Title

A Novel Approach Inducing Transplant Tolerance by Activated Invariant Natural Killer T Cells with Co-stimulatory Blockade

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Abbreviations

α-GalCer: α-galactsylceramide, **αGC-liposomes**: liposomal formulation of α-galactsylceramide, **APCs**; antigen presenting cells, **BMCs**: bone marrow cells, **BMT**: bone marrow transplantation, **CD40L**: CD40 ligand, **CFSE**: carboxyfluorescein succinimidyl ester, **CTLA4**: cytotoxic T-lymphocyte associated protein 4, **DCs**: dendritic cells, **DPBS**: Dulbecco's Phosphate-Buffered Saline, **FITC**: fluorescein isothiocyanate, **GvHD**: graft versus host disease, **HBSS**: Hanks' Balanced Salt Solution, **ICOS**; inducible T cell co-stimulator, **IFN-γ**: Interferon-gamma, ***i*NKT cells**: invariant natural killer T cells, **i.p.**: intraperitoneally, **i.v.**: intraveneously, **KLRG-1**: Killer cell lectin-like receptor subfamily G, member 1, **mDCs**: myeloid dendritic cells, **NK cells**; natural killer cells, **PBMCs**: peripheral blood mononuclear cells, **PD-1**; programmed cell death-1, **pDCs**: plasmacytoid dendritic cells, **PE**: phycoerythrin, **TBI**: total body irradiation, **TCD mAb**: T cell depletion monoclonal antibody, **Teffs**: effector T cells, **Th1**: Type 1 helper T, **Th2**: Type 2 helper T, **Tregs**: regulatory T cells,

Abstract

Invariant natural killer T (*i*NKT) cells are one of the innate lymphocytes that regulate immunity, although it is still elusive how *i*NKT cells should be manipulated for transplant tolerance. Here we describe the potential of a novel approach using a ligand for *i*NKT cells and sub-optimal dosage of antibody for CD40-CD40 ligand (L) blockade as a powerful method for mixed chimerism establishment after allogenic bone marrow transplantation in sub-lethally irradiated fully-allo recipients. Mixed chimera mice accepted subsequent cardiac allografts in a donor-specific manner. High amounts of Th2-cytokines were detected right after *i*NKT-cell activation, while subsequent IFN-γ production by NK cells was effectively inhibited by CD40/CD40L blockade. Tolerogenic-components, such as $CD11c^{low}mPDCA1^{+}$ plasmacytoid dendritic cells and activated regulatory T cells (Tregs) expressing CD103, KLRG-1 and PD-1, were subsequently augmented. Those activating Tregs seem to be required for the establishment of chimerism because depletion of the Tregs one day before allogenic cell-transfer resulted in a chimerism brake. These results collectively suggest that our new protocol makes it possible to induce donor-specific tolerance by enhancement of the innate ability for immune tolerance in place of the conventional immunosuppression.

Introduction

Invariant natural killer T (*i*NKT) cells are a small subset of lymphocytes that is characterized by Vα14-Jα18 invariant TCR, which recognizes glycolipid molecules, e.g. a synthetic ligand α-galactsylceramide (α-GalCer)—to date the most potent *i*NKT-cell ligand—loaded on a non-classical MHC class I-like molecule, CD1d(1, 2). Upon stimulation by α-GalCer, *i*NKT cells quickly secrete enormous amounts of cytokines within hours(3, 4), both type 1 helper T (Th1) cytokines (e.g. IFN-γ, IL-12), which activate cytotoxic reactions, and less harmful type 2 helper T (Th2) cytokines (e.g. IL-4, IL-10), which are thought to regulate immunity(5).

In the field of transplantation, *i*NKT cells are considered to be crucial in some experimental models of transplant tolerance, such as cornel allograft survival(6, 7), rat xen-islets acceptance induced by anti-CD4 mAb(8), thymic chimerism induction with cyclophosphamide(9), cardiac graft tolerance induced by anti-CD40L(10, 11), and mixed hematopoietic chimerism induction(12, 13). Even though it has been accepted that *i*NKT cells are responsible for the promotion and/or maintenance of transplant tolerance, how *i*NKT cells should best be activated for tolerance induction still remains unclear. Indeed, some negative effects of *i*NKT-cell activation have been reported in several transplant experiments(10, 14-16). These results are caused by the enhancement of cytotoxicity and/or Th1-cell responses including IFN-γ production generated by *i*NKT-cell activation. Indeed, the skewing of Th2-cytokines by *i*NKT-cell activation using some α-GalCer analogues that do not enhance IFN-γ production(17), resulted in the delay of cardiac allograft rejection(18). Taken together, it is suggested that the treatment of *i*NKT cells should be controlled to enhance production of Th2-cytokines and to reduce the production of Th1-cytokines.

It is well known that presentation of α-GalCer by dendritic cells (DCs) to *i*NKT cells leads to the production of large amounts of IFN-γ(19). In contrast to aqueous α-GalCer, the liposomal formulation of α-GalCer (αGC-liposomes) could be preferentially incorporated into the B-cell subset of $CD21^{high}CD23^{low}$ phenotype and play a role in the suppression of helper T-cell priming and the development of IL-10-producing regulatory T cells (Tregs)(20). Based on these results, αGC-liposomes were assessed by using murine models of acute graft versus host disease (GvHD). It was confirmed that αGC-liposomes had a significant prophylactic effect in parallel with allo-specific Tregs expansion in vivo(21).

In this study, αGC-liposomes were tested using a mixed chimerism model that is a reliable method for transplant tolerance induction. Treatment with αGC-liposomes resulted in the complete acceptance of transplanted donor bone marrow without GvHD and the cardiac allograft from the same donor permanently when combined with sublethal total body irradiation (TBI) followed by co-stimulation blockade. This novel protocol made it possible to regulate *i*NKT cell-mediated cytokine production and to expand Tregs in vivo.

Materials and Methods

Animals

WT BALB/c (H-2^d), C57BL/6 (B6, H-2^b), and C3H/He (H-2^k) mice were purchased from Japan Slc. Inc. Vα14 NKT-deficient mice (BALB/c Jα18−/−), described elsewhere(1), were backcrossed for more than nine generations. All mice were bred and maintained in our animal facilities under specific pathogen-free conditions. An internal committee on the use and care of laboratory animals approved of all experiments (IRB12-76).

Reagents

Aqueous α-GalCer (KRN7000) was purchased from Kirin Brewery Co. Ltd, Japan. Liposomal formulation of α-GalCer was provided by REGiMMUNE(Japan). Doses of both formulations of α-GalCer were expressed based on the dose of contained α-GalCer and calculated assuming a mouse body weight of 20g. Both formulations of α-GalCer were diluted in Hanks' Balanced Salt Solution (HBSS; Life Technologies, USA) for intravenous (i.v.) injection. Anti-CD40L mAb (MR-1), anti-CD25 mAb (PC61.5.3), anti-IL4 mAb (11B11), and anti-IL-10 mAb (JES5-2A5) were purchased from BioXcell (USA) and diluted in Dulbecco's Phosphate-Buffered Saline (DPBS; Life Technologies) for intraperitoneal (i.p.) administration.

Bone Marrow Transplantation (BMT)

Femur and tibia were harvested from B6 donor mice and flushed with HBSS using a 21-gauge needle to collect bone marrow cells (BMCs). Age-matched (8-12 week) BALB/c recipients underwent non-myeloablative TBI (3 Gy) using an X-ray irradiation system (Hitachi *MBR-1520R-3*; Hitachi Medical Co. Japan) 3-4 h prior to i.v. injection of 2.0×10^7 unseparated B6 BMCs.

