Original Article

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Expression of glucocorticoid receptor shows negative correlation with human B-cell engraftment in PBMC-transplanted NOG-hIL-4-Tg mice

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Summary **The humanized mouse system is a promising tool for analyzing human immune responses *in vivo*. Recently, we developed a new humanized mouse system using the severely immunodeficient NOD/Shi-*scid*-IL2rγ^{null} (NOG)-hIL-4-Tg mouse, which enabled us to evaluate the human humoral immune response after peripheral blood mononuclear cell (PBMC) transplantation. However, the mechanism by which hIL-4 enhances antigen-specific IgG production in these mice is not clear. In this study, we analyzed the relationship between human lymphocyte subsets and the expression level of the glucocorticoid receptor (GR) to clarify the humoral immune condition in human PBMC-transplanted NOG-hIL-4 mice. The results showed that the human GR mRNA level was significantly lower in NOG-hIL-4-Tg splenocytes than in conventional NOG splenocytes after immunization. Whereas no obvious difference of the proportion of T helper-cell subsets was observed between the NOG and NOG-hIL-4-Tg mouse strains, the B-cell proportion and antigen-specific IgG concentration in plasma showed strong negative correlations with the GR mRNA level. These results suggest that the GR expression level was changed in PBMCs in the humanized NOG-hIL-4-Tg mice, which may support B-cell survival and function in the mouse system.**

Keywords: Humanized mouse, IL-4, Th subset, glucocorticoid receptor

1. Introduction

The humanized immune system mouse model is a promising tool in personalized medicine because the system can be used to anticipate the efficiency of each treatment before the actual drug administration (*1*). There are three main systems to reconstruct human immunity in the immunodeficient mice (*2-7*). The human immune cells used for transplantation are categorized as hematopoietic stem cells (*8-10*), peripheral blood mononuclear cells (*11-13*), and immune-related tissues, *i.e.*, bone marrow, thymus and/or fetal liver (*14,15*). Severely immunodeficient mice transplanted with human peripheral blood mononuclear cells (PBMCs) are affected by graft-versus-host disease (GVHD) within a short period (*12*). Previously, we developed NOD/Shi-*scid*-IL2rγ^{null} (NOG)-hIL-4-transgenic (Tg) mice, which systemically express human interleukin-4 (hIL-4) in the NOG mouse (*16*). This mouse strain suppressed GVHD after PBMC transplantation. In these mice, human lymphocytes

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were engrafted for a longer period without GVHD symptoms. Moreover, use of this mouse system enabled the induction of antigen-specific IgG production after immunization. The detailed mechanism of enhancing antibody production in hIL-4 Tg mice has not been clarified, but IL-4 was reported to support expansion of plasmablasts (*17*) and modify the proportion of T helper (Th)-cell subsets, especially Th2 and T follicular helper (Tfh) cells (*18*). While Th2 cells have been believed to function mainly in specific antibody production, Tfh, a subset existing in the germinal centers, has recently been shown to be necessary for the production of high-affinity IgG antibodies (*19-21*). Furthermore, the increase in regulatory T (Treg), which suppresses the immune reaction, may be observed to suppress the onset of GVHD (*22,23*).

The glucocorticoid receptor (GR), a steroid hormone receptor (*24-26*), is expressed in lymphocytes and is well known as a powerful immune regulator that can control GVHD symptoms (*27-29*). Therefore, glucocorticoid (GC) might play an important role in the suppression of GVHD symptoms in NOG and NOG-hIL-4-Tg mice transplanted with human PBMCs. On the other hand, GC is reported to suppress B-cell survival (*30*) or class switching by the suppression of activation-induced cytidine deaminase (AID) expression (*31*). This finding suggests that the highly expressed GC may inhibit specific IgG production in the NOG-based humanized mouse. Moreover, the effect of GC is still controversial on Treg- or Tfh-cell development (*29,32*).

In this study, we examined the correlation between GR expression level and lymphocyte cellularity to contribute to the clarification of the mechanism of enhancement of antibody production and suppression of GVHD in the NOG-hIL-4-Tg mouse system.

2. Materials and Methods

2.1. *Ethical approval*

Human PBMCs from healthy volunteer donors were obtained after receiving written informed consent based on Institutional Review Board-approved protocols according to institutional guidelines. This work was approved by the Tokai University Human Research Committee (12R-002) and Central Institute for Experimental Animals (CIEA) Human Research Committee (08-01). These studies were conducted in accordance with the Declaration of Helsinki protocols and all Japanese federal regulations required for the protection of human subjects. The use of immunodeficient mice for xenotransplantation studies was approved in compliance with the Guidelines for the Care and Use of Laboratory Animals, and all animal studies were approved by the committees of CIEA and the Tokai University School of Medicine.

2.2. *Mouse strains*

NOD/Shi-scid-IL2rγ^{null} (NOG; formal name, NOD.Cg-*Prkdc*^{scid}*il2rγ*^{tm1Sug}/ShiJic) mice (*3*) were purchased from InVivo Science, Inc. (IVS, Kawasaki, Japan). NOG-hIL-4 Tg (formally, NOD.Cg-*Prkdc*^{scid}*il2rγ*^{tm1Sug}/ShiJic *Tg*(*CMV-IL4)3-2*/Jic) mice (*16*) were previously generated by CIEA and maintained at Tokai University School of Medicine under specific-pathogen-free (SPF) conditions. The offspring with the inserted transgene were selected as reported previously (*16*).

