$cells recovered from the BDF1 spleen cells were calculated. The results summarized in Table 1 indicated that the treatment with αGC-liposomes induced a marked increase in $CD11c^{low}CD45RB^{high}$ cells without affecting the number of $CD11c^{high}CD45RB^{low}$ cells, whereas the treatment with aqueous αGalCer rather induced a significant increase in $CD11c^{high}$ DCs.

Characteristics of the DCs developed by the αGC-liposome-treatment were determined. The $CD11c^{low}CD45RB^{high}$ cells and $CD11c^{high}CD45RB^{low}$ cells were recovered by cell sorting from the splenocytes of BDF1 mice 3 days after the injection of αGC-liposomes. Microscopic examination revealed that the $CD11c^{low}CD45RB^{high}$ cells had plasmacytoid-like morphology, while the $CD11c^{high}CD45RB^{low}$ cells were typical dendritic cells (Figure 3C). Analysis of the cell surface molecules of the sorted cells by flow cytometry showed that the expression level of $I\text{-}A^d$, CD40 or CD80 on the $CD11c^{low}CD45RB^{high}$ cells were significantly lower than those on the $CD11c^{high}CD45RB^{low}$ cells, while the expression of CD86 was comparable in the two populations (Figure 3C). Upon stimulation with LPS, the $CD11c^{low}CD45RB^{high}$ population produced IL-10 but no IL-12, while the $CD11c^{high}CD45RB^{low}$ population secreted IL-12 but no IL-10 (Figure 3D). The results collectively indicated that the DC population of the $CD11c^{low}CD45RB^{high}$ phenotype might represent tolerogenic DCs described by Wakkach *et al.* (15), and that this population expanded *in vivo* by the injection of αGC-liposomes, but not aqueous αGalCer.

4.4. Differentiation of Treg cells *in vitro* by antigen-pulsed $CD11c^{low}CD45RB^{high}$ cells

Since the injection of αGC-liposomes promoted the development of $CD11c^{low}CD45RB^{high}$ tolerogenic DCs in the spleen, the next experiments were carried out to confirm that the cells developed by the liposome-treatment facilitate the differentiation of Treg cells. BALB/c mice received an intraperitoneal injection of αGC-liposomes, and the $CD11c^{low}CD45RB^{high}$ cells and $CD11c^{high}CD45RB^{low}$ DCs in their spleen were purified by cell sorting following the procedures described in the previous section (cf. Figure 3C). Naïve $CD4^+$ T cells were purified from the spleen cells of RAG2-/-DO11.10 mice, and the T cells were cultured either with the $CD11c^{low}CD45RB^{high}$ cells or with $CD11c^{high}CD45RB^{low}$ DCs in the presence of $OVA_{323\text{-}339}$ peptide. After 7 days culture, $CD4^+$ T cells were recovered, and stimulated again for 7 days with the peptide in the presence of freshly isolated DCs from the αGC-liposome-treated mice. T cells recovered after three cycles of the culture were stained with anti-CD4 and anti-CD25 mAbs on their cell surface, and then with anti-Foxp3 mAb intracellularly. Fow cytometric analysis showed that $CD4^+CD25^+$ T cells co-cultured with $CD11c^{low}CD45RB^{high}$ cells expressed Foxp3 protein much

more than those with $CD11c^{high}CD45RB^{low}$DCs (Figure 4A). Possible expression of Foxp3 mRNA was also studied. The total RNA extracted from aliquots of the $CD4^+$ T cells were analyzed by RT-PCR method using primers for Foxp3, IL-21 or G3PDH. As shown in Figure 4B, Foxp3 mRNA was detected in the $CD4^+$ T cells cultured with $CD11c^{low}CD45RB^{high}$ cells, but not in the $CD4^+$ T cells cultured with conventional mature $CD11c^{high}CD45RB^{low}$DCs, despite both $CD4^+$ T cells expressed the comparable level of IL-21 mRNA which is induced in the activated $CD4^+$ T cells (16). As shown in Figure 4C, the majority of the $CD4^+$ cells proliferated *in vitro* in the presence of tolerogenic DCs expressed CD25, CD28 and ICOS, but not CD152 (CTLA-4). It was also found that more than 50% of the $CD4^+$ T cells possessed intracellular IL-10 or both IL10 and IFN-γ (Figure 4D). After the transfer of the $CD4^+$ T cells cultured with $CD11c^{low}CD45RB^{high}$ cells into normal BALB/c mice prior to the priming with DNP-OVA in alum, antibody responses were significantly suppressed with the transfer of 2×10^6 cells (Figure 4E). The results collectively indicated that $CD11c^{low}CD45RB^{high}$ cells were capable of developing the regulatory $Foxp3^+CD4^+CD25^+$ T cells producing IL-10 alone or both IL-10 and IFN-γ.

4.5. Differentiation of Treg cells *in vivo* by administration of αGalCer-liposomes encapsulated an antigen

Development of Treg-like cells from the RAG2-/-DO11.10-naïve $CD4^+$ T cells by the repeated stimulation with antigen (epitope)-pulsed $CD11c^{low}CD45RB^{high}$ cells from the αGC-liposome-treated mice suggested the possibility that injections of αGC-OVA-liposomes might induce the development of antigen-specific Treg cells *in vivo*. The next experiment was undertaken to prove the presence of the antigen-specific Treg cells. Three groups of BDF1 mice received three weekly injections of αGalCer-liposome or αGalCer-OVA-liposome. Their spleen cells were obtained 7 days after the last injection of the liposome, and the $CD4^+$ T cells were recovered. Ten million $CD4^+$ T cells from each group or normal $CD4^+$ T cells were intravenously transferred into the BDF1 mice, which had been immunized with alum-absorbed DNP-OVA and boosted with DNP-OVA. As shown in Figure 5A, the $CD4^+$ T cells from the αGC-OVA-liposome-treated mice significantly suppressed not only the IgE anti-DNP antibody response after the second boosting but also the on-going antibody formations and the antibody responses of both IgG1 and IgG2a isotypes in the recipients, while the $CD4^+$ T cells from the αGC-liposome-treated mice failed to do so.