FACS Staining

Heparinized blood or tissue samples were hemolyzed with ACK Lysing Buffer (Lonza, USA) to remove red cells. The cell pellets were incubated at 4ºC for 20 m with saturating concentrations of mAb mixtures: TCR-β, B220, Mac-1, Gr-1, CD4, CD8, CD25, CD44, CD49b, CD62L, CD69, CD103, ICOS, KLRG-1, PD-1 (BD Pharmingen, USA), CD11b, CD11c (eBioscience, USA), mPDCA1 (Miltenyi Biotech, Germany), and α-GalCer pre-loaded CD1d-tetramer (Proimmune, UK). FITC-conjugated anti-H-2K^{b} and PE-conjugated anti-H-2K^{d} (eBioscience) were used for chimerism analysis. All mouse cells were incubated with purified CD16/32 (BD Pharmingen) to block unspecific staining. For the detection of intracellular antigen, anti-Foxp3 (Miltenyi Biotech), anti-IFN-γ, anti-Ki-67, anti-CTLA4, and anti-Bcl-2 (BD Parmingen) were stained after fixation/permeabilization using the Foxp3 Staining Buffer Set (eBioscience). Cell samples were analyzed with a BD FACSCanto^{TM} II flow-cytometer (BD Biosciences, USA). Chimerism was analyzed in mononuclear cells gated by forward and orthogonal light scatter. The percentage of donor cells was calculated as follows: (%H-2K^{b} donor cells) / (%H-2K^{b} donor cells + %H-2K^{d} host cells).

Heterotopic Cardiac Transplantation

All transplant procedures were performed with the animals under general anesthesia. Fully vascularized heterotopic cardiac grafts from B6 donors were transplanted into BALB/c recipients using microsurgical techniques(22). Postoperatively, graft function was assessed daily by palpation for evidence of contraction. Rejection was defined as complete cessation of contractions and confirmed by direct visualization. Cardiac allografts of tolerant mice were removed 100 d after transplantation and studied histologically. Formalin-fixed sections (4-μm thick) were cut, mounted on silane-coated slides and stained with hematoxylin-eosin

or Masson-trichrome stain.

Mixed Lymphocyte Reaction (MLR)

As a responder, $CD4^+$ or $CD8^+$ T cells were negatively isolated with CD4/CD8 negative isolation kit (Life Technologies) from the splenocytes obtained from recipient mice. As a stimulator, antigen presenting cells (APCs) were prepared from the splenocytes of naïve mice by T-cell depletion with mouse pan-T beads (Life Technologies) and then irradiated 30Gy. Isolated T cells were incubated with stimulator at a 1:2 responder:stimulator ratio in RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum, 10mM Hepes, 1 x L-glutamine (2mM), 1 x Na pyruvate (1μM), 1 x penicillin-streptomycin (1μM), and 0.5 x antibiotic/mycotic (all from Life Technologies). For ELISpot assay, 96 well Multi-Screen plates (Millipore,USA) were coated with mouse IFN-γ capture antibody (R&D system,USA), incubated at 4ºC overnight. Responder $CD8^+$T cells were added to wells containing irradiated APCs and incubated for 42 h. Mouse IFN-γ detection antibody was added and incubated at 4 ºC overnight and then visualized with ELISPot Blue Color Mudule (R&D).

Cytokine Measurements

Serum samples were collected from recipient mice at 2 h and 20 h after BMT. Serum IL-4, IL-10, IL-12, and IFN-γ were determined by multicytokine detection system (Bio-Rad, USA) following the manufacturer's instructions. They were measured using a Luminex System and calculated using Bio-plex software.

Statistical Analysis

Statistical data were analyzed with Prism 4 software (GraphPad Software, Inc. USA) using the Student's t test (unpaired, two-tailed). Kaplan-Meier survival curves were analyzed

using the log-rank test. P-value <0.05 was considered statistically significant.

Results

Combination treatment of *i*NKT-cell ligand with CD40-CD40L blockade induces durable mixed chimerism

Allogenic BMCs derived from B6 (H-2^b) mice were administered intravenously into sublethal TBI-treated BALB/c (H-2^d) mice, and a proportion of grafted donor cells expressing H-2^b in peripheral blood mononuclear cells (PBMCs) was analyzed by flow-cytometer (**Figure.1A**). Grafted donor cells were undetectable two weeks after BMT in untreated mice. Consistent with other transplant models that showed negative effects of α-GalCer(10, 14-16), mice treated with αGC-liposomes alone also showed immediate graft rejection. It has been proved that CD40-CD40L interaction is essential for the IFN-γ production of *i*NKT cells(23), which may promote graft rejection caused by α-GalCer stimulation. Therefore, we attempted a combination therapy of αGC-liposomes and anti-CD40L mAb. Anti-CD40L is a co-stimulation blockade commonly used to induce transplant tolerance and is generally combined with T-cell depletion (TCD) mAb(24), donor-specific transfusion(25-28), or other co-stimulation blockades such as CTLA4-Ig(29, 30). However, in our fully-allo combination, single administration of 0.5mg anti-CD40L on d 0 failed to engraft donor cells. Even when tested with 2.0mg anti-CD40L, mixed chimerism induction was observed in only 20% of mice. On the other hand, combination treatment of αGC-liposomes with 0.5mg anti-CD40L resulted in substantial preservation of the grafted cells. Grafted donor cells in these chimera mice remained durable for over 100 d (**Figure.1B**). The mice maintained the grafted donor cells not only in the peripheral blood but also in the spleen, lymph nodes, and bone marrow for over 100 d (**Figure.1C**). Substantial numbers of donor-derived multi-lineages including T cells, B cells, macrophages, and granulocytes were observed (**Figure.1D**). The result indicated that mixed chimerism could be established

in mice treated with a combination of αGC-liposomes with a sub-optimal dosage of anti-CD40L.

Mixed chimerism-established mice accept cardiac allograft from the same donor

To test the capability of graft survival extension, treated recipient mice were transplanted with heterotopic cardiac allografts from the same donor mice two weeks after BMT (**Figure.2A**). In the case of H-2^d–restricted recipients that were transferred H-2^b-restricted BMCs with 0.5mg anti-CD40L alone, the H-2^b-restricted cardiac graft was rejected immediately. Even in mice treated with 2.0mg anti-CD40L alone, the cardiac graft was rejected gradually. In contrast, H-2^d-restricted recipient mice transferred H-2^b-restricted cells with αGC-liposomes plus 0.5mg anti-CD40L completely accepted the H-2^b-restricted cardiac graft. Histopathlogical analysis of the tolerant mice at 100 d showed neither cellular infiltration nor vascular fibrosis, both of which were observed in the grafts obtained from mice treated with 2.0mg anti-CD40L (**Figure.2C**). To test whether the cardiac graft survival achieved by αGC-liposomes plus anti-CD40L was restricted to the allogenecity of transferred BMCs, a 3rd-party strain-derived cardiac graft was transplanted. As expected, all cardiac grafts of C3H/He (H-2^k) mice were rejected (**Figure.2B**). These data collectively suggest that the mixed chimerism established by a single injection of donor BMCs with αGC-liposomes plus a sub-optimal dosage of anti-CD40L could induce donor-specific, long-term transplant tolerance, whereas it could not be achieved by anti-CD40L monotherapy, even using optimal-dosage.

αGC-liposomes plus anti-CD40L treatment diminishes allo-reactivity toward both host and donor in vitro

We examined allo-reactivity of T cells derived from the treated mice by using an in vitro system of MLR. First, splenic $CD8^+$T cells were recovered 28 d after BMT and then co-cultured with irradiated APCs of splenocytes derived from BALB/c or B6 mice. The number of IFN-γ-producing cells was determined by ELISpot system. Splenic $CD8^+$T cells derived from untreated and either αGC-liposomes- or anti-CD40L-treated mice produced numerous IFN-γ-positive spots upon stimulation by H-2^b APCs, but not by H-2^d APCs (**Figure.3A**). On the other hand, the $CD8^+$T cells from mice treated with a combination of αGC-liposomes with anti-CD40L did not produce IFN-γ-positive spots upon either H-2^b or H-2^d APCs stimulation. In addition, splenic $CD4^+$T cells were enriched, labeled with CFSE, and then co-cultured with H-2^b or H-2^d APCs. As expected from the results of the $CD8^+$T cells, proliferation capability of $CD4^+$T cells of mice treated with both αGC-liposomes and anti-CD40L were deficient upon stimulation by either H-2^b or H-2^d APCs (**Figure.3B**). On the other hand, the proliferation of $CD4^+$T cells from mice treated with αGC-liposomes alone or anti-CD40L alone was comparable to that from untreated mice. Taken together, combination therapy that uses αGC-liposomes and anti-CD40L might make it possible to reduce allo-reactivity of the recipient mice toward host and donor in vitro, which is essential for graft survival as well as prevention of GvHD.

