Novel Immunological Approach to Asses Donor reactivity of Transplant Recipients using a Humanized Mouse Model

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Abstract

In organ transplantation, a reproducible and robust immune-monitoring assay has not been established to determine individually tailored immunosuppressants (IS). We applied humanized mice reconstituted with human (hu-) peripheral blood mononuclear cells (PBMCs) obtained from living donor liver transplant recipients to evaluate their immune status. Engraftment of 2.5×10^6 hu-PBMCs from healthy volunteers and recipients in the NSG mice was achieved successfully. The reconstituted lymphocytes consisted mainly of hu-$CD3^+$ lymphocytes with predominant $CD45RA^-CD62L^{lo}$ T_{EM} and $CCR6^-CXCR3^+CD4^+$ Th1 cells in hu-PBMC-NSG mice. Interestingly, T cell allo-reactivity of hu-PBMC-NSG mice was amplified significantly compared with that of freshly isolated PBMCs ($p<0.05$). Furthermore, magnified hu-T cell responses to donor antigens (Ag) were observed in 2/10 immunosuppressed recipients with multiple acute rejection (AR) experiences, suggesting that the immunological assay in hu-PBMC-NSG mice revealed hidden risks of allograft rejection by IS. Furthermore, donor Ag-specific hyporesponsiveness was maintained in recipients who had been completely weaned off IS (n=4), despite homeostatic proliferation of hu-T cells in the hu-PBMC-NSG mice. The immunological assay in humanized mice provides a new tool to assess recipient immunity in the absence of IS and explore the underlying mechanisms to maintaining operational tolerance.

Keywords

liver transplantation

alloreactivity

humanized mouse

Abbreviations:

Ag, antigen

AR, acute rejection

CFSE, Carboxyfluorescein diacetate succinimidyl ester

DC, dendritic cell

DSA, donor specific antibody

FCS, fetal calf serum

Foxp3, Forkhead boxprotein P3

GVHD, graft-versus-host disease;

hu-, human

IS, immunosuppressants

IRB, Institutional review board

LDLT, living donor liver transplant

MFI, mean fluorescence intensity

MLR, mixed lymphocyte reaction

MMF, mycophenolate mofetil

mPSL, methylprednisolone

NASH, non-alcoholic steatohepatitis

NK, natural killer

NSG, NOD.Cg-Prkdc^{scid}IL-2rg$^{tm/Wjl}$/Sz

PBMC, peripheral blood mononuclear cell

TAC, tacrolimus

T_{REG}, regulatory T cells

Th, T helper

VPD, violet proliferation dye

1. Introduction

The development of immunosuppressants (IS) has remarkably improved the short-term outcomes of organ transplantation [1] [2]. Transplant recipients require lifelong IS to prevent transplant rejection, but this inevitably exposes the recipient to various adverse effects, such as carcinogenesis, infection, as well as renal and metabolic disorders [3][4][5][6][7]. Clinically, it is desirable to individually tailor immunosuppressive regimens for each recipient to avoid over-immunosuppression and the risk of allograft rejection caused by insufficient immunosuppression. To date, various immune-monitoring assays have been established to evaluate the immune status of transplant recipients. Previous studies have demonstrated that lymphocyte proliferation assays, such as the mixed lymphocyte reaction (MLR), can predict alloreactivity against donor antigens (Ag) and optimize IS in clinical settings [8][9]. In addition, MLR using carboxyfluorescein diacetate succinimidyl ester (CFSE) or violet proliferation dye (VPD) and IFNγ enzyme-linked immunospot assays provide precise quantification of donor Ag reactivities [10][11][12]. Thus, previous approaches have demonstrated real-time evaluation of the immune status in immunosuppressed recipients. However, these approaches do not indicate the immune status after weaning off IS and bona fide immune responses under non-immunosuppressed conditions. Additionally, *in vitro* immune assays may not reveal an *in vivo* comprehensive immunity by diverse T lymphocyte populations with individual heterogeneous behaviors.

The humanized mouse model has been reported as a useful tool to evaluate *in vivo* hu-immunity in hu-PBMC-engrafted immunodeficient mice [13]. In addition, the recent development of mice reconstituted with

various functional hu-immune cells has led to investigations of hu-specific molecules, evaluation of newly emerging drugs, and analysis of hu-immunity such as the mechanisms underlying the post-transplant organ rejection without risking the recipients [14]. Indeed, the humanized mouse model has been used as a tool to evaluate alloreactivities by transplanting hu-skin, pancreatic islets, and cardiac tissues into immunodeficient mice reconstituted with hu-PBMCs [15][16][17].

Here, we hypothesized that hu-PBMCs from post-transplant recipients could be reconstituted in immunodeficient mice and mimic the immune condition without the influence of IS. To test this hypothesis, we investigated whether hu-PBMCs from post-living donor liver transplant (LDLT) recipients could be applicably reconstituted in immunodeficient mice and how the reconstituted hu-PBMCs harvested from humanized mice respond to allo-Ag. Furthermore, we compared immunological assessment of LDLT recipients in the humanized mouse with previous assays.

2. Materials and methods

2.1. Mice

NOD.Cg-Prkdc^{scid}IL-2rg$^{tm/Wjl}$/Sz (NOD/SCID/IL-2rg^{null}; NSG) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in Hokkaido University in accordance with the guidelines provided by the Institutional Office of Animal Care and Use. Eight- to sixteen week-old mice, which were housed in microisolator cages under specific-pathogen-free conditions, were used for all experiment.

2.2. Hu-PBMCs

Hu-PBMCs were obtained from five healthy adults (Table 1) and 14 LDLT recipients and donors (11 blood relatives and three spouses) followed up at Hokkaido University (Tables 2 and 3). These individuals signed the informed consent approved by the Institutional Review Board (IRB) at Hokkaido University (#008-0129). No recipients with viral hepatitis were enrolled because of ethical and safety considerations. The median age of healthy volunteers was 36 (23–40) years, which was not significantly different from the age at blood collection of other groups (Table 1). Ten recipients (three males and seven females) had undergone LDLT at the age of 44.0 (0.5–70.0) years, who had been maintained on IS including tacrolimus (TAC), mycophenolate mofetil (MMF), methylprednisolone (mPSL), or sirolimus for 5.6 (1.9–24.0) years post-LDLT (Table 2). There were no re-LT cases. The data for sensitization pre- and post-LDLT are shown in Tables S1 and S2. All recipients were assessed by the lymphocyte cross-match test using donor lymphocytes and their sera. When

lymphocyte cross-match tests were positive, donor-specific anti HLA-A, -B, -Cw, -DR, and -DQ antibodies were assessed using the LABScreen® single antigen test (One Lambda Inc., Canoga Park, CA, USA). A normalized mean fluorescence intensity (MFI) greater than 1,000 was considered as positive. During post-transplant periods, anti-HLA antibodies were assessed annually. The anti-HLA antibodies matched two or more alleles of donor HLA loci-defined donor-specific antibodies (DSA). The timelines of AR episodes are shown in Figure S1. AR episodes greater than three are described as `multiple' in Table 2. Four recipients (two males and two females), who had undergone LDLT at the age of 53.8 (0.5–59.3) years, had achieved completed cessation of IS, and median IS-free years were 2.8 (2.5–13.5) (Table 3). Three of four patients (cases #I, #II and #III) who achieved complete cessation of IS were enrolled in the clinical study for weaning off IS approved by the IRB at Hokkaido University (#010-0070). The number of HLA mismatches against donors or third party Ag were greater than three (Tables 1-3) except for those against donor Ag in cases #6, #8 (Table 2), #II, and #III (Table 3).