2.3. *ELISA/EIA*

Peripheral blood (PB) samples were collected from the orbital venous plexus of 4- to 6-week-old mice using heparin (Novo-heparin; Mochida Pharmaceutical Co., Tokyo, Japan)-coated capillaries (Drummond Scientific, Broomall, PA, USA) under anesthesia. The level of human IL-4 protein was measured using a Human IL-4 ELISA Set BD OptEIA™ (BD OptEIA™, BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Corticosterone was measured using a YK240 Corticosterone EIA kit (Yanaihara Institute Inc, Shizuoka, Japan) according to the manufacturer's instructions. The protocol for specific IgG antibody detection has been previously described (*33*). Briefly, microwells in microtiter plates (Sumiron, Tokyo, Japan) were coated with CH401MAP peptide (1 μg/mL) diluted in carbonate buffer (pH 9.5), and the antigens were adsorbed to the microwells overnight at 4°C. The wells were washed with phosphate-buffered saline (PBS)-Tween (0.05%, v/v) and blocked with 3% bovine serum albumin (BSA)-PBS at room temperature (RT) for 2 h. After three washes with PBS-Tween, 10-fold serial dilutions of mouse plasma were added to the wells and incubated for 2 h at RT. The plates were washed three times before addition of biotin-conjugated mouse anti-human IgG monoclonal antibody (mAb) (BD Pharmingen, San Diego, CA, USA) (1:3,000, v/v). After a 2 h incubation at 37°C, the plates were washed 3 times, followed by the addition of streptavidin-horseradish peroxidase (1:50,000, v/v; BD Pharmingen). The plates were incubated for 1 h at RT, and unbound conjugates were removed by washing. EIA substrate kit solution (Bio-Rad Laboratories, Hercules, CA, USA) was then added to each well. The reaction was stopped with 10% HCl, and the absorbance was measured at 450 nm.

2.4. *Preparation and transplantation of human PBMCs into NOG mice and NOG-IL-4-Tg mice*

The healthy donors (HD) enrolled in the experiments and all of mice transplanted with HD PBMC are listed in Table 1. A total of 7.5 mL of PB from healthy donors was drawn into Vacutainer ACD tubes (NIPRO Corporation,

Table 1. List of NOG and NOG-IL-4-Tg mice transplanted with HD PBMCs (The percentage was estimated by FCM-analyzed lymphocyte-gated data)

Mouse No.	Donor	Cell No. ($\times 10^6$)	hIL-4 (pg/mL)	GC (ng/mL)	GR (relative amount)	Specific Antibody (ng/mL)	CD19+ (%)	% of CD45			% of CD4				
								CD3+ (%)	CD8+ (%)	CD4+ (%)	Th1 (%)	Th2 (%)	Th17 (%)	Tfh (%)	Treg (%)
PBS-NOG-hIL-4-Tg															
#1	HD1	230	221	91	0.118	1.6	0.9	99.1	75.9	20.3	38.6	0.98	0.13	0.89	4.63
#2	HD2	62	240	12	0.061	1	1.0	99.00	64.5	30.2	15.9	1.41	0.14	1.01	5.82
#3	HD3	110	251	131	0.123	14.6	12.9	87.1	13.5	68.3	31.4	1.13	0.18	1.91	5.46
#4	HD3	91	431	140	0.034	1.6	9.9	90.1	21.9	62.6	24.0	0.54	0.07	0.90	3.50
#5	HD4	46	404	286	0.114	29.5	11.4	88.8	14.7	60.1	6.9	0.25	0.02	0.16	3.68
#6	HD5	200	157	9	0.056	–*	7.5	92.5	38.0	50.7	48.1	0.91	0.18	0.35	1.56
CH401MAP-NOG-hIL-4-Tg															
#7	HD1	230	220	131	0.074	40.2	22.9	77.1	11.4	60.2	43.2	0.34	0.03	0.95	4.10
#8	HD2	120	181	64	0.114	7.6	12.5	87.5	35.8	47.8	40.0	0.52	0.08	1.03	2.92
#9	HD3	45	268	112	0.152	9.6	14.6	85.4	17.6	58.7	25.4	0.75	0.13	1.59	5.23
#10	HD3	74	494	137	0.063	–	21.7	78.2	20.3	55.2	31.6	0.65	0.12	0.88	4.16
#11	HD4	69	402	181	0.051	15.2	21.5	79.1	2.20	69.9	10.3	0.17	0.01	0.65	2.46
#12	HD5	120	196	82	0.069	–	13.3	86.7	16.0	59.6	30.6	0.44	0.04	1.59	8.31
PBS-NOG															
#13	HD1	130	nd**	188	0.462	–	1.1	98.9	33.2	58.7	22.6	1.25	0.04	1.19	3.77
#14	HD1	63	nd	188	0.041	32.3	3.7	96.4	37.4	47.1	25.3	0.26	0.08	1.43	5.46
#15	HD3	71	nd	195	0.187	22	1.3	98.7	36.8	53.6	20.3	5.55	0.64	0.40	1.25
#16	HD4	200	nd	162	0.110	11	0.6	99.4	16.3	78.1	42.4	0.52	0.12	1.03	2.78
CH401MAP-NOG															
#17	HD1	95	nd	157	0.247	–	2.4	97.7	48.3	35.8	26.8	1.02	0.05	0.24	3.88
#18	HD2	29	nd	75	0.183	1.5	0.6	99.4	48.4	35.2	24.8	1.15	0.12	0.43	3.97
#19	HD3	46	nd	69	0.165	8	7.3	92.7	14.0	74.5	34.4	0.10	0.03	1.90	1.74
#20	HD4	88	nd	187	0.095	32.1	12.6	87.4	11.0	72.0	43.5	0.53	0.07	2.07	6.21