αGC-liposomes plus anti-CD40L treatment deviates immunity toward Th2 and facilitates engraftment of donor cells

The administration of *i*NKT-cell ligand, α-GalCer, is known to result in the immediate secretion of multivalent cytokines. In our previous study, the production capability of Th2-cytokines such as IL-4 and IL-10 in *i*NKT cells was enhanced by treatment with αGC-liposomes rather than aqueous α-GalCer(20). To elucidate the correlation of the cytokine production and the establishment of mixed chimerism, representative cytokines such as IL-

4, IL-10, IL-12, and IFN-γ in the serum of untreated- and treated-mice were assessed (**Figure.4A**). The production of all cytokines was clearly elevated 2 h after BMT in the recipient mice treated with both formations of α-GalCer, but not with anit-CD40L alone. The amounts of all cytokines tested in αGC-liposomes-treated mice were much higher compared to those in aqueous α-GalCer-treated mice. As a result, the rate of chimerism establishment and the proportion of engrafted donor cells in PBMCs of the BMT-mice treated with αGC-liposomes plus anti-CD40L were significantly higher compared to those with aqueous α-GalCer plus anti-CD40L (**Figure.4B**). At 20 h after BMT, IFN-γ production was enhanced in recipient mice treated with either αGC-liposomes alone or aqueous α-GalCer alone. By contrast, the expression of IFN-γ was remarkably diminished 20 h after BMT in the recipient mice treated with either αGC-liposomes plus anti-CD40L or aqueous α-GalCer plus anti-CD40L.These data collectively suggest that the production of IL-4 and/or IL-10, but not IFN-γ generated by a ligand for *i*NKT cells might be relevant to the establishment of mixed chimerism. CD40-CD40L blockade antibodies could be indispensable for the reduction of IFN-γ production after *i*NKT-cell activation.

Anti-CD40L is required for abrogation of interaction between *i*NKT cells and NK cells

It is known that *i*NKT-cell activation is followed by high amounts of IFN-γ production by NK cells. To clarify whether anti-CD40L inhibited production of IFN-γ from *i*NKT cells or NK cells, splenocytes obtained from mice treated with αGC-liposomes with/without anti-CD40L were analyzed by intracellular IFN-γ cytostaining (**Figure.5**). At 90 m after αGC-liposome administration, production of IFN-γ in *i*NKT cells was already observed in mice treated without anti-CD40L. This production of IFN-γ was also shown even in mice treated with anti-CD40L. In NK cells, production of IFN-γ was not observed yet at this time in all animals (data not shown). At 6 h after treatment, IFN-γ production of *i*NKT cells had already

vanished in both mice treated with/without anti-CD40L (data not shown). Instead, NK cells in mice treated with αGC-liposomes alone started to produce IFN-γ. On the other hand, mice treated with αGC-liposomes plus anti-CD40L lacked this IFN-γ production of NK cells. Taken together, we presumed that anti-CD40L blocked NK-cell activation following *i*NKT-cell activation, although it could not directly inhibit IFN-γ production from *i*NKT cells themselves.

αGC-liposomes plus anti-CD40L treatment augments plasmacytoid DCs

To elucidate the relevance of soluble mediators for mixed chimerism establishment, neutralization mAb of IL-4 or IL-10 was administrated one day before BMT (**Table 1**). Contrary to expectation, achievement rate of mixed chimerism was still stably observed after those treatments. The data suggested there were other factors which facilitated engraftment caused by αGC-liposomes.

We previously reported that mice treated with αGC-liposomes but not aqueous α-GalCer showed augmentation of plasmacytoid DCs (pDCs), which could induce activating Tregs in vivo and in vitro(20). Thus, we focused on the change of those cellular components in untreated- and treated-mice by flow-cytometer. In our current study, conventional myeloid DCs (mDCs) were defined as $CD11b^{+}CD11c^{high}$ population among the lineages (CD3, CD19, CD49b, NK1.1, and TER-119)-negative population (**Figure6.A**). As $mPDCA1^{+}$ subpopulation mostly consisted of $CD11c^{low}$ cells and showed characteristics of immature DCs on their cell surface (**Figure.6B**), the cells corresponded to pDCs. In the splenocytes obtained from mice treated with αGC-liposome and/or anti-CD40L 3 d before, the injection of αGC-liposomes preferentially augmented the proportion of splenic pDCs rather than that of mDCs (**Figure.6C&D**). This augmentation of pDCs was not abrogated or enhanced by anti-CD40L.

αGC-liposomes plus anti-CD40L treatment activates and expands Tregs

Next, we analyzed $CD4^{+}CD25^{high}Foxp3^{+}$T-cell population in splenocytes of untreated- and treated-mice 30 d after BMT by flow-cytometer (**Figure.7A**). Consistent with previous reports, the proportion of Treg phenotype cells was increased with statistical significance in both mice treated with αGC-liposomes alone and in those with αGC-liposomes plus anti-CD40L treatment (**Figure.7B**). To elaborate on the proliferation activity of those expanded Tregs, intracellular Ki-67 expression of Tregs was compared among each group. Strikingly, Tregs in chimera mice treated with αGC-liposome plus anti-CD40L showed remarkable up-regulation of Ki-67 compared to those of other treated groups that had rejected the donor graft. Further analysis showed that Tregs in the chimera mice were fully activated because the expression of CD44, CD69, CD103, ICOS, KLRG-1, and PD-1 on the cell surface and intracellular CTLA-4 were up-regulated, while that of CD62L in the cell surface and intracellular Bcl-2 were down-regulated (**Figure.7C**). These results collectively suggest that activated Tregs could be expanded in the mixed chimera mice generated by combination therapy with αGC-liposomes plus anti-CD40L.

Tregs activated by *i*NKT cells are indispensable for induction of mixed chimerism

To clarify the importance of Tregs in the process of the establishment of mixed chimerism, we administered anti-CD25 mAb to deplete Tregs at the day before BMT. Mixed-chimera establishment was completely abrogated by Tregs depletion at the day before BMT (**Figure.8A**). To confirm that establishment of mixed chimerism and expansion of activating Tregs in our model is induced by *i*NKT cells, experiments were performed using Jα18−/− *i*NKT cell-deficient mice as a recipients. As expected, *i*NKT cell-deficient recipient mice failed to retain donor-derived cells and to activate Tregs (**Figure.8A&B**). Taken together,

this suggests that activation of Tregs just after BMT might be indispensable for establishment of mixed chimerism and may be regulated by recipient-derived *i*NKT cells upon stimulation by αGC-liposomes in the presence of anti-CD40L.

Discussion

The induction of mixed hematopoietic chimerism is a powerful and effective means to achieve transplant-tolerance. A growing body of the literature suggests that inhibition of T-cell function using TCD mAb and/or co-stimulatory blockade is fundamental for engraftment of allogeneic hematopoietic cells(12, 24, 25, 29, 31-33). However, such approaches non-specifically inhibit T-cell dependent immunity, including the regulatory mechanism that is important for establishment of tolerance. We reported here a novel approach that could establish mixed chimerism by boosting regulatory mechanisms with *i*NKT-cell activation under CD40/CD40L blockade.

Anti-CD40L in this study seems to be a switch that could control immune-direction of *i*NKT cells after α-GalCer activation. However, Matsuda *et al* reported that CD40/CD40L signaling is not crucial for the initial IFN-γ production from *i*NKT cells because IFN-γ production from *i*NKT cells could be detected at 2 h after α-GalCer administration in both WT and CD40-/- mice(3). Consistent with this, administration of anti-CD40L in our study also could not abrogate IFN-γ production of *i*NKT cells at 90 m after α-GalCer administration (**Figure.5**). It is thought that following initial activation, *i*NKT cells express CD40L and then cross-link with CD40 on DCs(34, 35), which results in the production of IL-12 by DCs. Therefore, we speculated that anti-CD40L blocked this process and abrogated DCs maturation. Indeed, IFN-γ production from NK cells, which follows after IL-12 production of $CD40^{high}$ mature-DCs, was inhibited by anti-CD40L in the current study (**Figure.5**). NK cells are considered to be deeply related to hematopoietic cell rejection(36-

40), and have a reciprocal relationship with Tregs(41, 42). Hence, we presume that blockade of NK-cell activation with anti-CD40L is indispensable for tolerance induction.