The rest of the $CD4^+$ T cells were cultured for 6 days with OVA-pulsed irradiated splenocytes, and were analyzed for intracellular Foxp3, IL-10 and IFN-γ. Most of $CD4^+$ $CD25^{high}$ cells from mice treated with αGC–liposomes expressed high level of Foxp3 but not IL-10 or IFN-γ (a in Figure 5B). In contrast, the treatment with αGC-OVA-liposomes induced the development of $CD4^+CD25^{high}$ cells, of which the majority expressed the low level of Foxp3 protein and both IL-10 and IFN-γ (b in Figure 5B). In the spleen of non-treated mice, $CD4^+CD25^{medium}$ cells which expressed high level of Foxp3, but no cytokines, were only detected (c in Figure 5B). The results suggested that $CD4^+$ T cells producing both IL-10 and IFN-γ might represent inducible Treg cells generated by the treatment with αGC-OVA-liposomes.

4.6. Antigen-specific suppression by the treatment with αGC-OVA-liposomes

BDF1 mice were immunized with alum-absorbed DNP-OVA and, after detection of the primary anti-DNP IgE antibody responses at 2 weeks, either αGC-liposomes or αGC-OVA-liposomes was injected intraperitoneally into the DNP-OVA-primed mice at day 21, 28 and 35. Seven days after the last injection of the liposomes, booster immunization of DNP-OVA was given to all groups, and the secondary anti-DNP-antibody response was assessed at 7 days after the booster. As shown in Figure 6, anti-DNP antibody response of all Ig isotypes examined was markedly suppressed by the treatment with αGC-OVA-liposomes. The treatment with αGC-liposomes slightly suppressed both the IgG1 and IgG2a antibody responses, but failed to affect the IgE antibody response. To determine the carrier-specific nature of the suppression by the treatment with αGC-OVA-liposomes, separate groups of BDF1 mice were immunized with alum-absorbed DNP-KLH; one group was treated by three intraperitoneal injections of αGC-OVA-liposomes, and another group was treated with αGC-liposomes. The results of the secondary antibody responses to DNP-KLH, given at 7 days after the liposome treatment, indicated that the treatment with αGC-liposomes slightly suppressed the secondary IgG1 and IgG2a antibody responses, but encapsulation of OVA in the liposomes did not enhance the suppressive effect. Neither αGC-liposomes nor αGC-OVA-liposomes suppressed the secondary IgE antibody response to DNP-KLH (Figure 6).

5. DISCUSSION

Data presented in this study clearly showed that pretreatment of BDF1 mice with αGC-liposomes suppressed the T cell priming, the primary antibody response to the antigen used for subsequent immunization, and the development of IL-10-producing $CD4^+CD25^+$ T cells, while the pretreatment of the mice with the same dose of aqueous αGalCer rather enhanced the T cell priming (cf. Figure 1). The difference between the effects of αGC-liposomes and those of aqueous αGalCer on the immune response is probably ascribed to the difference in the antigen-presenting cells involved. It is well known that presentation of αGalCer by DCs to iNKT cells induces the production of large amounts of IFN-γ and IL-4 (17) , and that the production of IFN-γ by the NKT cells in this system requires the formation of IL-12 by the DCs (18, 19) . Since aqueous α-GalCer was presented by DCs, but not B cells (cf Figure 2A), naïve $CD4^+$ T cells were preferentially differentiated into helper T cells by virtue of cytokine productions from activated iNKT cells and DCs. As expected from previous reports on liposomes (20), formulation of αGalCer in liposome appears to enhance the uptake of αGalCer by DCs, and resulted in an increase in the formation of the cytokines by iNKT cells (Figure 2A).