* Not determined. The measurement failed because of nonspecific cross-reactivity. ** Not detected.

Osaka, Japan) containing heparin. The collected PB was immediately placed in 10 mL of Ficoll-Hypaque (Sigma-Aldrich, London, UK), and mononuclear cells were isolated by density centrifugation (500 × g, 30 min, 20°C). The cells were washed with PBS for 5 min at 300 × g, 4°C. Numbers of PBMCs ranging from 2.5 to 5 × 10^6 were intravenously transplanted into 8- to 12-week-old NOG or NOG-hIL-4-Tg mice. Two mice from each strain were transplanted with the same donor PBMCs, to use one for peptide immunization and the other for negative control (PBS and Freund's adjuvant).

2.5. *Peptide immunization*

A CH401 peptide, which includes the epitope sequence of the anti-human epidermal growth factor receptor 2 (HER2) mAb, was determined using multiple antigen peptides (MAP) with a partial amino acid sequence of HER2/neu previously (*34*). The peptide was synthesized using a Rink amide resin (0.4-0.7 mmol/g), an ACT357 peptide synthesizer (Advanced Chemtech, Louisville, KY, USA) and a multiple antigen peptide named CH401MAP, a 20-mer peptide of the HER2 molecule that was synthesized as an antigen peptide. The HER2 peptide was emulsified with complete Freund's adjuvant (CFA) (Wako Pure Chemical Industries, Ltd, Osaka, Japan) (50 μg/head, 100 μL, 1:1, v/v) and administered intraperitoneally to the PBMC-transplanted NOG-hIL-4-Tg mice in which the hIL-4 level was greater than 100 pg/mL in the plasma. For the negative control, an equal volume of PBS was emulsified and injected into PBMC-transplanted NOG-hIL-4-Tg mice. Boosters were performed using incomplete Freund's adjuvant (IFA) (Wako Pure Chemical Industries, Ltd) at 2 weeks after the first immunization. Two weeks after the booster, the mice were sacrificed for analyses.

2.6. *Analysis of engrafted human cells in human PBMC-transplanted mice*

At 4 weeks after transplantation, the mice were euthanized for analysis. PB was collected in the presence of heparin *via* retro-orbital bleeding under inhalation anesthesia at 4 weeks after transplantation. The mice were sacrificed and analyzed for human T- and B-cell development and antibody production. Engraftment and differentiation of human cells in the spleen cells were analyzed by flow cytometry (FCM) staining with anti-human antibodies as described below. Fluorochrome-conjugated anti-human mAbs were used to identify human immune cells. The cells were incubated with appropriate dilutions of fluorescently labeled mAbs for 15 min at 4°C and were then washed with PBS containing 1% (w/v) BSA. The cells were analyzed using FACS Fortessa, Verse, or Canto (BD Bioscience, Franklin Lakes, NJ, USA). For each analysis, the live gate with white blood cells or lymphocytes was further gated based on hCD45 expression. The mouse anti-human mAbs used in this study are listed in Table S1 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=24*).

2.7. *Real time RT PCR*

A total of 5 × 10^5 cells from NOG or NOG-IL-4-Tg mouse spleens were collected, stored in TRIzol (Invitrogen, Carlsbad, CA, USA) at –80°C and extracted according to the manufacturer's instructions. The total RNA concentration was determined by measuring absorbance at 260 nm. The purity was estimated by the relative ratio of the absorbance at 260/280 nm. Integrity was assayed by agarose gel electrophoresis. The purity and integrity were greater than 95%. cDNA was synthesized from the total RNA (2 μg) using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, CA, USA). TaqMan probes for human common GR and β-actin (Thermo Fisher Scientific Co., Ltd., MA, USA), were used and RT-PCR was conducted by Applied Biosystems Step One Plus Real-Time PCR Systems in all analyses. A commercially available Taqman Fast Universal PCR Master mix (Thermo Fisher Scientific) was used for PCR amplification and detection of GR (Hs00353740_m1). All samples were assessed in triplicate. Quantification of the expression of the target gene in relation to the respective housekeeping gene was carried out employing the comparative (ΔΔ) Ct method.

2.8. *Statistics*

Statistical analysis was performed with Microsoft Excel (Microsoft, Redmond, WA, USA). The data are shown as the mean ± SD. Significant differences between groups were determined by two-sided Student's *t*-test analysis.