2.3. Hu-PBMC-NSG mouse model

NSG mice were irradiated with 2 Gy, followed by intraperitoneal (i.p.) injection of 2.5×10^6 freshly isolated hu-PBMCs resuspended in RPMI-1640 on day 0. Hu-PBMCs were separated from approximately 30 ml peripheral blood using Ficoll solution (GE Healthcare Japan, Tokyo, Japan). We used approximately 30 ml blood collection that appears to be a sufficient amount considering the additional 15 ml blood sampling for routine

outpatient medical checks and follow-up post-transplantation. To monitor hu-PBMCs reconstitution in mice, peripheral blood was collected via the tail vein using heparin-coated capillary tubes on day 14, and the ratio of $hCD45^+$ lymphocytes in blood was assessed. We defined >1% $hu\text{-}CD45^+$ lymphocytes in mouse peripheral blood as successful reconstitution in hu-PBMC-NSG mice [18]. Three weeks after the injection, the mice were sacrificed and lymphocytes were collected from peripheral blood, spleen, and bone marrow.

2.4. Flow cytometry

The following antibodies against hu-Ag were used: FITC-conjugated anti-CD3, -CD14, and -CD62L, PE-conjugated anti-CD19, -CD16, -CD56, -CD45RA, -CCR4, and -CCR6, PerCP-Cy5.5-conjugated anti-HLA-DR, PE-Cy7-conjugated anti-CD3, APC-conjugated anti-CD3, -CD45, and -CCR6, Alexa-Fluor 647-conjugated anti-CD127, Alexa-Fluor 488-conjugated anti-Foxp3, APC-H7-conjugated anti-CD8 and -CD45RO, BV421-conjugated anti-CD25 and -CXCR3, and V500-conjugated anti-CD4 (all from BD Pharmingen, San Diego, USA). Dead cells were excluded by 7AAD staining. For intracellular staining, Fixation/Permeabilization solution (eBioscience, San Diego, USA) was used. Multicolor cytometric analysis was performed on a FACS Canto II (BD Bioscience, New Jersey, USA) and data were analyzed by FlowJo software (FLOWJO LLC).

2.5. Cell proliferation assay

The alloreactivities of VPD (BD Horizon, New Jersey, USA)- or CFSE (eBioscience)- labeled freshly isolated hu-

PBMCs or hu-CD3$^+$ T cells purified from hu-PBMC-NSG mice at 21 days after injection were investigated by coculture with irradiated (30 Gy) allogeneic hu-PBMCs from living liver donors and third party healthy volunteer or dynabeads CD3/CD28 T Cell Expander (Invitrogen, San Diego, USA) in a complete medium for 5 days at 37 °C with 5% CO_2. Proliferated huCD3$^+$ T cells were evaluated by the difference compared with the control (no stimulus). To eliminate contamination of mouse cells, hu-CD3$^+$ T cells were isolated using anti-CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in which the purity was confirmed to be >90%. Donor-specific alloreactivities were evaluated by comparison with third party Ag from the only one healthy volunteer in all experiments and calculated by the following formula: (Donor – third party)/third party × 100%.

2.6. Human leukocyte antigen (HLA) typing

All enrolled individuals were assessed by HLA typing (A-B-DR) using a WAKFlow HLA typing kit (Wakunaga Pharmaceutical Co. Ltd, Osaka, Japan).

2.7. Statistical analysis

Data were evaluated by one-way analysis of variance (ANOVA) and the Student's *t*-test using Graphpad Prism version 6.0 (GraphPad Software, Inc.). Data were considered to be significantly different at $P<0.05$, $P<0.01$, and $P<0.001$ represented in the figures and tables as *, **, and ***, respectively.

3. Result

3.1. Engraftment of hu-PBMCs into NSG mice

To investigate whether hu-PBMCs could be reconstituted in the NSG mouse, 2.5×10^6 hu-PBMCs from five healthy individuals (Table 1) were i.p. injected into irradiated (2 Gy) NSG mice (n=3–8). We found 20.9±2.7% hu-CD45$^+$ lymphocytes in the peripheral blood of NSG mice at 2-weeks post-injection. Eventually, all cases successfully achieved hu-PBMC reconstitution in NSG mice, which was defined as more than 1% of hu-CD45$^+$ lymphocytes (Fig. 1A). As shown in Fig. 1B and C, when hu-PBMCs from LDLT recipients maintained on IS (on IS, n=10, Table 2) or completely weaned off IS (off IS, n=4, Table 3) were i.p. injected into NSG mice, a sufficient number of hu-CD45$^+$ lymphocytes were detected in the mouse peripheral blood (40.9±6.5% and 39.8±11.8%, respectively). There was a tendency that the reconstitution rate of hu-CD45$^+$ lymphocytes from healthy volunteers was lower than that of LDLT recipients (vs. on IS, $p = 0.16$, vs. off IS, $p = 0.32$, Fig. 1D).

3.2. Engraftment kinetics of hu-CD45$^+$ lymphocytes and T cell subsets in hu-PBMC-NSG mouse model

hu-CD45$^+$ lymphocytes from mouse peripheral blood and spleen at 3 weeks post-injection were assessed to investigate dynamic changes in lymphocytes subsets in hu-PBMC-NSG mice. No significant difference in the lymphocytes subsets was observed among healthy volunteer, on IS, and off IS groups (Fig. 2A). In hu-PBMC-NSG mice, significantly higher percentages of hu-CD4$^+$ T cells were found in the peripheral blood (Fig. 2B) and spleen (Fig. 2C) of IS recipients (vs. blood and spleen of healthy volunteers, $p<0.05$, and vs. spleen of off

IS patients, p = 0.008). The subsets of hu-CD3$^-$CD19$^+$ B cells, hu-CD3$^-$CD19$^-$CD56$^+$ natural killer (NK) cells, hu-CD3$^-$CD19$^-$HLA-DR$^+$ dendritic cells (DCs) and hu-CD3$^-$CD19$^-$CD14$^+$ monocytes were reduced markedly to nearly undetectable levels in the peripheral blood of hu-PBMC-NSG mice, although small subsets were detected in the spleens of hu-PBMC-NSG mice in all groups (Fig. 2A–C). We also found a significantly increased percentage of hu-CD3$^+$ T cells in hu-PBMC-NSG mice compared with those of fresh hu-PBMCs in all groups (Fig. 2D and E). In addition, the percentages of hu-CD8$^+$ T cells in hu-PBMC-NSG mice were increased significantly, although no significant differences in the percentages of hu-CD4$^+$ T cells were observed between fresh hu-PBMCs and hu-PBMC-NSG mice (Fig. 2D and E).