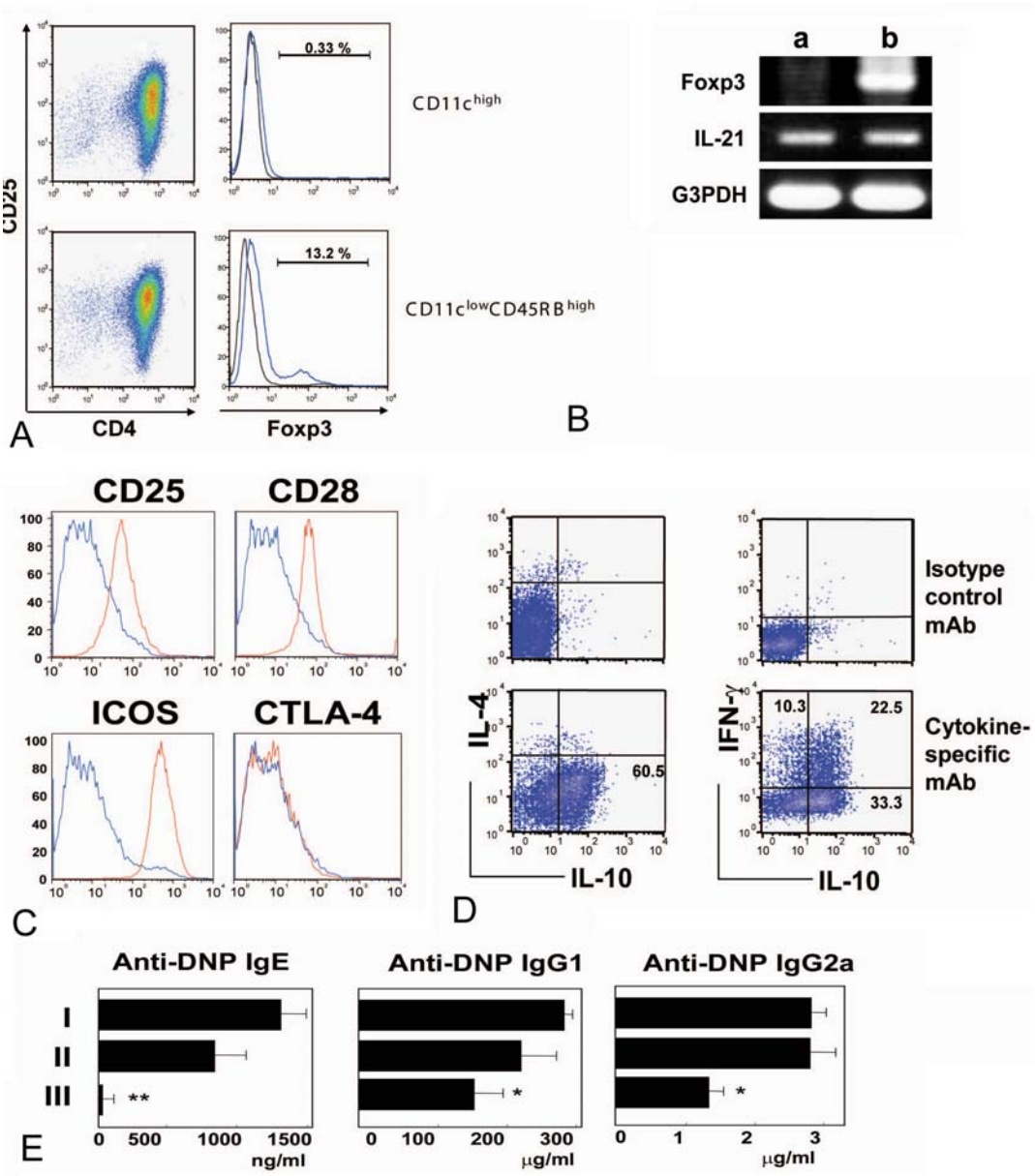

Figure 4. Differentiation of antigen-specific IL-10-producing T cells *in vitro* by antigen-pulsed CD11c^{low}CD45RB^{high} cells. (A) CD4$^+$ T cells from RAG2-/-DO11.10-mice were stimulated three rounds with CD11c^{high} conventional DCs or CD11c^{low}CD45RB^{high} cells in the presence of $OVA_{323-339}$ peptide OVA peptide (600 nM). CD4$^+$ cells were enriched from the cultured cells, and stained with FITC-conjugated CD4 mAb and APC-conjugated CD25 mAb. The cells were permeabilized, and then stained with PE-conjugated anti-Foxp3 mAb. Results of cytometric analysis are shown. (B) Total RNA of CD4$^+$ T cells cultured with CD11c^{high} conventional DCs (lane a) or CD11c^{low}CD45RB^{high} cells (lane b) in the presence of the OVA peptide was analyzed by RT-PCR method using primers for Foxp3, IL-21 or G3PDH. (C) The CD4$^+$ T cells co-cultured with CD11c^{low}CD45RB^{high} cells in the presence of the OVA peptide were stained with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD25, anti-CD28, or anti-ICOS. Aliquots were carried out intracellular staining with anti-CTLA-4 mAb. Histograms show FITC-positive cells among CD4$^+$ cells. (D) The cells were stained with biotinylated anti-CD4 mAb and streptavidin-conjugated PerCP Cy5.5. After the stimulation with both PMA and ionomycin for 4 h, intracellular cytokines were stained by using FITC-anti-IFN-γ, PE-labeled anti-IL-4 and APC-labeled anti-IL-10 mAbs (Cytokine-specific mAb), or FITC-IgG1 (rat), PE-IgG1 (rat) and APC-IgG2b (rat) mAbs (Isotype control mAb). Figure shows intracellular staining of CD4$^+$ cells. (E) BALB/c mice received an intravenous transfer of naïve CD4$^+$ T cells (2 X 10^6 cells; I) or the cultured CD4$^+$ T cells with CD11c^{low}CD45RB^{high} cells (4 X 10^5 cells; II, 2 X 10^6 cells; III) and then immunized with alum-adsorbed DNP-OVA (10 μg). The concentration of anti-DNP Abs in their sera was determined by ELISA at day 14. * $p < 0.05$ and ** $p < 0.001$ for the cultured CD4$^+$ T cell-transferred group versus naïve CD4$^+$ T cell-transferred group.