3. Results

3.1. *Correlation between human IL-4 and Th and B cell proportions in humanized NOG-hIL-4-Tg mice*

First, we purified donor PBMCs and transplanted them into 2 to 3 NOG and NOG-hIL-4-Tg mice. After the transplantation, CH401MAP, a breast cancer vaccine candidate that is a part of the HER2 molecule (*34,35*), emulsified with FCA was injected into the mice, and a booster was administered after 2 weeks. A mouse transplanted with PBMCs from the same donor as those transplanted into the CH401MAP-vaccinated mouse was injected with PBS/FCA and used as a control mouse. After 4 weeks, mice were sacrificed, and spleen cells were obtained as shown in Figure 1A and Table 1. The mean spleen cell number was 1.23 ± 0.69 (× 10^8) in PBS-NOG-hIL-4-Tg mice, 1.10 ± 0.60 (× 10^8) in CH401-NOG-hIL-4-Tg mice, 1.16 ±

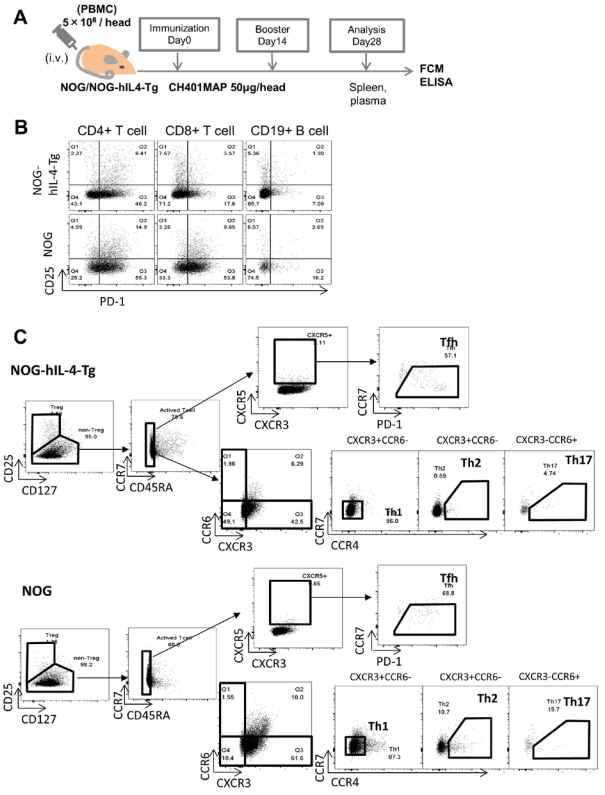

Figure 1. Characterization of human lymphocyte subsets in PBMC-transplanted humanized mice. (A) Protocol for analysis of the human PBMC-transplanted humanized mouse. **(B)** FCM analysis of CD25 and PD-1 expression in human CD4+ and CD8+ T cells and B cells in spleens of NOG-hIL-4-Tg and NOG mice at 4 weeks after PBMC transplantation. **(C)** FCM analysis of human Th subsets in spleens of NOG-hIL-4-Tg and NOG mice at 4 weeks after PBMC transplantation. Representative data from 12 NOG-hIL-4-Tg and 8 NOG are shown.

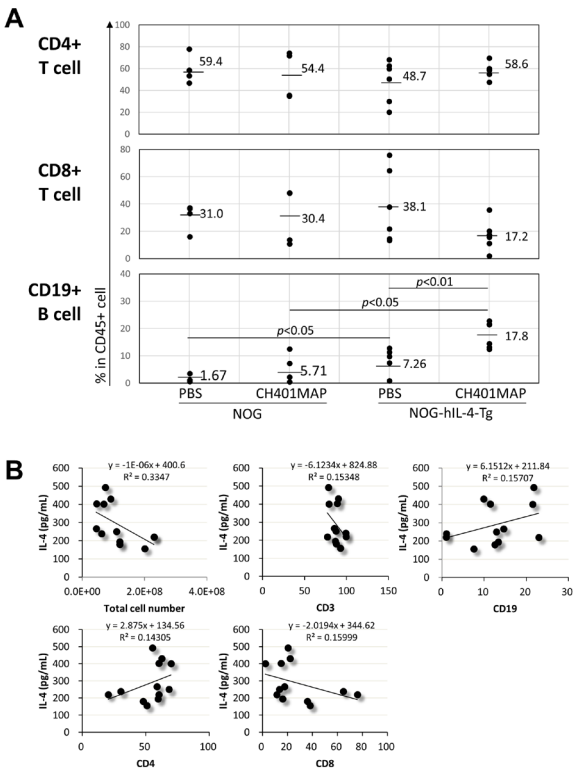

Figure 2. Relationship between the human IL-4 level and lymphocyte subsets in PBMC-transferred humanized mice. (A) The percentage of human CD4+ and CD8+ T cells and B cells in CD45+ cells in spleen cells from NOG-hIL-4-Tg and NOG mice at 4 weeks after PBMC transplantation. PBS; the mice treated with adjuvant alone. CH401MAP; the mice treated with CH401MAP emulsified with Freund's adjuvant. PBS-NOG (n = 4), CH401MAP-NOG (n = 4), PBS-NOG-hIL-4-Tg (n = 6), CH401MAP-NOG-hIL-4-Tg (n = 6). **(B)** Comparison of the correlation between the plasma hIL-4 level and total cell number and proportions of CD3+ T cells, CD19+ B cells, CD4+ T cells, and CD8+ T cells in CD45+ cells in NOG-hIL-4-Tg mice. PBS-NOG-hIL-4-Tg (n = 6), CH401MAP-NOG-hIL-4-Tg (n = 6). Correlation equations and R2 are shown in each panel.