Although some mice treated with a combination of aqueous α-GalCer plus anti-CD40L showed engraftment of donor cells, the achievement rate of mixed chimerism observed in those mice is significantly poorer as compared to mice treated with αGC-liposomes plus anti-CD40L. The difference between liposomal and aqueous formulations of α-GalCer on the immune response is ascribed to the profile of the APCs incorporated. As reported previously, αGC-liposomes are incorporated into splenic B-cell fractions more than $CD11c^{+}$DCs and involved in IL-10 production of B cells(20). Smedt *et al* showed that IL-10 affected during the maturation of DCs down-regulated their capacity of producing IL-12 and activating Th1 cells(43). Bezbradica *et al* demonstrated that α-GalCer -presenting B cells weakened not only DCs maturation but also DCs-mediated activation of *i*NKT cells and NK cells(44). Furthermore, Veronique *et al* also reported that production of IL-12 by DCs was reduced after the interaction with B cells(45). Together, one might speculate that B cell-derived IL-10 induced by αGC-liposomes could inhibit maturation of DCs in the micro-environment. These results collectively suggest that αGC-liposomes and anti-CD40L work complementally to block DCs maturation and to relatively augment pDCs (**Figure.6C&D**), which are designated as immature tolerogenic DCs producing IL-10 and functionally different from the $CD11c^{hi}$ mDCs that produce IL-12(46).

It has been reported that pDCs could develop the antigen-specific IL-10 producing Tregs in vivo and in vitro(26, 46). In our regimen, the development of $CD4^{+}CD25^{high}Foxp3^{+}$ T cells was observed (**Figure.7A&B**). It is noteworthy that the $CD4^{+}CD25^{high}Foxp3^{+}$ T cells expressed activation markers like CD103 and KLRG-1 (**Figure.7C**). KLRG-1^{+} Tregs are

considered to be recent Ag-responsive and highly activated phenotypes with enhanced suppressive function(47-49). Furthermore, in our study, the $CD4^{+}CD25^{high}Foxp3^{+}$ T cells expressed a high level of PD-1 (**Figure.7C**), which is considered to be linked to IL-10 production(13). Since the depletion of $CD25^{+}$ T cells on the day before BMT abrogated the mixed chimerism induction (**Figure.8A**), we conclude that the $CD4^{+}CD25^{high}Foxp3^{+}$ T cells expanded by our regimen could be activated functional Tregs.

The importance of Tregs for peripheral tolerance induction has been proved(26, 28). However, the role of Tregs in the mixed chimerism induction model still remains unclear. In several reports, mixed chimerism induction was not abrogated by anti-CD25 mAb treatment(30, 50). In those studies, the impact of Treg-depletion might be insufficient to induce allogeneic hematopoietic cell rejection because they used stronger immune suppression or lower immunological barrier models than ours. Other supportive studies described the abrogation of mixed chimerism induction with anti-CD25 mAb in the lower invasive protocol setting of fully-allo combination(27, 51). It is presumable that there is a delicate balance between Tregs and effector T cells (Teffs) in allograft tolerance.

Tregs themselves are thought to have the ability to induce chimerism because adoptive transfer of *ex vivo* expanding donor-specific or even polyclonal Tregs enables mixed chimerism and graft tolerance(52). In those protocols, however, preconditioning or immunosuppression, e.g. 5Gy TBI(53) combined with anti-CD40L plus CTLA4-Ig—with(54) or without(55) rapamycin—are needed to some extent to suppress the allo-reactivity of Teffs. The ratio of Tregs to antigen-specific Teffs has been previously identified as a predictor of the potential protective effects of Tregs(28, 56). From those data, it is speculated that allo-reactivity could be regulated, and allo-hematopoietic cell engraftment would be facilitated

when the number and activity of Tregs overcome those of Teffs. This might be achieved by inhibiting Teffs with TCD-mAbs or co-stimulation blockades. Another approach is to augment the number of functional Tregs, which could be achieved by the regime we present in this study.

Concerning the clinical indication of our protocol, clinical application of anti-CD40L has been hampered by thrombolic complication(57). However, development of anti-CD40 (e.g. 4D11(58, 59), 3A8(60), and more recently, 2C10(61)) has progressed. Those antibodies are not only alternatives for anti-CD40L, but rather more suitable for our protocol, because they can directly affect DCs, which are responsible for unfavorable Th1-immunity caused by α-GalCer stimulation.

Because the amino acids of CD1d and invariant TCR, which are important for binding with α-GalCer, are well conserved among species such as the mouse and human(62-64), α-GalCer can be used to activate both mouse and human *i*NKT cells. Thus, our protocol could be applied to clinical trials just as it is. α-GalCer has already been used in clinical studies for patients with advanced cancer and chronic hepatitis B/C(65, 66). Immunological effects induced by the administration of α-GalCer were observed, especially in the subgroup of patients with relatively high pre-treatment circulating *i*NKT-cell numbers. Meanwhile, the low number of *i*NKT cells likely contributed to the lack of immunoreactivity after α-GalCer administration. For those unresponsive patients, novel approaches such as transfer of in vitro α-GalCer -pulsed DCs(67-69) or autologous *i*NKT cells generated in vitro(65, 70) have shown to be more potent than α-GalCer administration alone. Those data are valuable for clinical indication of αGC-liposomes, although the effect of αGC-liposomes on human cells should be investigated closely.

Our data suggest a new insight—that the immune direction of *i*NKT cells is controlled through a type of APCs presenting α-GalCer and co-stimulation signals, and that it is possible to enhance our innate ability of immune tolerance by appropriate activation of *i*NKT cells. We hope that not only transplant tolerance but also prevention of other immune disorders might be achieved by using our regimen.

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Financial Disclosure

Emi Fukuda is an employee of REGiMMUNE. Yasuyuki Ishii is the founder of the company and has ownership interests in REGiMMUNE. Other authors don't have any conflict of interest related to this work.

Author Contributions

All authors contributed extensively to the work presented in this paper; T.H. designed the study, performed experiments, analyzed data and wrote the first draft; Y.I. assisted the study design and revised the manuscript; M.I. performed experiments and analyzed data; E.F. prepared and generated Vα14 NKT-deficient mice and analyzed cytokine assays; K.O., M.N., M.T., and K.T. supervised the project.

Figure Legends

Figure 1. Combination treatment of αGC-liposomes plus anti-CD40L establishes multi-organ/multi-lineage durable mixed hematopoietic chimerism. BALB/c mice were grafted with B6 2.0x10^7 bone marrow cells after 3Gy total body irradiation. Single dose of anti-CD40L mAb i.p. injected right after BMT at the dose indicated; 10 μg/kg αGC-liposomes injected via tail vein within 30 m after BMT. **(*A*)** FACS analysis of PBMCs carried out two weeks after BMT to assess the establishment of mixed chimerism. Summary of percentage of donor (H-2^b) cells among PBMCs calculated from individual mice from five independent experiments. **(*B*)** Percentage of donor cells in PBMCs of individual mice treated with αGC-liposomes plus anti-CD40L followed on d 30, 60, and 100 after BMT. **(*C*)** Mononuclear cells isolated from peripheral blood, spleen, superficial lymph nodes, and bone marrow obtained from chimera mice 100 d after BMT. Left, representative figures of dot plots of H-$2K^b$ versus H-$2K^d$ staining in each organ; Right, mean percentages ± SD of H-$2K^b$ positive donor cells in each organ. **(*D*)** Multilinage chimerism in PBMCs as analyzed from chimera mice 100 d after BMT. Left, representative figures of dot plots in each linage. T cells, B cells, macrophages, and granulocytes indicated as TCR-β, B220, MAC-1, and Gr-1 positive cells, respectively. Right, mean percentage ± SD of H-$2K^b$ positive donor cells in each linage. Data are representative of five independent experiments. αGC-liposomes: liposomal formulation of α-galactsylceramide; PBMCs: peripheral blood mononuclear cells; BMT: bone marrow transplantation.