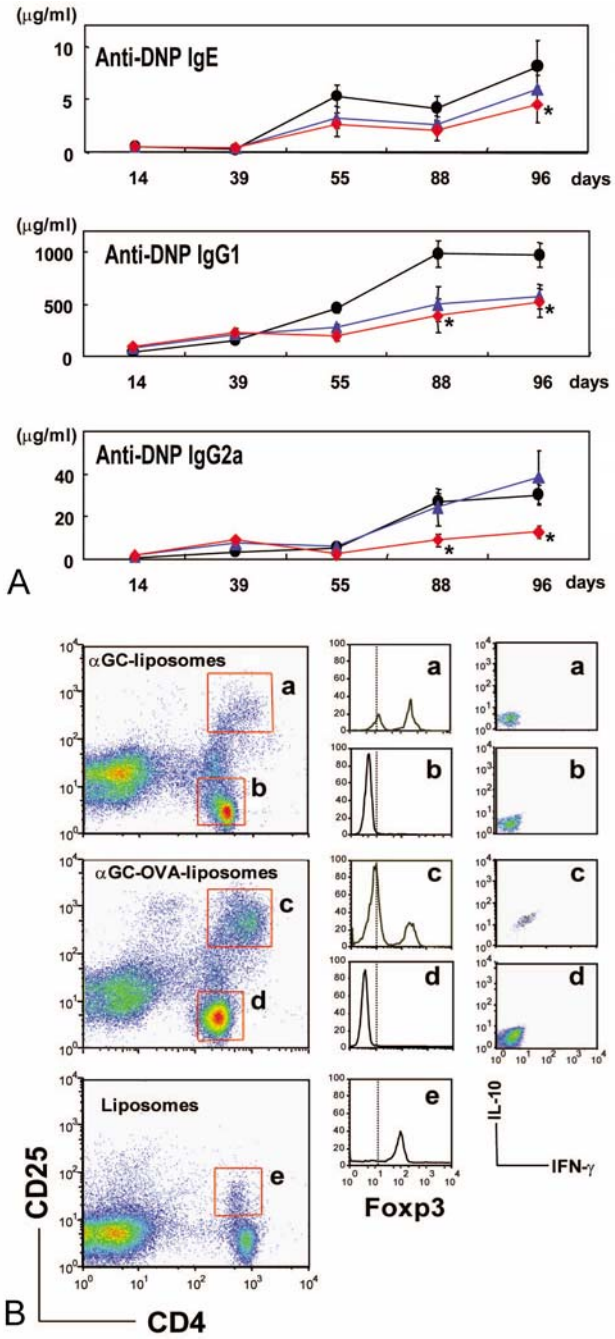

Figure 5. Generation of antigen-specific Treg *in vivo* by repeated injections of αGC-OVA-liposomes. (A) The $CD4^+$ T cells (1 X 10^7 cells/mouse) from the mice treated with αGC-OVA-liposome (□) or those of mice treated with αGC-liposome (▲), or $CD4^+$ T cells of untreated mice (●) were intravenously transferred into the treated BDF1 mice (n=5) at day 42, which had been immunized with alum-absorbed DNP-OVA at day 0 and boosted with DNP-OVA at day 39. The mice were then boosted with DNP-OVA at day 88. Anti-DNP Abs were measured by ELISA at day 0, 14, 39, 55, 88 and 96. * $p < 0.05$ αGC-OVA-liposome group versus saline group. (B) The $CD4^+$ T cells were cultured with irradiated syngenic splenocytes in the presence of 100 μg/ml OVA for 6 days, and were stained with biotinylated anti-CD4 mAb plus streptavidin-conjugated PerCP Cy5.5 and PE-conjugated anti-CD25 mAbs. The cells were permeabilized, stained with PE-conjugated anti-Foxp3 mAb, or with FITC-conjugated anti-IFN-γ and APC-conjugated anti-IL-10 mAbs, and then analyzed by flow cytometry. Expression of Foxp3 and cytokine production were analyzed in each of the squared cell populations.

However, the present experiments also showed that αGC-liposomes, injected intraperitoneally, was captured not only by splenic DCs but also by LD-B cells, and that the presentation of αGalCer by the B cells to iNKT cells resulted in the formation of IL-10 (Figure 2B). Since marginal zone B cells, which are included in the LD-B cell fraction, express high levels of CD1d and CD21 (21), one may expect that αGalCer associated with CD1d molecules on the B cells will be presented to iNKT cells. Indeed, co-culture of sub-cellular fractions in LD-B cells from the αGC-liposome-treated mice with normal spleen cells resulted in the formation of IL-10 mainly from $CD21^{high}$ $CD23^{-}$ cell and $CD21^{low}$ $CD23^{-}$ cell fractions, but not from $CD21^{middle}$ $CD23^{high}$ cell fraction which includes follicular B cells (Figure 2C). It is known that marginal zone B cells produce IL-10 upon bacterial stimulation (22). The present experiments actually showed that IL-10 was derived from LD-B cells rather than iNKT cells, when LD B cells presented αGalCer to NKT cells (Figure 2E). In view of the finding by Colgan *et al* (23) that ligation of intestinal epitherial CD1d induced the formation of IL-10, we suspect that ligation of CD1d-associated αGalCer on the B cells by invariant Vα14 TCR induced the formation of IL-10 by the B cells.

It is well known that blood-borne particulate antigen is transported to marginal zone of the spleen, where DCs, marginal zone B cells and macrophages exist (24, 25). Fujii *et al.* (26) have shown that intravenously injected αGalCer was captured by splenic DCs within 1 to 4 hrs after the injection, and that adoptive transfer of the splenic DCs into naïve mice induced selective activation of iNKT cells and maturation of DCs in the recipients. However, pretreatment of the recipient mice with aqueous αGalCer prior to the adoptive transfer of the αGalCer-pulsed DCs rendered iNKT cells unresponsive to the DCs, suggesting that presentation of αGalCer to iNKT cells by non-DCs inhibited the response of IFN-γ producing iNKT cells to αGalCer-bearing DCs (27). Direct evidence for the hypothesis was obtained by using DC-depleted mice. Bezbradica *et al* (28) demonstrated that B cell-iNKT cell interactions *in vivo* appear to suppress DC-mediated αGalCer-induced activation of NKT cells and DC maturation. We suspect that immunoregulatory effects of αGC-liposomes are due to uptake of the liposomes by $CD1d^{high}$ B cells, such as marginal zone B cells.