3.3. Allo-reactivity of hu-CD3$^+$ T cells is amplified in hu-PBMC-NSG mice

To evaluate the functions of reconstituted hu-CD3$^+$ T cells in NSG mice, we performed cell proliferation assays using CFSE or VPD. A sufficient number of proliferated hu-CD3$^+$ T cells following CD3/CD28 stimulation was observed in fresh hu-PBMCs and hu-PBMC-NSG mice of all groups (Fig. 3A and B). No significant differences in the percentage of responding hu-CD3$^+$ T cells following CD3/CD28 stimulation were observed between fresh PBMCs and hu-PBMC-NSG mice, although there was a slightly lower rate of hu-CD3$^+$ proliferation in hu-PBMC-NSG mice of off IS recipients (p=0.06, right column, Fig. 3B). Similar findings were observed between the hu-CD4$^+$ and CD8$^+$ T cells (upper row, right column, Fig. 3E and F, respectively). In contrast, a significant increase in the percentage of responding hu-CD3$^+$ T cells against allo-Ag was observed in hu-PBMC-NSG mice

compared with those in fresh PBMCs of all groups (p<0.05, Fig. 3D), and hu-CD4$^+$ and CD8$^+$ T cells (lower row in Fig. 3E, F, respectively). Importantly, there were absolutely no dividing hu-T cells from hu-PBMC-NSG mice in the absence of stimulation (open line in histogram, Fig. 3C) as well as following coculture with splenocytes of NSG mice (data not shown), suggesting that the amplifying alloreactivity in humanized mice may not be directly caused by a xeno-reaction.

3.4. Dynamic changes in naïve and memory phenotypes of hu-CD3$^+$ T cells in hu-PBMC-NSG mice

To clarify the underlying mechanisms of amplified of alloreactivities in hu-PBMC-NSG mice, we evaluated the subsets of reconstituted hu-CD3$^+$ T cells. Consistent with previous evidences in humanized mice [19][20][21], a significant decrease in the percentage of CD45RA$^+$CD62L^{hi} naïve (T_N) cells and a significant increase in the percentage of CD45RA$^-$CD62L^{lo} effector memory T (T_{EM}) cells among hu-CD4$^+$ and hu-CD8$^+$ T cells were observed in all groups (Fig. 4A–F). In addition, the percentage of CD45RA$^-$CD62L^{lo} central memory subset (T_{CM}) of CD8$^+$ cells, but not CD4$^+$ T cells, was increased in all groups of hu-PBMC-NSG mice (Fig. 4A–F). These findings suggest that phenotypic conversion from the T_N to T_{EM} subset may be the mechanism underlying the amplification of alloreactivities during the process of hu-PBMC reconstitution in NSG mice.

3.5. Phenotype of the Th1 population increased predominantly in hu-PBMC-NSG mice

Th1 and Th17 cells play a major role in mediating allograft rejection [22][23][24]. To identify the role of Th

subsets in hu-PBMCs-NSG mice, we investigated the expression of CXCR3 and CCR6 in hu-$CD4^+$ T cells, which allowed categorization of $CD4^+$ Th cell subsets. The predominant cell fraction was converted dramatically from the $CCR6^-CXCR3^-$ Th2 to $CCR6^-CXCR3^+$ Th1 cell subset in the hu-PBMC-NSG mice (Fig. 5A–C). Moreover, there was no significant difference in the frequencies of $CD25^{hi}CD127^{lo}Foxp3^+$ regulatory T (T_{REG}) cells among hu-$CD4^+$ T cells between fresh PBMCs and hu-PBMC-NSG mice in all groups (Fig. 5D). This result suggests that the predominance of the Th1 cell subset is involved in the amplification of alloreactivities in hu-PBMC-NSG mice.

3.6. Evaluation of donor Ag reactivity in the hu-$CD3^+$ T cells of LDLT recipients in hu-PBMC-NSG mice

The amplified alloreactivity in humanized mice raised the clinical issue of whether the immune responses to donor Ag in hu-PBMC-NSG mice were different from those in fresh PBMCs of organ transplant recipients. To address this issue, CFSE- or VPD-labeled hu-T cells were cocultured with irradiated allo-PBMCs from living donor or third party Ag. In this experiment, to compare the immune responses to third party Ag as a standard response to allo-Ag, we used freshly isolated PBMCs from the same person for each experiment. In addition, we confirmed that the numbers of HLA -A, -B and -DR mismatches between recipient and third party Ag, or donor and third party Ag were greater than three. First, alloreactivities of fresh PBMCs against both donor and third party Ag were suppressed strongly in seven of 10 recipients who were maintained on IS (#1, #3, #4, #5, #6, #7, and #8, upper panel, Fig. 6A). Moreover, two of 10 recipients on IS, presented a donor Ag-specific

hyporesponsiveness (#9 and #10). hu-T cells failed to respond to donor Ag despite the maintained response to third party Ag (upper panel, Fig. 6A). Additionally, only recipient #2 maintained on IS responded robustly to donor and third party Ag (upper panel, Fig. 6A). Consistent with the previous finding, magnified alloreactivities of hu-T cells were observed in all cases of hu-PBMC-NSG mice. The evaluation of large scale reactivities in hu-PBMC-NSG mice demonstrated that eight of 10 cases maintained on IS had lower reactivities against donor Ag than those against third party Ag (upper panel, Fig. 6B). However, the other two recipients (#1 and #2) responded highly to donor Ag relative to third party Ag in hu-PBMC-NSG mice (upper panel, Fig. 6B). Indeed, these two cases had frequent AR episodes (Table 2). It is likely that the accelerated response to donor Ag in hu-PBMC-NSG mice is an indicator of a high risk of AR in patients. Moreover, in the off IS group (n=4), we found donor-specific hyporesponsiveness in both fresh PBMCs and hu-PBMC-NSG mice (lower panel, Fig. 6A and B, respectively). In addition, the differences between the alloreactivities against donor and third party Ag was 30% greater (third party - donor)/third party × 100) in both fresh PBMCs and hu-PBMC-NSG mice of the off IS group (lower panel in Fig. 6). This suggests that the sustained donor-specific hyporesponsiveness observed in hu-PBMC-NSG mice proves the capability of the post-transplant recipient to maintain withdrawal of IS. Next, we evaluated the differences in alloreactivity against donor- and third party Ag in recipients maintained on IS. The 30% greater differences in alloreactivity against donor and third party Ag were observed in hu-PBMC-NSG mice of the four recipients (case #3, #5, #7, and #9) who appeared to reduce their IS (similar to off IS, Fig. 6C).

4. Discussion

In this study, we evaluated the alloreactivity of hu-PBMCs in humanized mice and the hu-immune status of LDLT recipients.

The humanized mouse was developed to analyze individual hu-immunity. Recent advances in the development of humanized mice have provided a suitable mouse model for hu-cell engraftment [13]. To reconstitute the hu-immune system, NSG mice bearing targeted mutations, including IL-2Rγc, have been reported as reproducible and robust [13]. It has been reported that $1–2\times10^7$ PBMCs from healthy volunteers were engrafted in NSG mice with 15%–35% of hu-CD45$^+$ lymphocytes on day 14–21 following hu-PBMC administration [25][26][27]. In addition, sub-lethal irradiation (2 Gy) led to reconstitution of a high level of hu-CD45$^+$ lymphocytes (20%–50%), despite the small number of PBMCs ($1–5\times10^6$) in NSG mice [19][28][29]. Recently, a humanized mouse model using clinical samples has been reported, in which hu-PBMCs from lung transplant recipients under 3 weeks of post-transplant IS administration were engrafted in NOD rag-/-IL-2Rγc-/- mice. In this previous study, 5.0×10^6 hu-PBMCs from recipients were administered to mice and 30%–40% hu-CD45$^+$ lymphocytes were confirmed in peripheral blood at 4 weeks after i.p. injection [30]. In our study, we applied a relatively small number of hu-PBMCs (2.5×10^6) to irradiated (2 Gy) NSG mice from healthy volunteers and LDLT recipients with a post-transplantation period of more than 1 year and treated with or without IS. We achieved 20%–40% hu-PBMC engraftment in NSG mice at 14 days following i.p. injection, although variabilities in the levels of hu-PBMCs engraftment from a single recipient and between transplant

recipients were observed compared with healthy volunteers (Fig. 1). Importantly, neither weight loss nor mouse death caused by graft-versus-host disease (GVHD) were observed within 3 weeks. These data suggest that the humanized mouse using hu-PBMCs isolated from transplant recipients is reproducibly useful as a surrogate to assess an individual immune statuses. This immunological tool supports a basic *in vivo* approach for the complex immunity of transplant recipients, which allows a profound understanding of an individual's immune status and precise management with an appropriate amount of IS.