Figure 2. Combination therapy of αGC-liposomes plus anti-CD40L induces cardiac allograft tolerance in donor-specific manner.

(*A*) BALB/c (H-2^d) mice-transplanted heterotopic cardiac allograft from B6 (H-2^b) donor 14 d

after B6 bone marrow transfer with 0.5mg anti-CD40L alone (N=6, black dot line), with 2.0mg anti-CD40L alone (N=6, blue dashed line), or with combination therapy of αGC-liposomes and 0.5mg anti-CD40L (N=6, red solid line). **(*B*)** Chimera mice-transplanted heterotopic cardiac allograft from B6 (H-2^b) donor (N=6, red solid line) or C3H/He (H-2^k) donor (N=8, blue dot line) after B6 bone marrow transfer with αGC-liposomes plus 0.5mg anti-CD40L. Graft survival curves of each group; P-values calculated by log-rank test. **(C)** All mice with beating grafts were sacrificed on d 100 after cardiac transplantation and examined histopathologically. Representative histopathology of cardiac grafts obtained from mice grafted from syngenic donors (left column), grafted from B6 donors after B6 bone marrow transfer with 2.0mg anti-CD40L alone (middle column) and with αGC-liposomes plus 0.5mg anti-CD40L (right column).

Figure 3. T cells of mixed chimera mice show hypo-responsiveness toward donor antigen as well as toward host antigen

(A) $CD8^+$T cells obtained from bone marrow-transplanted mice co-cultured with H-2^b or H-2^d-restricted APCs. IFN-γ secreting cells were captured and recognized as spots by ELISpot assay. Left: data presented are mean number ± SD of IFN-γ secreting cells among 1 x 10^5 $CD8^+$T cells calculated from triplicated wells of each treated group. Right, photographs are representative of one in triplicated wells of each group. **(B)** $CD4^+$T cells obtained from bone marrow-transplanted mice stained with CFSE, then co-cultured with H-2^b or H-2^d-restricted APCs. Four days later, dilution of CFSE in $CD4^+$T cells was compared among each group. Data represent one of three independent experiments. APC: antigen presenting cell; CFSE: carboxyfluorescein succinimidyl ester.

Figure 4. αGC-liposomes plus anti-CD40L treatment enhances Th2 cytokine

production and donor cell engraftment compared to aqueous α-GalCer plus anti-CD40L. (A) Serum samples collected from recipient mice of each treatment group at 2 h (white bar) and 20 h (black bar) after BMT, and serum concentration of IL-4, IL-10, IL-12, and IFN-γ as measured using Bio-Plex cytokine assay. N.D: not determined; data presented are mean cytokine concentrations ± SD (pg/mL). Data represent one of three independent experiments. **(*B*)** BALB/c mice-transplanted B6 bone marrow with administration of αGC-liposomes or aqueous α-GalCer with 0.5mg anti-CD40L; the α-GalCer contained in 10 μg/kg in both formulations. Two weeks after BMT, the establishment of chimerism was assessed by flow-cytometer. Percentage of donor cells in PBMCs of individual mice from three independent experiments and p-value as calculated by unpaired t-test. α-GalCer: aqueous α-galactsylceramide; αGC-liposome: liposomal formulation of α-galactsylceramide.

Figure 5. IFN-γ production of NK cells after *i*NKT-cell activation are inhibited by anti-CD40L

Splenocytes were obtained from mice administrated with αGC-liposomes with or without anti-CD40L 90 m or 6 h before, then stained with cell surface molecules to define *i*NKT cells (as double positive of α-GalCer pre-loaded CD1d-tetramer and TCR-β) and NK cells (as $CD49b^{+}$ and $TCR\text{-}\beta^{-}$). Representative histogram of IFN-γ expression of each mouse detected in *i*NKT cells (top, 90 m after treatment) and NK cells (bottom, 6 h after treatment). Gray filled line: isotype control. Data represent one of two independent experiments.

Figure 6. αGC-liposomes augment the proportion of splenic $CD11c^{low}mPDCA1^{+}$ cells rather than that of $CD11c^{hi}$ DCs. αGC-liposomes and/or anti-CD40L were injected into

BALB/c mice, of which splenocytes were then obtained after 3 d. For DCs staining, collagenase D was injected into the spleen obtained from recipient mice and incubated at 37 ºC for 30 m before suspension. **(A)** The following biotinated antibodies were stained with streptavidin and used to gate out lineages: CD3e, CD19, CD49b, NK1.1, and TER-119 (left dot plots). Then, mDCs were defined as $CD11b^{+}CD11c^{high}$ population (mid dot plots) and pDCs were defined as $CD11c^{low}mPDCA1^{+}$ population (right dot plots). **(B)** To confirm characteristics of both pDCs and mDCs defined in this study, expression level of $I\text{-}A^{d}$, CD62L, CD80, CD86, and CD40 on their cell surface were analyzed by flow-cytometer. Representative histograms of $CD11b^{+}CD11c^{high}$ cells (top) and mPDCA1+$CD11c^{low}$ cells (bottom) obtained from mice treated with αGC-liposomes plus anti-CD40L (black line); naïve BALB/c mice controls (gray dashed line); Isotype controls (filled histogram). **(C)** Representative dot plots of CD11C(+)Lineage(-) population in each treated group. Proportion of mDCs and pDCs indicated as % $CD11b^{+}$ cells and % $mPDCA1^{+}$ cells, respectively. **(D)** Proportion of the mDC (left) and that of pDC (right) among the CD11c(+)lineage(-) cells in the each treatment group. The data summarizes 4 or 5 mice from each group covering three independent experiments; P-values as calculated by unpaired t-test.

Figure 7. Expansion and activation of $CD4^{+}CD25^{high}Foxp3^{+}$ Tregs in chimera mice induced by αGC-liposomes plus anti-CD40L treatment. (A) Splenocyte obtained from each group 30 d after BMT stained with CD4/CD25/Foxp3/Ki-67. Top, representative dot plots in each group gated by CD4-positive cells; $CD4^{+}CD25^{high}Foxp3^{+}$ cells confirmed as Tregs. Bottom, histograms representative of Ki-67 expression of $CD4^{+}CD25^{high}Foxp3^{+}$ gated cells in each group. **(B)** Statistical analysis of quantitative and qualitative difference of Tregs among each group. Mean percentage of Tregs in $CD4^{+}$ T cells (left) and mean

population of Ki-67^{high} in Tregs (right). Data are summarized from three independent experiments; P-values calculated by unpaired t-tests. **(C)** Splenocytes obtained from chimera mice at 30 d after BMT stained with CD4/CD25/Foxp3 together with each cell surface or intracellular molecules. Representative histograms of each molecule expressed on the cells gated with CD4^{+}CD25^{high}Foxp3^{+}. As a control, splenocytes obtained from naïve BALB/c mice were stained. Chimera mice: black unfilled line; control naïve BALB/c mice: gray filled line. Data are representative of one of three independent experiments. ICOS: inducible T cell co-stimulator; KLRG-1: killer cell lectin-like receptor subfamily G, member-1; PD-1: programmed cell death-1; CTLA4: cytotoxic T-lymphocyte associated protein 4.

Figure 8. Both Tregs and *i*NKT cells are essential for mixed chimera induction.

(A) WT or Jα18-/- (*i*NKT-cell deficient) BALB/c mice were transplanted with WT B6 bone marrow with αGC-liposomes plus anti-CD40L. In the Treg-depleted group, WT recipient mice were injected interaperitoneally with 0.5mg anti-CD25 mAb (PC61.5.3) one day before BMT to deplete Tregs. Two weeks after BMT, establishment of chimerism was assessed by flow-cytometer indicating % of donor cells in PBMCs of individual mice. **(B)** Both WT recipient mice (N=4) and *i*NKT-deficient recipient mice (N=4) were sacrificed 30 d after BMT; Tregs in the splenocytes were analyzed. Top, representative dot plots of cells gated by CD4 positive cells. Bottom, representative histogram of Ki-67 expression in the cells gated with CD4^{+}CD25^{high}Foxp3^{+}.