Exact mechanisms for the B cell-mediated regulation of DC maturation are unknown. It was suggested, however, that B cells interact with DCs, and lower the level of IL-12 released by the DCs (29). Since IL-10 inhibits the production of IL-12 by DCs (30), one might speculate that B cell-derived IL-10 is involved in the regulatory function of B cells. Indeed, production level of IL-12 from splenic $CD11c^{+}$ DCs treated with αGC-liposomes was much lower than that with aqueous αGalCer (data not shown). When both DCs and marginal zone B cells captured αGC-liposomes and presented αGalCer to iNKT cells, formation of IL-10 by the B cells preceded the formation of IFN-γ and IL-4 by NKT cells (Figure 2A). Absence of IFN-γ and IL-4 in the culture supernatant of LD-B cells suggests that iNKT cells were not activated in the culture, probably due to failure of marginal zone B cells to produce IL-12. We speculate that early release of IL-10 in the marginal zone may affect maturation of DCs in the environment. Such cellular mechanisms of immune suppression by αGC-liposomes may be shared by the mechanism of immune tolerance in ACAID, in which antigen injected into anterior chamber is transferred exclusively through blood stream to the marginal zone in the spleen (31). Accumulated evidence indicated that both iNKT cells (8) and marginal zone B cells (32, 33) are required for the induction of immune tolerance by the injection of protein antigen into anterior chamber.

The present experiments have shown that both an adoptive transfer of αGC-liposome-pulsed LD-B cells and an injection of αGC-liposomes could induce the development of $CD11c^{low}CD45RB^{high}$ DCs in the spleen (Figure 3A & 3B). Yield of splenic DCs from the αGalCer-treated mice suggested that the treatment with aqueous αGalCer increased the number of $CD11c^{high}$ DCs in the spleen, whereas the treatment with αGC-liposome induced the selective increase in $CD11c^{low}CD45RB^{high}$ cells (cf. table 1). These cells were similar to the tolerogenic DCs described by Wakkach *et al* (15), in terms of plasmacytoid morphology, cell surface markers, low expression levels of co-stimulatory molecules, such as CD80, CD40 and I-A, and production of IL-10, rather than IL-12, upon stimulation with LPS (Figure 3C). In view of their findings that the $CD11c^{low}CD45RB^{high}$ tolerogenic DCs could be obtained by culture of bone marrow cells with GM-CSF in the presence of IL-10, and that the proportion of this subset in all DCs was significantly high in the spleen of IL-10 transgenic mice, one may suspect that IL-10 released from the marginal zone B cells and iNKT cells in the spleen of the αGalCer-liposome-treated mice promoted the development of the tolerogenic DCs. However, marked increase in the number of $CD11c^{low}CD45RB^{high}$ tolerogenic DCs in their spleens may not be explained by IL-10 alone. Svensson *et al* (34) reported that spleen-derived stroma cells promoted selective development of $CD11c^{low}CD45RB^{high}$ tolerogenic DCs from progenitor cells, and that the stroma cells from the mice injected with Leishmania donovani were more effective than normal stroma cells for directing the development of the tolerogenic DCs. In the course of the present experiments for the enrichment of DCs in the spleen, we realized that the number of non-lineage cells, which include both DCs and stroma cells, markedly increased after an injection of αGalCer-liposome, and that the increase of the non-lineage cells could not be explained by the increase of $CD11c^{low}CD45RB^{high}$ tolerogenic DCs alone (cf. Table I). One might speculate that the liposome either increase or activate the stroma cells through unknown mechanisms, such as active TGF-β1 production, and promoted the differentiation of progenitor cells towards tolerogenic DCs. Further studies are required for elucidation of the mechanisms for the selective increase in the population of the tolerogenic DCs by an injection of αGC-liposomes. Nevertheless, the findings that transfer of αGC-liposome-pulsed LD-B cells into normal mice induced the development of tolerogenic DCs in the spleen

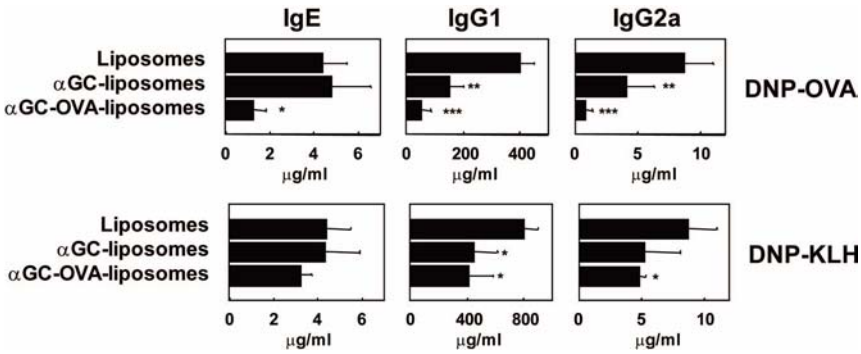

Figure 6. Carrier-specific suppression of the secondary antibody responses by administration of αGC-OVA-liposomes. Three groups of BDF1 mice (n=5) were immunized with alum-adsorbed DNP-OVA (0.1 μg) or alum-adsorbed DNP-KLH (1 μg) at day 0. They received intraperitoneal injections of "Liposome", "αGC-liposome" or "αGC-OVA-liposome" at day 21, 28 and 35, and then boosted with either DNP-OVA (0.1 μg) or DNP-KLH (1 μg) at day 42. The concentrations of anti-DNP IgE, IgG1 and IgG2a antibodies in their sera at day 49 were measured by ELISA. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.001$ for the αGC-liposome or αGC-OVA-liposome group versus liposome group.

of the recipients (cf. Figure 3A) indicate that the presentation of αGalCer by marginal zone B cells to iNKT cells was responsible for the generation of $CD11c^{low}CD45RB^{high}$ DCs in the spleen.