Previous studies have shown that the majority of lymphocytes reconstituted in humanized mice are the hu-$CD3^+$ T cell population and subsets of hu-B cells, and that monocytes are rare [19][27]. Consistently, we observed approximately 90% hu-$CD3^+$ T cells and 1%–20% of hu-B cells, monocytes, DCs, and NK cells in the peripheral blood and spleen of hu-PBMC-NSG mice. In addition, hu-$CD8^+$ T cells consisted predominantly of $CD3^+$ T cells in hu-PBMC-NSG mice of healthy volunteer and off IS groups with a CD4/CD8 ratio of 0.28 (0.07–0.87). However, previous studies have demonstrated that a greater number of $CD4^+$ T cells were reconstituted in hu-PBMC-NSG mice compared with our study (CD4/CD8 ratio = 0.5-2.0) [21][29][31]. These studies have supported our data because, when a lower numbers of lymphocyte (5×10^6) were reconstituted in NSG mice, more variability and a lower CD4/CD8 ratio were observed [29]. Our study applied a small and acceptable amount of blood (30 ml) from outpatients, where we collected $15–25 \times 10^6$ hu-PBMCs. To avoid variation, we administered a small number of hu-PBMCs (2.5×10^6) per mouse to multiple NSG mice and calculated the average in reconstituted NSG mice. Such a relatively small number of hu-PBMCs administered to an NSG

mouse may lead to a lower CD4/CD8 ratio in reconstituted hu-PBMC-NSG mice. Moreover, relatively low percentages of hu-CD8$^+$ T cells were observed in hu-PBMC-NSG mice of the on IS group (Fig. 2B and C). These data could be interpreted to influence the on IS group because a previous study has shown that the engraftment of CD8$^+$ T cells in NSG mice depends on the support of CD4$^+$ T cell functions [32][33], suggesting that CD4$^+$ T cells exposed to IS might inhibit immune functions in NSG mice.

Here, we confirmed that both hu-CD4$^+$ and CD8$^+$ T cell subsets downregulated CD45RA expression, resulting in a predominant memory phenotype of lymphocytes in hu-PBMC-NSG mice (Fig. 4). Previous studies have demonstrated that 80%–90% of hu-CD4$^+$ and CD8$^+$ T cells in the peripheral blood, spleen, and peritoneal cavity of humanized mice are CD45RO$^+$, a memory phenotype, at 2–3 weeks after administration of hu-PBMCs [19][20][21][32]. During an adoptive transfer of T cells into immunodeficient mice, homeostatic proliferation occurs as a physiological process. In this phenomenon, T_N cells acquire a memory phenotype in an Ag-independent manner [34]. Additionally, another study revealed that T cell clones with the greatest potential for homeostatic expansion had a high level of TCR expression and avidity for peptides [35]. T cells with a memory phenotype, which have undergone homeostatic proliferation, respond to Ag with a low activation threshold. Ovalbumin (OVA)-specific T cells that have undergone homeostatic proliferation more quickly and profoundly engage OVA-Ag even in the absence of costimulation compared with freshly isolated OVA-specific T cells [34]. Thus, accumulating evidence has revealed the magnitude of effector responses following homeostatic proliferation. In addition, a study has demonstrated that female C57BL/6 (B6)

splenocytes that have undergone homeostatic proliferation in B6 RAG-/- mice promptly reject male B6 kidney grafts, despite a single minor Ag difference [36]. Furthermore, in an Ag-independent process, T cells that undergo homeostatic expansion include a bona fide memory population that favors AR [37]. Moreover, humanized mice have demonstrated that engrafted hu-PBMCs, particularly hu-$CD4^{+}CD25^{-}$ T effector cells, respond to an allogeneic hu-skin graft, but autologous hu-PBMCs did not show a response [18][20][26][38]. In addition, allografts of hu-pancreatic islet, cardiac tissues and vessels are rejected by allogeneic hu-PBMCs in humanized mice [21][39][40][41]. Thus, hu-PBMCs reconstituted in NSG mice are functionally capable of responding to allo-Ag and rejecting allogeneic hu-tissues. In this study, we compared alloreactivities between fresh PBMCs and hu-PBMC-NSG mice. Consistent with previous studies on homeostatic proliferation, we found significantly amplified alloreactivity of hu-$CD3^{+}$ T cells following homeostatic proliferation in hu-PBMCs-NSG mice compared with that of fresh PBMCs. Considering the large scale immune response in hu-PBMCs-NSG mice, we detected dramatic changes in hu-T cell alloreactivity against donor- and third party Ag. Of note, in cases #1 and #2, extraordinary T cell expansion against donor Ag relative to third party Ag in hu-PBMCs-NSG mice may predict the incidence of AR in the absence of IS. Indeed, these cases had experienced multiple AR episodes (Table 2). Case #1 had repeated non-adherence to IS, which caused multiple AR (Fig. S1) and developed de novo DSA with C4d positivity in liver biopsy samples (Table S1). In addition, case #2 was enrolled a clinical study to wean off IS. However, AR episodes were observed during IS weaning (Fig. S1). Therefore, these two cases had clinical evidence showing that IS reduction led to AR episodes, which support

our data in hu-PBMC-NSG mice shown in Fig. 6B and C. However, case #4, who also had multiple AR episodes, did not display donor reactivities in the hu-PBMC-NSG mouse. In the clinical course of patient #4, stopping IS for some time following reactivation of varicella zoster virus caused multiple AR. Viral infection might affect the occurrence of graft rejection in case #4. In addition, the immunological assay using the humanized mouse model focuses on T cell immune responses. The model may have a limitation to mimic the clinical findings in the absence of assessment of humoral or innate immune systems. Taken together, the assessment of alloreactivities in hu-PBMC-NSG mice may reveal subtle changes in the immune status of recipients.

Previous efforts in developing immune-monitoring assays have highlighted *in vitro* alloimmune responses by coculture with irradiated donor lymphocytes. It has been shown that donor-specific hyporesponsiveness was associated with a low number of AR episodes, but it could not indicate successful withdrawal of IS [42]. In addition, recipients who had an AR episode within 3 months post-transplantation demonstrated significantly enhanced donor Ag reactivity in an MLR and cytokine production depending on the TAC concentration [8]. Additionally, a CFSE-MLR classifies the immune status of recipients into hyper-, normo- and hypo-responsiveness, and succeeds in adjusting IS. Hence, AR episodes can be reduced significantly [9]. Such immune monitoring assays have shown utility in prediction and monitoring of AR post-transplantation [43]. However, the previous assays provide a `snap shot' of the immune status under IS treatment and may not predict performance after reduction or cessation of IS. In humanized mice, PBMCs from post-transplant recipients can be isolated after weeks of IS exposure. Hence, it is speculated that the immunological assay

without an IS influence on immunodeficient mice can predict donor Ag reactivities after withdrawal off IS. Alternatively, the homeostatic proliferation-driven PBMCs in humanized mice might indicate a risk of lymphopenia mediated by viral infection or drugs. Thus, augmented donor Ag reactivities after homeostatic proliferation, such as in cases #1 and #2, probably require caution for AR, particularly under lymphopenia in clinical settings.