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Figure 1 A

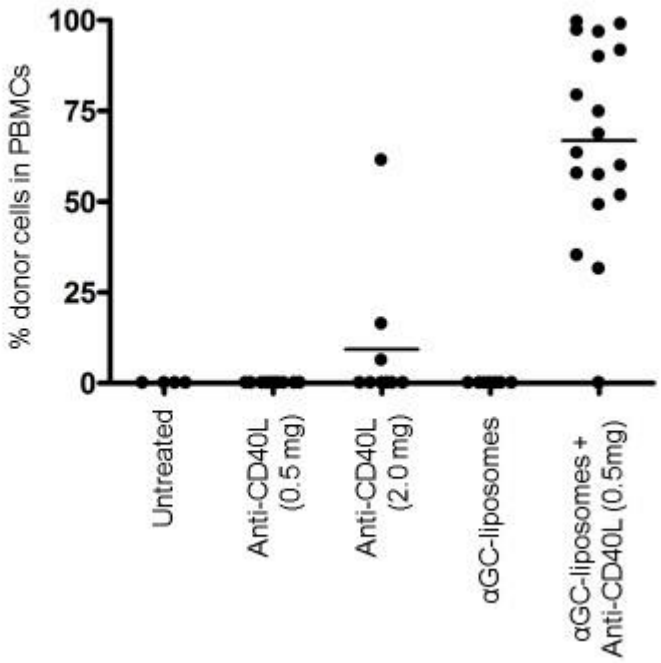

Figure 1 B

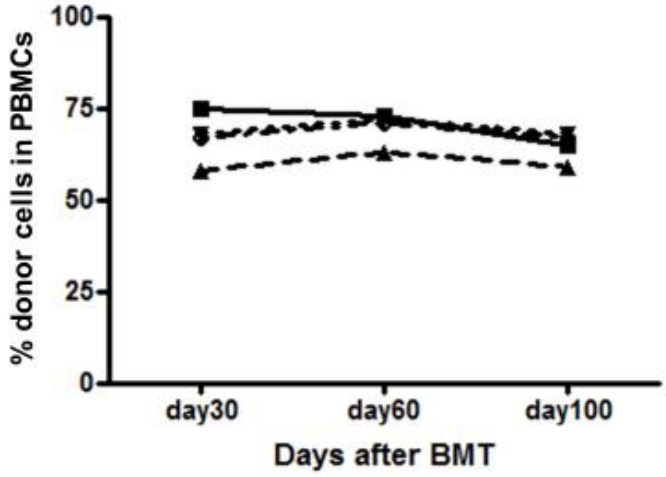

Figure 1 C

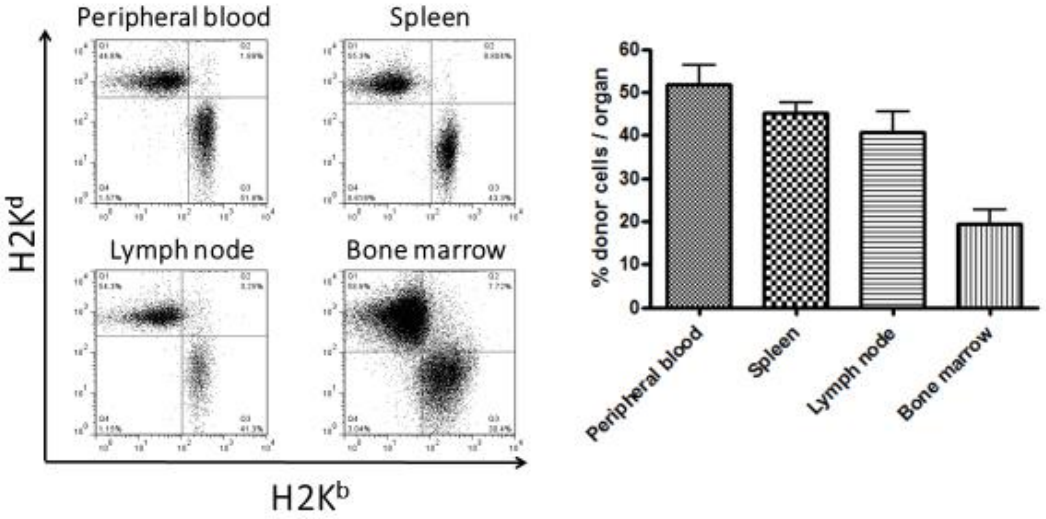

Figure 1 D

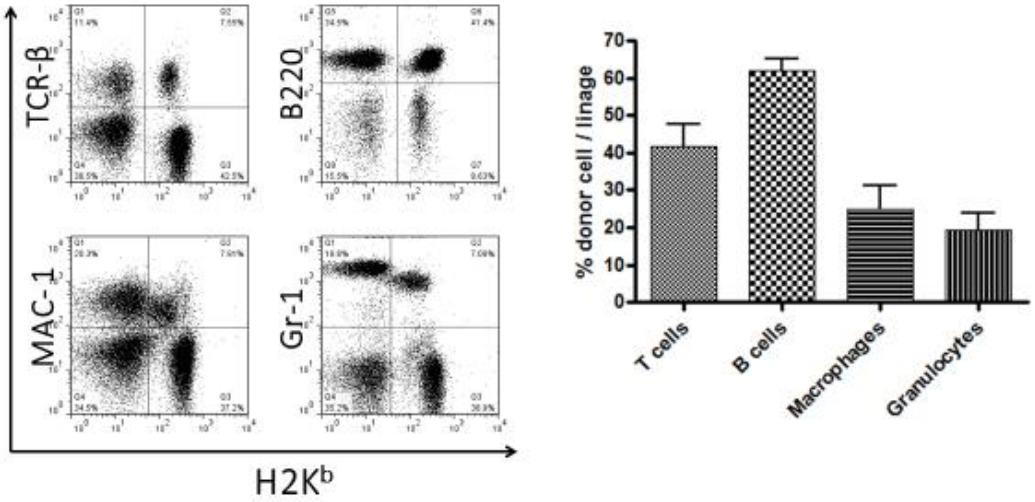

Figure 2

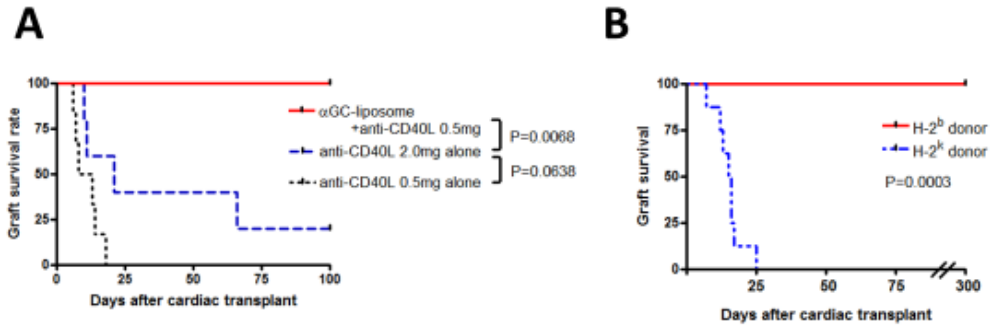

Figure 2 C

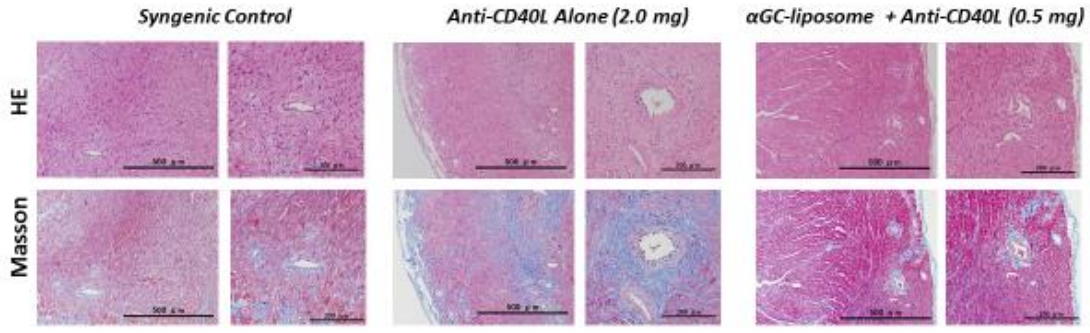

Figure 3 A

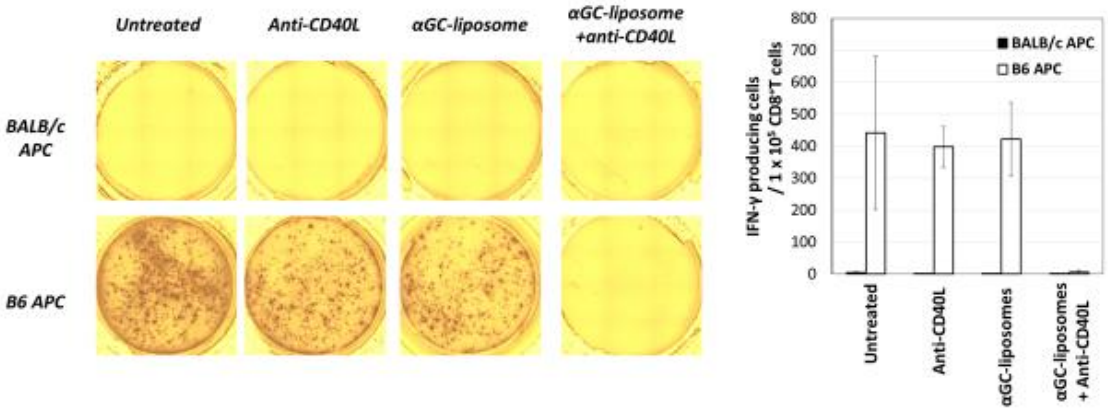

Figure 3 B

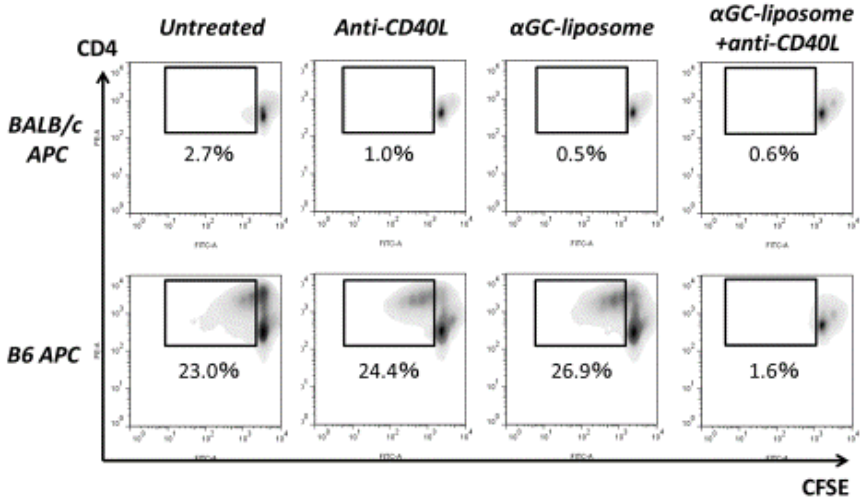

Figure 4 A

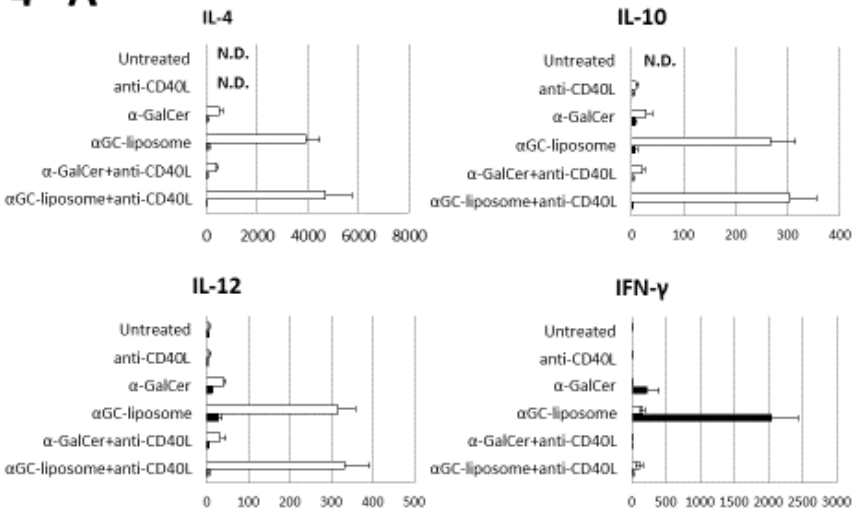