0.55 (× 10^8) in PBS-NOG mice and 6.45 ± 0.28 (× 10^7) in CH401-NOG mice as shown in Table 1. Thus, the spleen cell number in the CH401MAP-immunized NOG mouse was significantly lower than that in the other three groups. In the NOG-hIL-4-Tg mice, the IL-4 concentration in the plasma showed a negative correlation with total spleen cell number (Figure 2B, upper left panel).

These results suggest that IL-4 expression affects the cell number in the transplanted mouse spleen and that this effect may be maintaining the splenic cell number after immunization.

3.2. *Flow cytometry analysis of human lymphocytes transplanted into NOG and NOG-hIL-4-Tg mice*

To compare the lymphocyte subsets of the mice, we conducted multicolor-flow cytometry using the antibodies shown in Table S1 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=24*). At first, we analyzed human CD4+ and CD8+ T cells, B cells and expression of CD25, an activation/regulatory marker in the spleen of NOG and NOG-hIL-4-Tg mice. As shown in Figure 1B and Figure 2A (upper two panels), no significant difference was observed in the proportions of CD4+ T cells and CD8+ T cells between NOG and NOG-hIL-4 Tg mice with or without immunization, whereas CD19+ B cell levels were significantly higher in NOG-hIL-4-Tg mice than in conventional NOG mice (Figure 2A, lower panel). This result was consistent with our previous report (*16*). On the other hand, immunized NOG-hIL-4-Tg mice possessed the highest proportion of B cells (17.8%).

Next, we compared the mean fluorescent intensity (MFI) of the programmed death-1 (PD-1) molecule on CD4+ T cells between immunized and nonimmunized mouse spleen cells because PD-1 is an activation/exhaustion marker of lymphocytes (Figures 1B and S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=24*). As a result, in the NOG-hIL-4-Tg mouse, the PD-1 MFI was higher in CD4+ T cells than in adjuvant-alone CD4+ T cells ($p < 0.05$), while in the conventional NOG mouse, no significant difference was observed between the

adjuvant-alone and peptide-immunized CD4+ T cells (Figure S1, upper panel, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=24*). For CD8+ T cells, while PD-1 expression was lower than that in CD4+ T cells overall, PD-1 MFI tended to increase after peptide immunization, but the expression was lower in the NOG-hIL-4-Tg mouse than in the conventional NOG mouse ($p < 0.01$) (Figure S1, lower panel, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=24*). These results suggest that the NOG-hIL-4-Tg mouse possessed a higher number of B cells, but the CD4+ T cells and CD8+ T cells are not significantly different compared with those of the conventional NOG mouse. The activation/exhaustion of T cells was indicated after antigen-immunization in the NOG-hIL-4-Tg mouse.

The total spleen cell number was compared with the IL-4 level in the plasma of the NOG-hIL-4-Tg mice. As a result, IL-4 expression was shown to have a negative correlation with the number of splenic cells in the NOG-hIL-4-Tg mice, irrespective of the treatment (Table 1 and Figure 2B, upper left panel). When the proportions of T cells and B cells were compared with IL-4 expression, the total T-cell proportion showed a weak and negative correlation ($R^2 = 0.15348$) to the plasma IL-4 level (Figure 2B, upper middle panel), while the B-cell proportion showed a weak and positive correlation ($R^2 = 0.15707$) with the plasma IL-4 level (Figure 2B, upper right panel). In the T-cell subsets, CD4+ T cells showed a positive correlation ($R^2 = 0.14305$), but CD8+ T cells showed a negative correlation ($R^2 = 0.15999$) (Figure 2B, lower panels). These results suggest that the proportion of CD4+ T cells but not CD8+ T cells may be increased along with GR expression and influence B-cell survival and/or function.