Previous studies on the tolerogenic DCs have shown that antigen-pulsed tolerogenic DCs facilitate the development of IL-10-producing Treg (IL-10 Treg or Tr1) cells *in vitro*, and antigen-specific tolerance *in vivo* (15, 34, 35). The present experiments actually showed that stimulation of naïve $CD4^+$ T cells from RAG2-deficient DO11.10 mice with antigen-pulsed $CD11c^{low}CD45RB^{high}$ tolerogenic DCs from the αGC-liposome-treated mice induced the differentiation of naïve T cells to $CD4^+CD25^+$ Treg cells, which produce IL-10 upon antigenic stimulation (Figure 4D). The Treg cells driven by the injections of αGalCer-OVA-liposomes do not express CTLA-4 (Figure 4C), which is consistently expressed on naturally occurring $CD4^+CD25^+$ Treg (nTreg) cells, however, they expressed Foxp3 (Figure 4A & B), which was believed to be specifically expressed in nTreg cells (36, 37). Expression of Foxp3 in Tr1 cells is controversial. Typical IL-10 producing Tr1 cells, which were developed by stimulating naïve DO11.10-$CD4^+$ T cells with anti-CD3 in the presence of vitamin D3 and dexamethasone, were $CD4^+CD25^+$ cells, but did not express Foxp 3 (38). However, the antigen-specific Tr1-like cells induced by neuropeptide-generated $CD11c^{low}CD45RB^{high}$ DCs expressed Foxp 3 (35). Indeed, adoptive or inducible Treg cells developed under different experimental conditions appear to be quite heterogeneous. Previous publications indicated that both the Treg cells induced by the pulmonary dendritic cells from the mice exposed to respiratory allergen (Th2-like Treg) (39) and those induced by CD8α DCs of the mice immunized with antigen and heat-killed Listeria monocytogenis (Th1-like Treg) (7), expressed Foxp3. Indeed, the Treg cells obtained in the present experiments by the injections of αGC-OVA-liposomes share common properties with the Th1-like Treg, with respect to the expression of CD25, ICOS and Foxp3, and the production of both IL-10 and IFN-γ upon stimulation with antigen-pulsed antigen presenting cells (Figure 4). It appears that the Treg cells induced by the treatment with αGC-OVA-liposomes represent one type of inducible Treg (iTreg) cells. The same $CD4^+$ Treg cells producing both IL-10 and IFN-γ were generated *in vivo* by repeated injections of αGC-OVA-liposomes, and transfer of $CD4^+$ T cells from the αGC-OVA-liposome-treated mice suppressed the secondary antibody responses of the recipients to DNP-OVA (Figure 5A & B). Since the repeated injections of αGC-OVA-liposomes to DNP-OVA-primed mice suppressed their secondary antibody responses to DNP-OVA in antigen (carrier)-specific manner (Figure 6), we believe that generation of the antigen-specific iTreg cells was responsible for the suppression of the secondary antibody response. In the recent studies, we clarified that the $CD11c^{low}CD45RB^{high}$ cell population was at least consisted of two populations, of which one is a NK-phenotype of CD49b-positive cells expressing both IL-10 and TGF-β1 but not MHC class II, and the other is an immature DC phenotype of CD180-positive cells expressing both TGF-β1 and MHC class II but not IL-10 (40). The result may suggest that both the $CD49b^+$ cells and $CD180^+$ cells in the $CD11c^{low}CD45RB^{high}$ cell population expanded by a single injection of αGC-liposomes cooperate in the generation of iTreg cells.

Recent clinical studies on allergy called much attention to possible role of iTreg cells in prevention of allergic diseases. Ling *et al* (41) have shown that $CD4^+CD25^-$ cells from the majority of non-atopic donors gave proliferative response and IL-5 production upon stimulation with allergen-pulsed antigen presenting cells, and that the responses were prevented by the addition of autologous $CD4^+CD25^+$ cells. In the active allergic pollinosis patients, however, the inhibitory effect of the autologous $CD4^+CD25^+$ cells was substantially less than that observed in non-atopic individuals. Subsequent studies by Akdis *et al* (42) identified allergen-specific Th1, Th2 and Tr1 $CD4^+$ cells, which produce IFN-γ, IL-4 and IL-10, respectively, upon antigenic stimulation in the peripheral blood of both atopic and healthy individuals, and found that Tr1 cells are dominant subset in healthy individuals. They

proposed the hypothesis that balance between allergen-specific Th2 and Tr1 cells would determine the development of healthy or allergic immune response. These findings support our expectations that αGC-liposomes containing an appropriate allergen would be a useful tool for the immunotherapy of allergic patients.

6. ACKNOWLEDGEMENTS

The authors thank Kimishige Ishizaka for helpful discussions and critical reading of the manuscript.