Figure 4 B

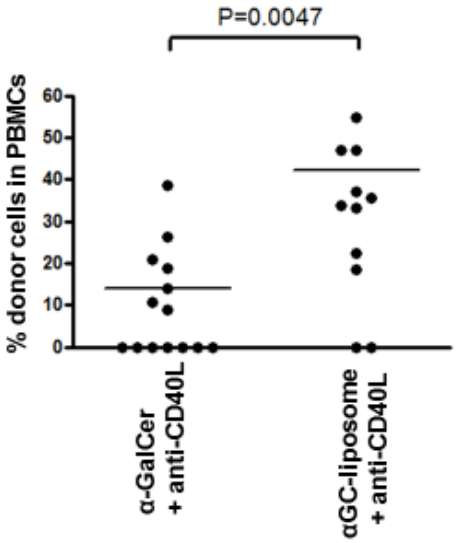

Figure 5

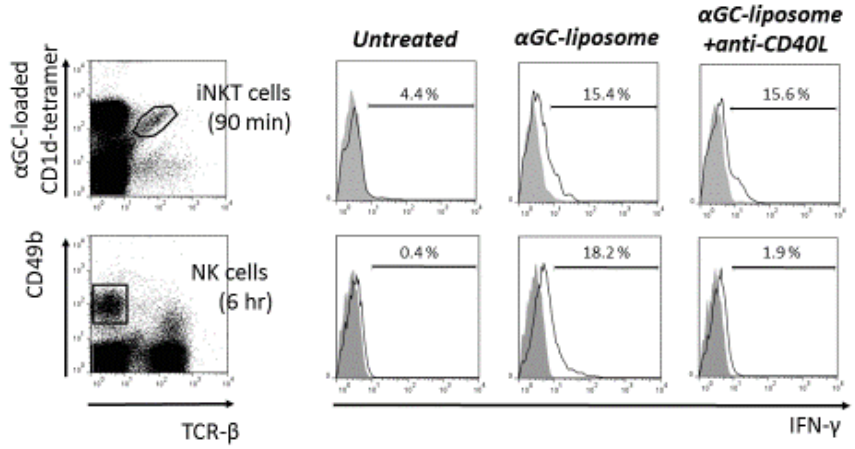

Figure 6 A

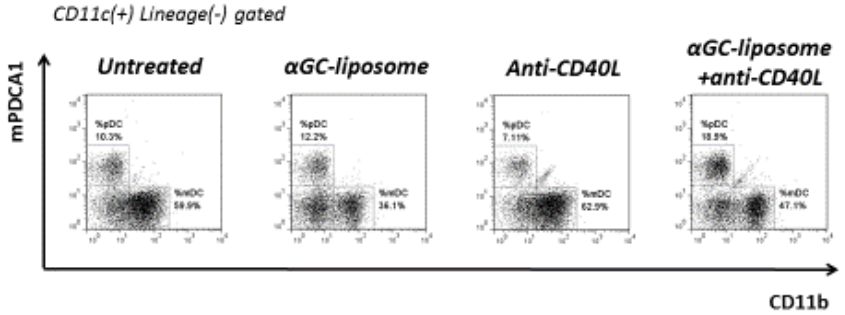

Figure 6 B

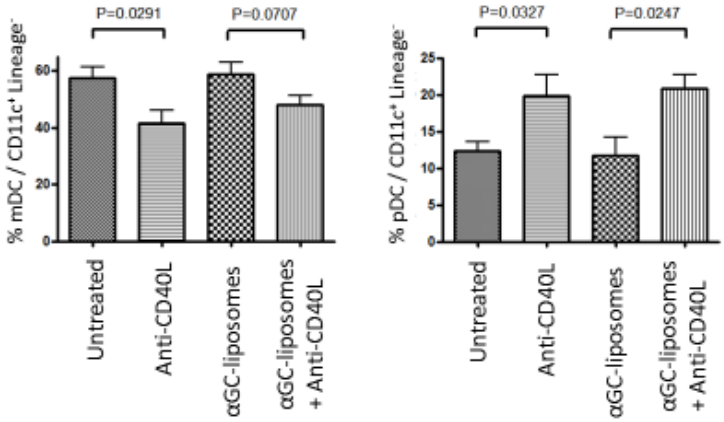

Figure 6 C

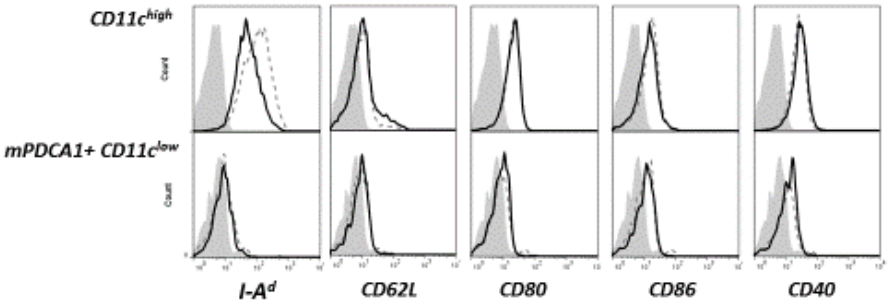

Figure 7 A

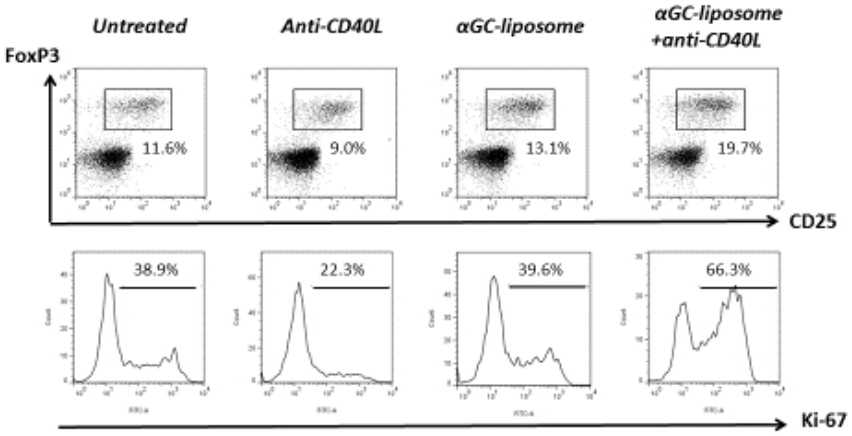

Figure 7 B

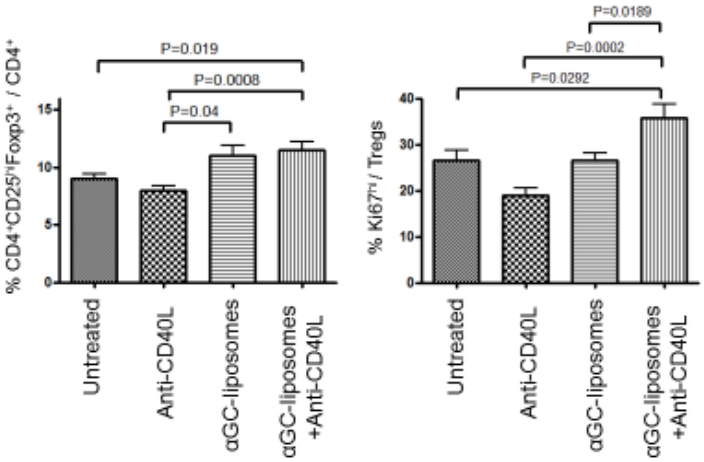

Figure 7 C

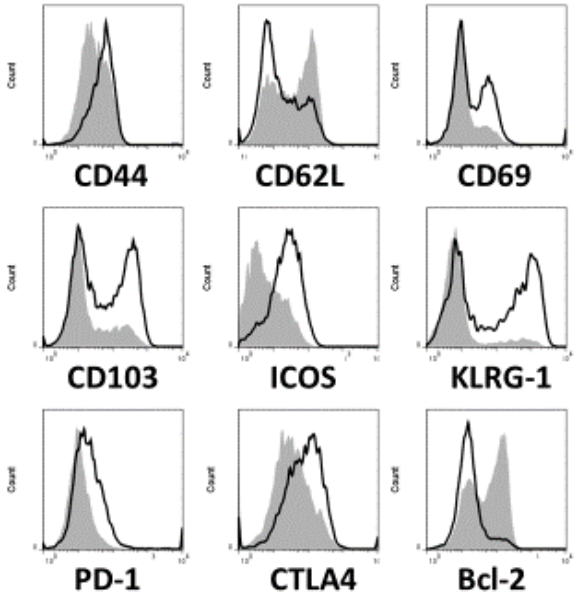

Figure 8 A

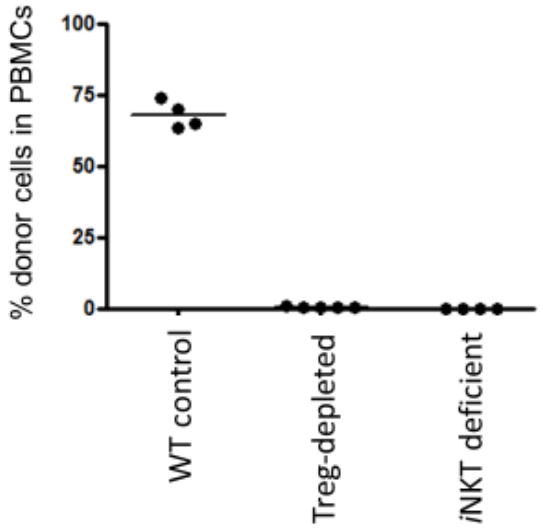

Figure 8 B