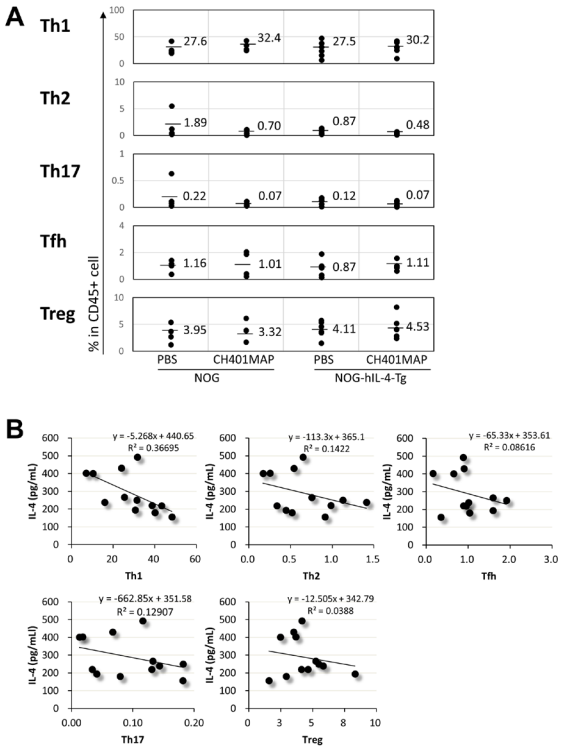

Figure 3. Relationship between human IL-4 level and Th subsets in PBMC-transferred humanized mice. (A) The percentage of Th-cell subsets in the spleen cells of NOG-hIL-4-Tg and NOG mice at 4 weeks after PBMC transplantation. The same mice described in Figure 2 were used. PBS-NOG ($n = 4$), CH401MAP-NOG ($n = 4$), PBS-NOG-hIL-4-Tg ($n = 6$), CH401MAP-NOG-hIL-4-Tg ($n = 6$). **(B)** Comparison of the correlation between plasma hIL-4 level and the proportions of Th1, Th2, Tfh, Th17, and Treg cells in CD45+ cells in NOG-hIL-4-Tg mice. PBS-NOG-hIL-4-Tg ($n = 6$), CH401MAP-NOG-hIL-4-Tg ($n = 6$). Correlation equations and R2 are shown in each panel.

3.3. *Flow cytometry of human Th cells transplanted into NOG and NOG-hIL-4-Tg mice*

Since the Th-cell proportion increase was dependent on IL-4 expression, we analyzed the Th-cell subset proportion and examined the correlation to IL-4 concentration in plasma. To compare the Th-cell subsets of the mice, we defined the subsets using the antibodies shown in Table S1 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=24*. As shown in Figure 1C, we analyzed the Th-cell subsets (Th1, Th2, Th17, Tfh and Treg) developed in the mice (*36*).

After the transplantation of human PBMCs into the NOG and NOG-hIL-4-Tg mouse models, the proportion of human Th-cell subsets was examined with/without peptide stimulation. Th1 cells were dominant in both NOG and NOG-hIL-4-Tg mice transplanted with human PBMCs as shown in Table 1, Figure 1C and Figure 3A. Th2 cells did not increase in NOG-hIL-4-Tg mice compared with NOG mice, irrespective of peptide immunization. In addition, Th17, Treg, and Tfh cells did not increase in either NOG or NOG-hIL-4-Tg mice. These results indicated that the proportion of Th-cell subsets are not affected by the plasma IL-4 level in the NOG-hIL-4-Tg mice.

Next, we examined the correlation between plasma IL-4 concentration and Th-cell subset. All the Th-cell subsets showed a negative correlation with IL-4 concentration (Figure 3B). For example, Th1, Th2 and Th17 proportions showed moderate correlations with IL-4 plasma concentration (Slope = –5.268, R2 = 0.36695 for Th1; Slope = –113.3, $R^2 = 0.1422$ for Th2; Slope = –662.85, $R^2 = 0.12907$ for Th17). Other Th-cell subsets had no correlation, while the B-cell proportion showed a moderate positive correlation with IL-4 ($R^2 = 0.15707$) (Figure 2B, upper right panel).

These results suggest that human IL-4 expressed in the NOG-hIL-4-Tg mouse was not sufficient for induction of Th2 cells or enhancement of Tfh cells but was sufficient to decrease Th1 cells. Therefore, the increase in B-cell number did not appear to be directly correlated with enhanced Th2 or Tfh cells.

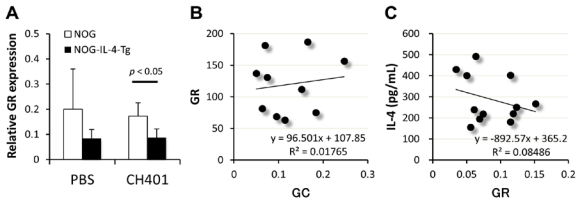

Figure 4. GR expression in PBMC-transferred humanized mice. (A) Relative GR expression in the spleen cells of the mice was shown. White bars represent NOG mice, and black bars represent NOG-hIL-4-Tg mice. Student's *t*-test was performed, and the significant difference ($p < 0.05$) is shown. PBS-NOG ($n = 4$), CH401MAP-NOG ($n = 4$), PBS-NOG-hIL-4-Tg ($n = 6$), CH401MAP-NOG-hIL-4-Tg ($n = 6$). **(B)** Comparison of the correlation between GC in the plasma and GR expression in the spleen cells of CH401MAP–NOG and CH401MAP-NOG-hIL-4-Tg mice were further analyzed. CH401MAP-NOG ($n = 4$), CH401MAP-NOG-hIL-4-Tg ($n = 6$). **(C)** The correlation between human IL-4 concentration in plasma and GR expression in the spleen cells of PBS-NOG-hIL-4-Tg and CH401MAP-NOG-hIL-4-Tg were analyzed. PBS-NOG-hIL-4-Tg ($n = 6$), CH401MAP-NOG-hIL-4-Tg ($n = 6$). Correlation equations and R2 are shown in each panel.