7. REFERENCES

1. T. L. Stevens, A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann and E. S. Vitetta: Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature*, 334 (6179), 255-258 (1988)

2. H. J. Cho, K. Takabayashi, P. M. Cheng, M. D. Nguyen, M. Corr, S. Tuck and E. Raz: Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat Biotechnol*, 18 (5), 509-14 (2000)

3. H. Tighe, K. Takabayashi, D. Schwartz, G. Van Nest, S. Tuck, J. J. Eiden, A. Kagey-Sobotka, P. S. Creticos, L. M. Lichtenstein, H. L. Spiegelberg and E. Raz: Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol*, 106 (1 Pt 1), 124-34 (2000)

4. J. D. Marshall, S. Abtahi, J. J. Eiden, S. Tuck, R. Milley, F. Haycock, M. J. Reid, A. Kagey-Sobotka, P. S. Creticos, L. M. Lichtenstein and G. Van Nest: Immunostimulatory sequence DNA linked to the Amb a 1 allergen promotes T (H)1 cytokine expression while downregulating T (H)2 cytokine expression in PBMCs from human patients with ragweed allergy. *J Allergy Clin Immunol*, 108 (2), 191-197 (2001)

5. K. Takatsu and K. Ishizaka: Reaginic antibody formation in the mouse. VII. Induction of suppressor T cells for IgE and IgG antibody responses. *J Immunol,* 116(5), 1257-1264(1976)

6. W. Y. Lee, A. H. Sehon and E. Akerblom: Suppression of reaginic antibodies with modified allergens. IV. Induction of suppressor T cells by conjugates of polyethylene glycol (PEG) and monomethoxy PEG with ovalbumin. *Int Arch Allergy Appl Immunol,* 64(1),100-114 (1981)

7. P. Stock, O. Akbari, G. Berry, G. J. Freeman, R. H. Dekruyff and D. T. Umetsu: Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immunol*, 5 (11), 1149-56 (2004)

8. F. Cottrez, S. D. Hurst, R. L. Coffman and H. Groux: T regulatory cells 1 inhibit a Th2-specific response *in vivo*. *J Immunol*, 165 (9), 4848-4853 (2000)

9. K. H. Sonoda, M. Exley, S. Snapper, S. P. Balk and J. Stein-Streilein: CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune-privileged site. *J Exp Med*, 190 (9), 1215-1226 (1999)

10. K. H. Sonoda, D. E. Faunce, M. Taniguchi, M. Exley, S. Balk and J. Stein-Streilein: NK T cell-derived IL-10 is essential for the differentiation of antigen-specific T regulatory cells in systemic tolerance. *J Immunol,* 166 (1), 42-50 (2001)

11. J. Cui, N. Watanabe, T. Kawano, M. Yamashita, T. Kamata, C. Shimizu, M. Kimura, E. Shimizu, J. Koike, H. Koseki, Y. Tanaka, M. Taniguchi and T. Nakayama: Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated Valpha14 natural killer T cells. *J Exp Med*, 190 (6), 783-792 (1999)

12. F. T. Liu, J. W. Bohn, E. L. Ferry, H. Yamamoto, C. A. Molinaro, L. A. Sherman, N. R. Klinman and D. H. Katz: Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. *J Immunol*, 124 (6), 2728-2737 (1980)

13. T. Tomura, H. Watarai, N. Honma, M. Sato, A. Iwamatsu, Y. Kato, R. Kuroki, T. Nakano, T. Mikayama and K. Ishizaka: Immunosuppressive activities of recombinant glycosylation-inhibiting factor mutants. *J Immunol*, 162 (1), 195-202 (1999)

14. T. Yasumi, K. Katamura, T. Yoshioka, T. A. Meguro, R. Nishikomori, T. Heike, M. Inobe, S. Kon, T. Uede and T. Nakahata: Differential requirement for the CD40-CD154 costimulatory pathway during Th cell priming by CD8 alpha+ and CD8 alpha- murine dendritic cell subsets. *J Immunol*, 172 (8), 4826-33 (2004)

15. A. Wakkach, N. Fournier, V. Brun, J. P. Breittmayer, F. Cottrez and H. Groux: Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation *in vivo*. *Immunity*, 18 (5), 605-617 (2003)

16. J. Parrish-Novak, S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Madden, W. Xu, J. West, S. Schrader, S. Burkhead, M. Heipel, C. Brandt, J. L. Kuijper, J. Kramer, D. Conklin, S. R. Presnell, J. Berry, F. Shiota, S. Bort, K. Hambly, S. Mudri, C. Clegg, M. Moore, F. J. Grant, C. Lofton-Day, T. Gilbert, F. Raymond, A. Ching, L. Yao, D. Smith, P. Webster, T. Whitmore, M. Maurer, K. Kaushansky, R. D. Holly and D. Foster: Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function, *Nature* 408 (6808), 57-63 (2000)

17. N. Burdin, L. Brossay, Y. Koezuka, S. T. Smiley, M. J. Grusby, M. Gui, M. Taniguchi, K. Hayakawa and M. Kronenberg: Selective ability of mouse CD1 to present glycolipids: alpha-galactosylceramide specifically stimulates V alpha 14+ NK T lymphocytes. *J Immunol*, 161 (7), 3271-3281 (1998)

18. H. Kitamura, K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, T. Kawano, M. Taniguchi and T. Nishimura: The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med*, 189 (7), 1121-1128 (1999)

19. M. Brigl, L. Bry, S. C. Kent, J. E. Gumperz and M. B. Brenner: Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol*, 4 (12), 1230-7 (2003)