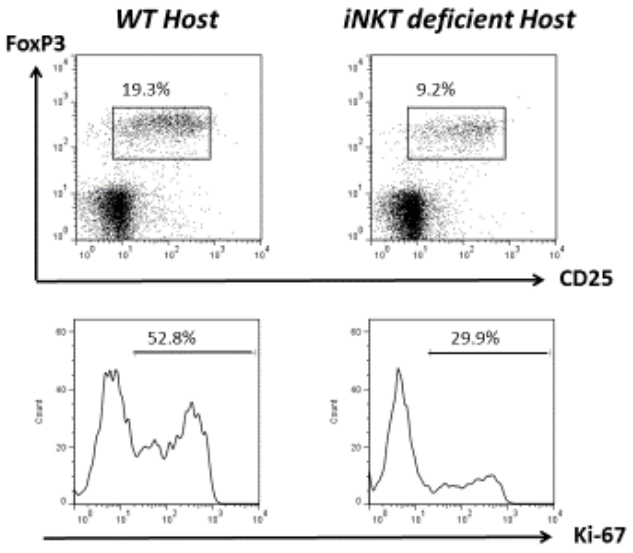

Table 1

Treatments	Fraction of chimera mice* / Total	% Engrafted donor cells
PBS	4 / 4 (100%)	64.6±18.9%
Anti-IL-4	4 / 4 (100%)	49.3±26.2%
Anti-IL-10	4 / 4 (100%)	48.6±16.7%

All mice were transplanted with bone marrow from B6 donor with administration of αGC-liposome+anti-CD40L mAb.
Anti-IL-4/ IL-10 was i.p. injected on d -1at 1.0mg.
% Engraftment was evaluated on d 14 after BMT with FACS scan.
*Chimera mice were defined as >6% of % engrafted donor cells on d 14 after BMT.