A recent report on adoptive virus-specific T cell transfer showed that the CD45RA-depleted population with loss of T_{EM}RA cells substantially lost donor-specific immune responses. In contrast, the CD62L-depleted population with loss of T_{CM} cells consistently yielded 3–5 folds higher donor Ag responses [44]. In addition, it has been reported that the main driving force behind AR is T_{EM} cells in the transplant field [45]. In humanized mice, T_{EM} cells mediate the destruction of hu-endothelial cells, while T_{CM} cells are not involved [46]. Additionally, $CD4^+$ T_{EM} cells produce more IFNγ than T_{CM} cells against allogeneic endothelial cells [46]. Furthermore, in humanized mice that received hu-$CD4^+$ T cells, but not hu-$CD8^+$ T cells, high concentrations of IFNγ, IL-2, and IL-12, and relatively lower concentrations of IL-4, IL-5, and IL-13 were observed [47]. Numerous studies of humanized mice have demonstrated an increase in hu-IFNγ production [41][48][49]. In addition, T cells undergo homeostatic proliferation that skews toward the Th1 phenotype [36]. Here, we showed that fresh hu-PBMCs consisting of a predominant Th2 subset were replaced with T_{EM} and Th1 subsets, that are critical lymphocytes of AR in transplantation of hu-PBMC-NSG mice (Fig. 4 and 5). It is presumed that the immunological assessment in humanized mice highlights lymphocyte subsets that are potently

associated with the AR. However, there were no differences in both populations (T_{EM} and Th1) and the immune balance between T_{REG} and T_{EM} cells or Th1 cells among patients with or without multiple AR episodes and off IS (Fig. S2), suggesting that the specific phenotypes of lymphocytes responding to donor Ag were not clarified, which warrants further study.

As a downside of homeostatic proliferation, it causes autoimmune disease and resistance to tolerance induction [37]. It has been reported that the adoptive transfer of T_N cells into lymphopenic mice causes development of a progressive inflammatory bowel disease [50]. Furthermore, T cells that undergo homeostatic proliferation are resistant to tolerance induction in a transplant model [37] and homeostasis-driven proliferation eliminates the tolerogenicity of T cells, which is vital to maintain the tolerant state [51]. This suggests that lymphopenia abrogates post-transplantation tolerance. However, in our study, we found that hu-PBMCs from a patient who was completely weaned off IS for a longtime (greater than 2.5 years) had maintained donor-specific hyporesponsiveness after homeostatic proliferation within humanized mice. In addition, our study revealed that the donor-specific hyporesposiveness was preserved consistently, despite undergoing homeostatic proliferation and the absence of a functioning allograft that provides the continuous donor Ag. Continuous Ag exposure, such as an allograft, renders Ag-specific T cell unresponsiveness [52]. A previous study showed that post-transplantation tolerance was abrogated upon removal of a functioning allograft, suggesting that continuous Ag exposure is important to maintain the tolerant states [53].In contrast, a recent study demonstrated that maintenance of T cell tolerance is not necessarily restored by removing

the tolerizing environment via transfer of tolerant T cells into another mouse without tolerogens [51]. Here, hu-PBMC-NSG mice suggested that a donor organ may not be associated with the mechanism of donor Ag hyporesponsiveness maintenance in T cells from off IS recipients. Alternatively, T_{REG} cells appear to be associated with the underlying mechanisms by which recipients can omit IS treatment. A previous humanized mouse study showed that AR was efficiently prevented by hu-T_{REG} cells [41]. However, in our study, no significant differences in the percentage of T_{REG} cells was observed between off and on IS groups (Fig. 5D and Fig. S2C) although a recent study found an increased number of T_{REG} cells following an allo-Ag stimulation in tolerant cases [54]. Additionally, multiple tolerant strategies and different success rates in organ transplantation suggests that the mechanisms underlying graft protection may vary from case to case [55]. Although it warrants further study, including a prospective clinical study of weaning off IS in patients with donor hyporesponsiveness similar to the off IS group of hu-PBMC-NSG mice, the humanized mouse model provides insights into the mechanisms of maintaining operational tolerance in each patient, which may improve immunological approaches for transplantation.

The development of lethal xenogeneic GVHD is also detrimental for evaluations in humanized mouse models. Injection of hu-PBMC usually induces GVHD within 4–8 weeks. However, the efficacy of an evaluation system for hu-$CD3^+$ T cell functions in a humanized mouse model has only been established in short-term experiments [56]. In addition, we confirmed that the xeno-reactivity of hu-PBMCs from NSG mice reconstituted with hu-PBMC was minimal in a CFSE-MLR (data not shown).

In conclusion, our novel immunological approach using hu-PBMCs-NSG mice can be a surrogate *in vivo* tool without IS with exaggeration of the memory Th1 lymphocyte population and alloreactivities. It may predictably recapitulate the immune status of a diverse transplant recipients after withdrawal of IS and contribute to personalized treatment while minimizing the risk of AR or overimmunosuppression in clinical settings.

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Tables

Table 1: Healthy volunteers' characteristics

Recipient #	Age	Sex	HLA mismatch vs. allo-Ag A-B-DR
I	33	M	1-2-2
Ii	37	M	1-1-2
Iii	36	M	1-2-2
Iv	23	M	0-2-2
V	40	M	1-1-1-

Table 2: Characteristics of recipients who maintained on immunosuppressants after LDLT

Recipient #	Age at LDLT (years)	Sex	Primary disease	Years after LDLT	Donor	HLA mismatch A-B-DR		Details of current immunosuppressants				AR episode
						vs. Donor	vs. 3rd party	TAC (mg/day) [trough (ng/ml)]	MMF (mg/day) [trough (μg/ml)]	mPSL (mg/day)	sirolimus (mg/day) [trough (ng/ml)]	
1	5.5	M	Fulminant hepatitis	17.3	grandmother	2-1-1	2-2-2	2.5 [5.0]	2000 [15.5]	5.0	-	multiple
2	58.3	F	PBC	4.0	son	1-1-1	1-1-1	4.0 [7.2]	-	-	-	multiple
3	43.2	M	PBC	1.9	brother	1-1-1	1-1-1	1.4 [4.4]	500 [1.6]	-	-	none
4	48.1	F	Cryptogenic hepatitis	4.0	husband	2-2-2	2-2-2	1.0 [6.5]	1500 [3.1]	5.0	1.0 [10.0]	multiple
5	44.7	F	Alcoholic hepatitis	7.9	husband	2-2-2	1-2-2	4.0 [4.8]	-	-	-	once
6	3.9	F	Byler disease	10.7	mother	0-1-0	0-1-2	1.0 [1.5]	-	-	-	twice
7	42.7	M	FAP	6.1	wife	1-2-1	2-2-0	4.0 [8.6]	1000 [3.0]	-	-	once
8	70.0	F	PBC	3.1	son	0-1-1	1-1-1	6.0 [3.9]	1000 [1.9]	-	-	none
9	55.5	F	PSC	5.0	daughter	1-1-1	1-2-1	-	1000 [1.7]	7.5	-	once
10	0.5	F	BA	24.0	mother	1-1-1	2-2-2	1.0 [2.9]	-	-	-	none

AR, acute rejection; PBC, primary biliary cholangitis; FAP, familial amyloid polyneuropathy; PSC, primary sclerosing cholangitis; BA, biliary atresia; TAC, tacrolimus; MMF, mycophenolate mofetil; mPSL, methylprednisolone

Table 3: Characteristics of recipients who had been completely weaned off immunosuppressants

Recipient #	Age at LDLT (years)	Sex	Primary disease	Years after LDLT	Donor	HLA mismatch A-B-DR		Years after off IS	AR episode
						vs. Donor	vs. 3rd party		
I	59.3	F	NASH	4.5	daughter	0-0-1	1-1-2	3.0	none
II	52.2	M	Alcoholic hepatitis	4.5	son	1-1-0	0-1-2	2.6	once
III	55.4	M	NASH	4.0	son	0-1-1	1-1-1	2.5	none
IV	0.5	F	BA	19.0	mother	1-1-1	1-2-2	13.5	once

AR, acute rejection; NASH, nonalcoholic steatohepatitis; BA, biliary atresia.