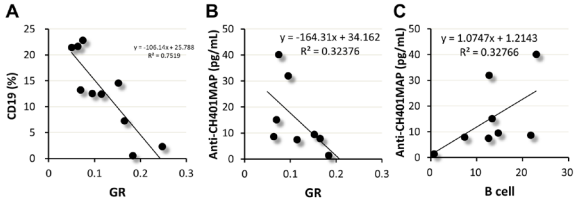

Figure 5. Relationship between GR expression and antibody production in PBMC-transferred humanized mice. (A) Comparison of the correlation between CD19+ B cells (% in CD45+ cells) and relative GR expression in the spleen cells of CH401MAP-NOG ($n = 4$) and CH401MAP-NOG-hIL-4-Tg mice ($n = 6$). **(B)** The correlation between anti-CH401MAP IgG antibody in plasma and GR expression in the spleen cells of CH401MAP-NOG ($n = 3$) and CH401MAP-NOG-hIL-4-Tg ($n = 5$) mice. **(C)** Comparison of the correlation between CD19+ B cells (% in CD45+ cells) and anti-CH401MAP IgG antibody in the plasma of CH401MAP-NOG ($n = 3$) and CH401MAP-NOG-hIL-4-Tg ($n = 5$) mice. Correlation equations and R2 are shown in each panel.

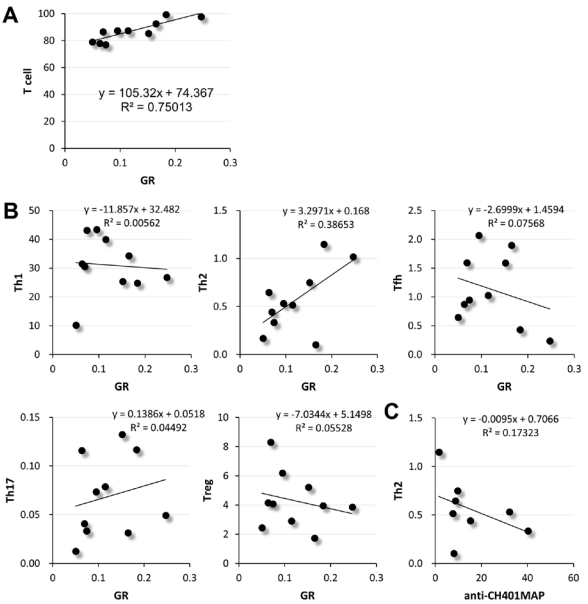

Figure 6. Relationship between GR expression and Th subsets in PBMC-transferred humanized mice. (A) Comparison of the correlation between CD3+ T cells (% in CD45+ cells) and relative GR expression in the spleen cells of CH401MAP-NOG ($n = 4$) and CH401MAP-NOG-hIL-4-Tg ($n = 6$) mice. **(B)** The correlation between each Th cell subset (Th1, Th2, Tfh, Th17, and Treg) and GR expression. **(C)** The correlation between Th2 cells and anti-CH401MAP IgG antibody. Correlation equations and R2 are shown in each panel.

3.4. *Glucocorticoid receptor expression negatively correlates with B cells*

As GC signal is important for T-cell regulation (*29,32*), we hypothesized that IL-4 affected the decrease in GC signal. Therefore, we measured the concentration of GC and the expression of the GR in human lymphocytes, which affects GC signaling in T cells and B cells.

Spleen cells were collected from PBMC-transplanted and immunized mice, mRNA was purified and the GR mRNA was measured by real-time PCR. The results showed that GR expression tended to be low in immunized NOG-hIL-4-Tg mice compared with that in conventional NOG mice in the nonimmunized group, but a significant difference was not observed (Figure 4A). However, a significant difference was observed between immunized NOG and NOG-hIL-4 Tg mice, as shown in Figure 4A. In the immunized group, NOG-hIL-4-Tg mouse GR expression was significantly lower than that in conventional NOG mouse. However, the plasma corticosterone (representative of mouse GC, selected as the GC in the humanized mouse mainly produced by mouse tissues) level neither was significantly different nor showed a correlation with GR expression (Table 1 and Figure 4B).

These results demonstrated that humanized NOG mouse lymphocytes produce GR, which is suppressed by human IL-4. However, IL-4 expression and GR expression showed no correlation in NOG-hIL-4-Tg mice (Figure 4C).

3.5. *Correlation of GR with Th and B cells*

As GR expression was significantly different between the NOG and NOG-hIL-4-Tg mice and no correlation was observed between the difference and the level of corticosterone produced by the mice, we tried to analyze whether the difference was correlated with the lymphocyte subset differentiation/survival.

As spleen B-cell number was increased with IL-4 expression (Figure 2B, upper right panel), we analyzed the specific IgG production in the mouse. PB was collected from these mice, and the plasma was subjected to anti-CH401MAP IgG ELISA. The result indicated that GR expression showed a strong inverse correlation with the proportion of B cells ($R^2 = 0.75192$) (Figure 5A) and anti-CH401MAP IgG level ($R^2 = 0.32376$) (Figure 5B). The slope on a graph was not significantly

different between the NOG and NOG-hIL-4-Tg mouse models (Slope of NOG; –72.441, NOG-IL-4-Tg; –77.183) (Figure S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=24*). As the specific IgG and the B cell proportion also showed a strong correlation (R^2 = 0.32776) (Figure 5C), it is suggested that GR expression suppresses B-cell survival or differentiation into antibody-secreting effector cells.