20. I. Gursel, M. Gursel, K. J. Ishii and D. M. Klinman: Sterically stabilized cationic liposomes improve the uptake

and immunostimulatory activity of CpG oligonucleotides. *J Immunol*, 167 (6), 3324-3328 (2001)
21. J. H. Roark, S. H. Park, J. Jayawardena, U. Kavita, M. Shannon and A. Bendelac: CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J Immunol*, 160 (7), 3121-3127 (1998)
22. F. Burke, A. J. Stagg, P. A. Bedford, N. English and S. C. Knight: IL-10-producing B220+CD11c- APC in mouse spleen. *J Immunol*, 173 (4), 2362-2372 (2004)
23. S. P. Colgan, R. M. Hershberg, G. T. Furuta and R. S. Blumberg: Ligation of intestinal epithelial CD1d induces bioactive IL-10: critical role of the cytoplasmic tail in autocrine signaling. *Proc Natl Acad Sci U S A*, 96 (24), 13938-13943 (1999)
24. F. Martin and J. F. Kearney: Marginal-zone B cells. *Nat Rev Immunol*, 2 (5), 323-335 (2002)
25. S. Pillai, A. Cariappa and S. T. Moran: Marginal zone B cells. *Annu Rev Immunol*, 23, 161-196 (2005)
26. S. Fujii, K. Shimizu, C. Smith, L. Bonifaz and R. M. Steinman: Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells *in vivo* and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med*, 198 (2), 267-279 (2003)
27. S. Fujii, K. Shimizu, M. Kronenberg and R. M. Steinman: Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol*, 3 (9), 867-874 (2002)
28. J. S. Bezbradica, A. K. Stanic, N. Matsuki, H. Bour-Jordan, J. A. Bluestone, J. W. Thomas, D. Unutmaz, L. Van Kaer and S. Joyce: Distinct roles of dendritic cells and B cells in Va14Ja18 natural T cell activation *in vivo*. *J Immunol*, 174 (8), 4696-4705 (2005)
29. V. Moulin, F. Andris, K. Thielemans, C. Maliszewski, J. Urbain and M. Moser: B lymphocytes regulate dendritic cell (DC) function *in vivo*: increased interleukin 12 production by DCs from B cell-deficient mice results in T helper cell type 1 deviation. *J Exp Med*, 192 (4), 475-482 (2000)
30. T. De Smedt, M. Van Mechelen, G. De Becker, J. Urbain, O. Leo and M. Moser: Effect of interleukin-10 on dendritic cell maturation and function. *Eur J Immunol*, 27 (5), 1229-1235 (1997)
31. G. Wilbanks and J. Streilein: Studies on the induction of anterior chamber-associated immune deviation (ACAID). 1. Evidence that an antigen-specific, ACAID-inducing, cell-associated signal exists in the peripheral blood. *J Immunol*, 146 (8), 2610-2617 (1991)
32. J. Y. Niederkorn and E. Mayhew: Role of splenic B cells in the immune privilege of the anterior chamber of the eye. *Eur J Immunol*, 25 (10), 2783-2787 (1995)
33. K. H. Sonoda and J. Stein-Streilein: CD1d on antigen-transporting APC and splenic marginal zone B cells promotes NKT cell-dependent tolerance. *Eur J Immunol*, 32 (3), 848-857 (2002)
34. M. Svensson, A. Maroof, M. Ato and P. M. Kaye: Stromal cells direct local differentiation of regulatory dendritic cells. *Immunity*, 21 (6), 805-816 (2004)
35. M. Delgado, E. Gonzalez-Rey and D. Ganea: The neuropeptide vasoactive intestinal peptide generates tolerogenic dendritic cells. *J Immunol*, 175 (11), 7311-7324 (2005)
36. J. D. Fontenot, M. A. Gavin and A. Y. Rudensky: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*, 4 (4), 330-336 (2003)
37. S. Hori, T. Nomura and S. Sakaguchi: Control of regulatory T cell development by the transcription factor Foxp3. *Science*, 299 (5609), 1057-1061 (2003)
38. P. L. Vieira, J. R. Christensen, S. Minaee, E. J. O'Neill, F. J. Barrat, A. Boonstra, T. Barthlott, B. Stockinger, D. C. Wraith and A. O'Garra: IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol*, 172 (10), 5986-5993 (2004)
39. O. Akbari, G. J. Freeman, E. H. Meyer, E. A. Greenfield, T. T. Chang, A. H. Sharpe, G. Berry, R. H. DeKruyff and D. T. Umetsu: Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med*, 8 (9), 1024-1032 (2002)
40. Y. Tamura, A. Teng, R. Nozawa, Y. Takamoto-Matsui and Y. Ishii: Characterization of the immature dendritic cells and cytotoxic cells botn expanded after activation of invariant NKT cells with α-galactosylceramide *in vivo*. *Biochem Biophys Res Commun* in press (2008)
41. E. M. Ling, T. Smith, X. D. Nguyen, C. Pridgeon, M. Dallman, J. Arbery, V. A. Carr and D. S. Robinson: Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet*, 363 (9409), 608-615 (2004)
42. M. Akdis, J. Verhagen, A. Taylor, F. Karamloo, C. Karagiannidis, R. Crameri, S. Thunberg, G. Deniz, R. Valenta, H. Fiebig, C. Kegel, R. Disch, C. B. Schmidt-Weber, K. Blaser and C. A. Akdis: Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med*, 199 (11), 1567-1575 (2004)

Abbreviations: αGalCer or αGC, α-galactosylceramide; DCs, dendritic cells; iNKT, invariant natural killer T cells; OVA, ovalbumin; Treg, regulatory T cells

Key Words: Innate immunity, IgE, Allergy, Dendritic Cells, DC, natural killer T cells, iNKT, α-galactocylceramide, GalCer, Immune Tolerance, Suppression, Anergy, Regulatory T cells, Treg, IL-10, Tolerogenic

Send correspondence to: Yasuyuki Ishii, Laboratory for Vaccine Design, RIKEN Research Center for Allergy and Immunology, 1-7-22, Suehiro, Tsurumi, Yokohama, Kanagawa, Japan 230-0045, Tel: 81-45-503-7016; Fax: 81-45-503-7015, E-mail: ishiiyas@rcai.riken.jp

http://www.bioscience.org/current/vol13.htm