Figure captions

Fig. 1: Human CD45-positive (hu-CD45$^+$) lymphocyte ratio in the peripheral blood of hu-PBMC-NSG mice.

Percentages of hu-CD45$^+$ lymphocytes in the peripheral blood of three to eight NSG mice (8–16-weeks-old) at 2 weeks after i.p. injection of 2.5 × 10^6 hu-PBMCs resuspended in RPMI-1640 (A-D) collected from five healthy volunteers labeled i–v (A), 10 liver transplant recipients maintained on IS labeled 1–10 (B), and four liver transplant recipients completely weaned off IS labeled I–IV (C). The vertical bars represent the mean of individual healthy volunteers and recipients (A-C). Patient details are described in Table 1. The average reconstitution rate (percentages of hu-CD45$^+$ cells) in each healthy volunteer (black circles) and recipient (on IS; blue squares, off IS; red triangles) are represented as scatter plots (D). The vertical bars represent the mean of each group (D). There was a tendency that the reconstitution rate of hu-CD45$^+$ lymphocytes in healthy volunteers was lower than that in LDLT recipients (vs. on IS; p = 0.16, vs off IS; *p* = 0.32, respectively, one-way ANOVA).

Fig. 2: Difference in the hu-CD45$^+$ lymphocytes population between fresh PBMCs and hu-PBMC-NSG mice.

Percentages of hu-CD3$^+$ T cells, hu-CD3$^+$CD4$^+$ T cells, hu-CD3$^+$CD8$^+$ T cells, hu-CD3$^-$CD19$^+$ B cells, hu-CD3$^-$CD19$^-$CD56$^+$ natural killer (NK) cells, hu-CD3$^-$CD19$^-$HLA-DR$^+$ dendritic cells (DCs), and hu-CD3$^-$CD19$^-$CD14$^+$ monocytes after gating on hu-CD45$^+$ lymphocytes in freshly isolated hu-PBMCs (fresh PBMCs) (A), peripheral

blood from hu-PBMC-NSG mice (B) and spleen from hu-PBMC-NSG mice (C). Hu-PBMCs were isolated from hu-PBMC-NSG mice at 3 weeks after i.p. injection (B and C). No significant differences in each lymphocyte subset were observed among healthy volunteers (n=5, black bars), IS recipients (n=10, blue bars) and off IS recipients (n=4, red bars) (A-C), except for hu-$CD4^+$ and $CD8^+$ T cell subsets in both peripheral blood (B) and spleen (C) of NSG mice reconstituted with hu-PBMCs from on IS recipients. Data are the mean±SEM of two or three independent experiments. Percentages of hu-$CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes were compared between fresh PBMCs (D and E, open circles) and hu-PBMCs isolated from peripheral blood (D, filled squares) or spleen (E, filled squares) of hu-PBMC-NSG mice in healthy volunteers (D and E, left, black plots), LDLT recipients on IS (D and E, middle, blue plots), and LDLT recipients off IS (D and E, right, red plots). Significantly higher percentages of hu-$CD3^+$ T cells and hu-$CD8^+$ T cells in hu-PBMC-NSG mice than that of fresh PBMCs were observed in all experiments (D and E). One-way ANOVA (A-C), paired *t*-test (D and E), *p<0.05, **p<0.01, and ***p<0.001.

Fig. 3: Reactivity of hu-T cells against CD3/CD28 stimulation or allo-Ag between fresh PBMCs and hu-PBMC-NSG mice.

Proliferated hu-$CD3^+$ (A and B), $CD4^+$ (E, upper panel) and $CD8^+$ (F, upper panel) T cells following CD3/CD28 stimulation were compared between fresh PBMCs (open circles) and hu-PBMC-NSG mice (filled squares) in healthy volunteer (n=5, left, white and black dots), on IS (n=10, middle, blue dots) and off IS (n=4, right, red

dots) groups. Hu-CD3$^+$ T cells in hu-PBMC-NSG mice were isolated from peripheral blood, spleen, and bone marrow. Representative histograms of hu-CD3$^+$ T cell proliferation are shown in A (Filled, following CD3/CD28 stimulation; Open, no stimulation). To assess alloreactivities of hu-CD3$^+$ (C and D), CD4$^+$ (E, lower panel) and CD8$^+$ (F, upper panel) T cells between fresh PBMCs and hu-PBMC-NSG mice, hu-PBMCs (1.5×10^5 cells per well) labeled with VPD were cocultured with irradiated (30 Gy) allogeneic hu-PBMCs (1.5×10^5 cells) labeled with CFSE for 5 days in complete RPMI-1640 medium containing 10% FCS (Sigma-Aldrich Japan), 1% penicillin/streptomycin, and 50 mM 2-ME in a 96-well round-bottom plate (BD Falcon). Representative histograms of proliferated hu-CD3$^+$T cells in response to allo-Ag in healthy volunteers (C). Augmented hu-T cell responses to allo-Ag were observed in hu-PBMC-NSG mice compared with fresh PBMCs in all groups (C, D, lower panels in E and F, healthy volunteers, n=5, left, black and white dots; on IS, n=10, middle, blue dots, off IS, n=4, right, red dots). Paired *t*-test was applied.

Fig. 4: Changes in the cell populations of hu-CD3$^+$ T cells between fresh PBMCs and hu-PBMC-NSG mice.

Dynamic changes in naïve and memory phenotypes of hu-CD4$^+$ (A–C) and CD8$^+$ (D–F) T cells between fresh PBMCs (open circles) and hu-PBMCs collected from splenocytes at 3 weeks after i.p. injection in hu-PBMC-NSG mice (filled squares) of healthy volunteer (n=5, A and D), on IS (n=10, B and E) and off IS (n=4, C and F) groups. Representative images show dot plots to categorize hu-CD45RA$^+$CD62L$^+$ naïve T (T_N) cells, hu-CD45$^-$CD62L$^+$ central memory T (T_{CM}) cells, hu-CD45RA$^-$CD62L$^-$ effector memory T (T_{EM}) cells, and hu-

$CD45RA^+CD62L^-$ terminally differentiated effector memory T ($T_{EM}RA$) cells (A–F). Paired *t*-tests were applied (**p*<0.05, ***p*<0.01, and ****p*<0.001).

Fig. 5: Changes in the helper T cell population and regulatory T cells among hu-$CD4^+$T cells between fresh PBMCs and hu-PBMC-NSG mice.