Next, we examined the correlation between GR and Th-cell subsets. The total number of T cells showed a high positive correlation (R^2 = 0.75013) with GR (Figure 6A). Surprisingly, among the five Th-cell subsets (Th1, Th2, Th17, Treg and Tfh), a significant high positive correlation was observed only between Th2 and GR (R^2 = 0.38653) (Figure 6B, upper middle panel). As shown in upper right panel of Figure 6B, GR and Tfh, a subset known to be important for germinal center reaction and highly specific IgG production, showed no correlation (R^2 = 0.07568), suggesting that the development/maintenance of Tfh is not involved in the GR effect.

These results suggest that the suppression of GR expression increased the B cell ratio, not the Tfh ratio, in the spleen of NOG-hIL-4-Tg mice, and eventually the specific IgG concentration is increased in the plasma.

4. Discussion

The NOG-hIL-4-Tg mouse model, which was developed by our group recently, enabled us to suppress GVHD after human PBMC transplantation and to produce specific anti-IgG production after immunization (*16*). This mouse system is promising for the evaluation of human vaccines. However, it has not been clarified how human IL-4 enables specific IgG production or suppresses GVHD in the mouse system. We hypothesized that GR expression may affect the humoral immunity of the humanized mouse, as GVHD is controlled by the glucocorticoid signal. Our results showed that GR expression was significantly lower in NOG-hIL-4-Tg spleen cells after CD401 immunization, and a strong negative correlation with the proportion of B cells and strong positive correlation with that of T cells were observed. Moreover, after the detailed analysis, a high negative correlation between specific IgG production and GR level was obtained, while a correlation with Tfh was not observed. These results suggest that the GR signal is decreased in the NOG-hIL-4-Tg mouse, which makes it difficult to inhibit B-cell function by GC.

IL-4 and GC crosstalk was reported previously, but according to the report, GC administration induced Th2 differentiation (*37*). However, our observation was that systemic expression of human IL-4 suppressed the GR expression of transplanted human PBMCs. It is curious that systemic expression of a high level of IL-4 suppressed GR expression, which may cause a decrease in Th2 differentiation, while GC concentration in plasma was not correlated with GR expression (R^2 = 0.01765) (Figure 4B). This mechanism should be clarified in the future.

There have been many reports involving GC and/or GR expression and immune suppression, mainly with T-cell differentiation and function (*29,38*). Meanwhile, the effects of B-cell differentiation and function are not as well investigated and are less controversial. While a report suggests that GC function showed a negative correlation to B-cell survival and proliferation, another group reports that GC does not show a correlation with B-cell survival and proliferation but affects class-switch from IgM to other isotypes by modifying AID expression (*31*). GC is also reported to increase Treg cells, but another group reported that it does not (*29,32*). Occasionally, GC is used as an anti-inflammatory drug after anticancer treatment, as reported for leukemia (*39*). Thus, the function and the mechanism are not clear, but in our results, GC clearly affects B-cell number and antigen-specific antibody production negatively. Therefore, it shows promise to become important evidence for improving the humanized mouse system based on our model. Moreover, it will become a good model animal to investigate how GC affects the regulation of human antibody production.

Between the NOG and NOG-hIL-4-Tg mouse systems, no significant difference in the proportion of Th-cell subsets was detected. However, the proportion of the Th1 subset also showed a negative correlation with IL-4, which was consistent with a previous report (*40*). Although it is well known that the *in vitro* treatment of high-dose IL-4 induced Th2-cell differentiation (*41,42*), our result demonstrated that the proportion of the Th2 subset did not increase in NOG-hIL-4 Tg mice. As we reported previously (*17*), PMA-Ionomycin (PMA/Io) stimulation induced high expression levels of IL-4 in human CD4+ T cells engrafted in NOG-hIL-4-Tg mice. This contradiction might be resolved by analysis of the differences between the mice with and without *in vivo* stimulation. Kumar *et al*. and Murray *et al*. demonstrated that high-affinity and/or high numbers of antigen signals were polarized into Th1 cells, even in an IL-4-abundant environment (*43,44*). Similar to these reports, our CH401MAP peptide/Freund's adjuvant or Freund's adjuvant alone used to immunize NOG-hIL-4 Tg mice strongly activated human T cells; hence, the majority of T cells might be shifted to Th1 cells in the present study. Further analysis is required to determine the Th status in NOG-hIL-4 Tg mice.

In our results, there was a very low correlation between IL-4 in the plasma and the GR expression level, although NOG-hIL-4-Tg mice showed a lower GR level than conventional NOG mice. Therefore, it may be possible that the concentration of 100 pg/mL in the plasma is sufficient for the suppression of GR expression and that higher concentrations do not affect

the GR expression level further. While GR expression is reported to be increased by dexamethasone, which mimics GC induction *via* the hypothalamic-pituitary-adrenal axis against inflammatory signals such as TNF-α and/or IL-1β (*45*), sufficient IL-4 may suppress the release of these inflammatory cytokines in the blood, resulting in the suppression of GR expression. As spleen B cells are highly sensitive to GC (*30*), suppression of GR and GC signals may be highly beneficial to B-cell survival.

In conclusion, GR expression is decreased in NOG-hIL-4-Tg mice transplanted with human PBMCs. The decrease in GR expression shows a correlation with the increase in B-cell number and specific IgG production.

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