Dynamic changes of the $CD4^+$ helper T cell (Th) population between fresh PBMCs (open circles) and hu-PBMCs isolated from splenocytes at 3 weeks after i.p. injection in hu-PBMC-NSG mice (filled squares) of healthy volunteer (n=5, A), on IS (n=10, B) and off IS (n=4, C) groups. Representative images showed dot plots to categorize $CXCR3^+CCR6^-$ Th1, $CXCR3^-CR6^-$ Th2, and $CXCR3^-CCR6^+$ Th17 cells (Left images, A-C). Dynamic changes in the percentage of $CD4^+CD25^{hi}CD127^{lo}Foxp3^+$ regulatory T (T_{REG}) cells between fresh PBMCs (open circles) and hu-PBMCs from spleens in hu-PBMC-NSG mice (filled squares) of healthy volunteer (n=5, left, D), on IS (n=10, middle, D) and off IS (n=4, right, D) groups (D). Paired *t*-test was applied (**p*<0.05, ***p*<0.01, and ****p*<0.001).

Fig. 6: Variation in alloimmune reactivity of hu-$CD3^+$T cells between fresh PBMCs and hu-PBMC-NSG mice in recipients that underwent LDLT.

Proliferated hu-$CD3^+$ T cells after coculture with irradiated hu-PBMC isolated from each donor (black bars) or a third party healthy volunteer (gray bars) (A and B). The alloimmune responses against donor (black bars)

and third party (gray bars) Ag in fresh PBMCs (A) and hu-PBMC-NSG mice (B) are shown for each individual maintained on IS (#1‐10, upper panels, A and B) and completely off IS (#I‐IV, lower panels, A and B). The donor-specific alloreactivities were compared with immune responses against third party Ag calculated by the following formula: ([Donor ‐ third party] / third party × 100) shown on the X axis in C. The donor Ag-specific hyporesponsiveness similar to off IS (>30% changes in rate between alloimmune responses against donor- and third party Ag, bottom panel in C) was categorized in patients who may be capable of IS reduction similar to the off IS group (n=4, #3, 5, 7, and 9 in C). In contrast, augmented donor-reactivities were compared with responses to third party Ag in cases #1 and 2 that were categorized as a high risk of AR (#1 and 2 in C). hu-$CD3^+$ T cells in hu-PBMC-NSG mice were isolated from mouse peripheral blood, splenocytes, and bone marrow at 3 weeks after i.p. injection.

Figure 1

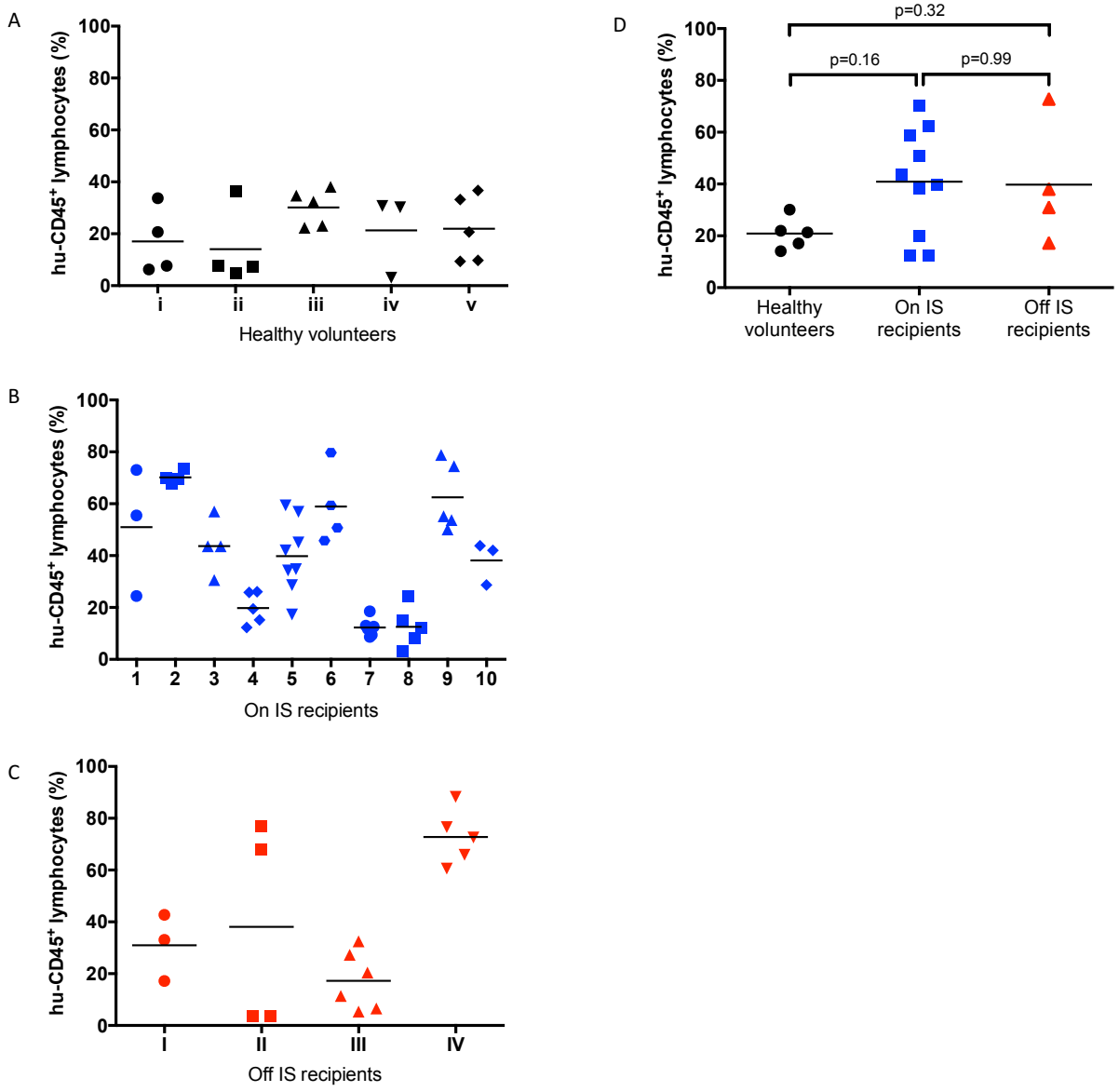

A
hu-CD45+ lymphocytes (%)
100
80
60
40
20
0
i
ii
iii
iv
v
Healthy volunteers
B
hu-CD45+ lymphocytes (%)
1
2
3
4
5
6
7
8
9
10
On IS recipients
C
hu-CD45+ lymphocytes (%)
I
II
III
IV
Off IS recipients
D
p=0.32
p=0.16
p=0.99
hu-CD45+ lymphocytes (%)
Healthy volunteers
On IS recipients
Off IS recipients

Figure 2:

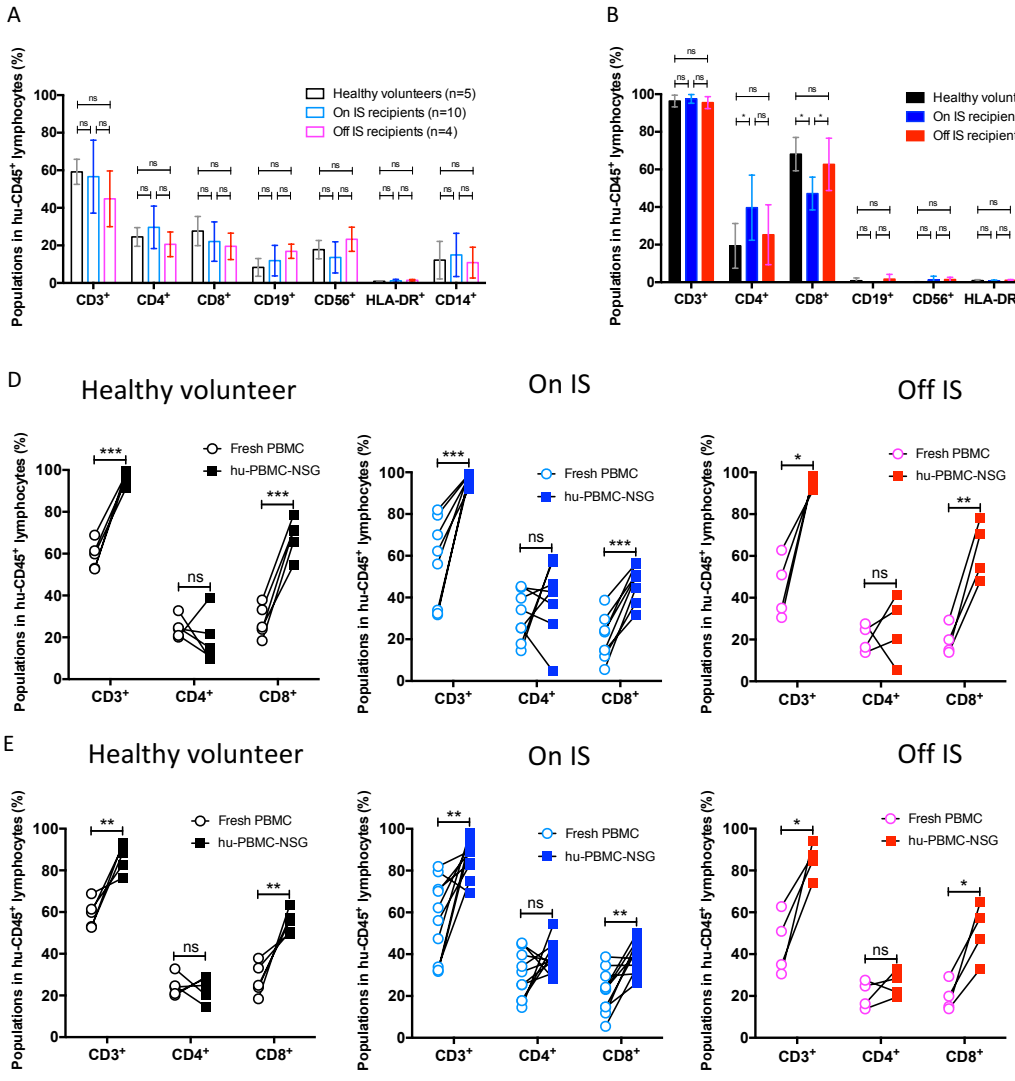
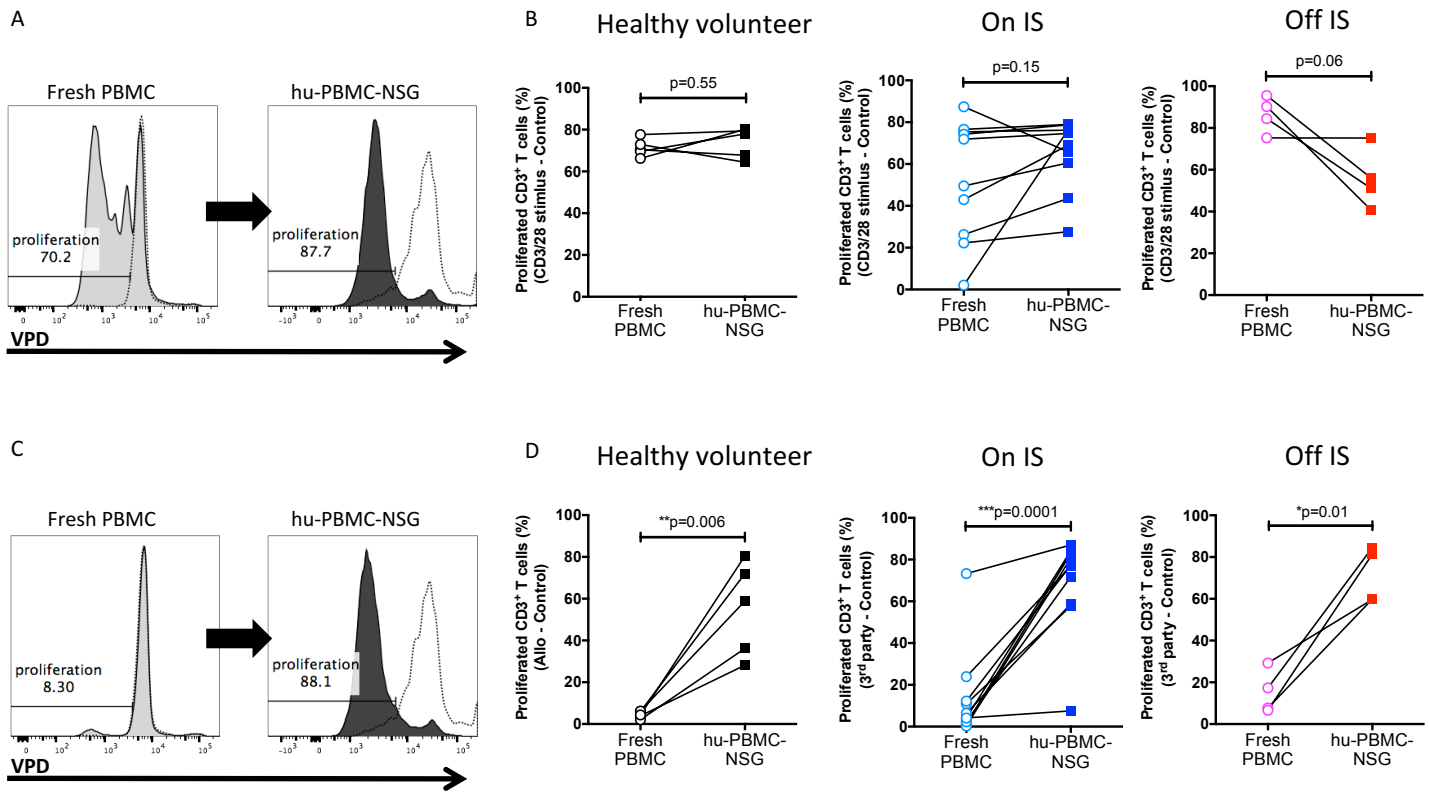

A
B
C
D
E
Healthy volunteer
On IS
Off IS
Healthy volunteers (n=5)
On IS recipients (n=10)
Off IS recipients (n=4)
Fresh PBMC
hu-PBMC-NSG
Populations in hu-CD45+ lymphocytes (%)
CD3+
CD4+
CD8+
CD19+
CD56+
HLA-DR+
CD14+
ns

Figure 3:


A
Fresh PBMC
hu-PBMC-NSG
proliferation
70.2
proliferation
87.7
VPD
B
Healthy volunteer
On IS
Off IS
Proliferated CD3+ T cells (%)
(CD3/28 stimlus - Control)
p=0.55
p=0.15
p=0.06
Fresh PBMC
hu-PBMC-NSG
C
Fresh PBMC
hu-PBMC-NSG
proliferation
8.30
proliferation
88.1
VPD
D
Healthy volunteer
On IS
Off IS
Proliferated CD3+ T cells (%)
(Allo - Control)
Proliferated CD3+ T cells (%)
(3rd party - Control)
**p=0.006
***p=0.0001
*p=0.01
Fresh PBMC
hu-PBMC-NSG

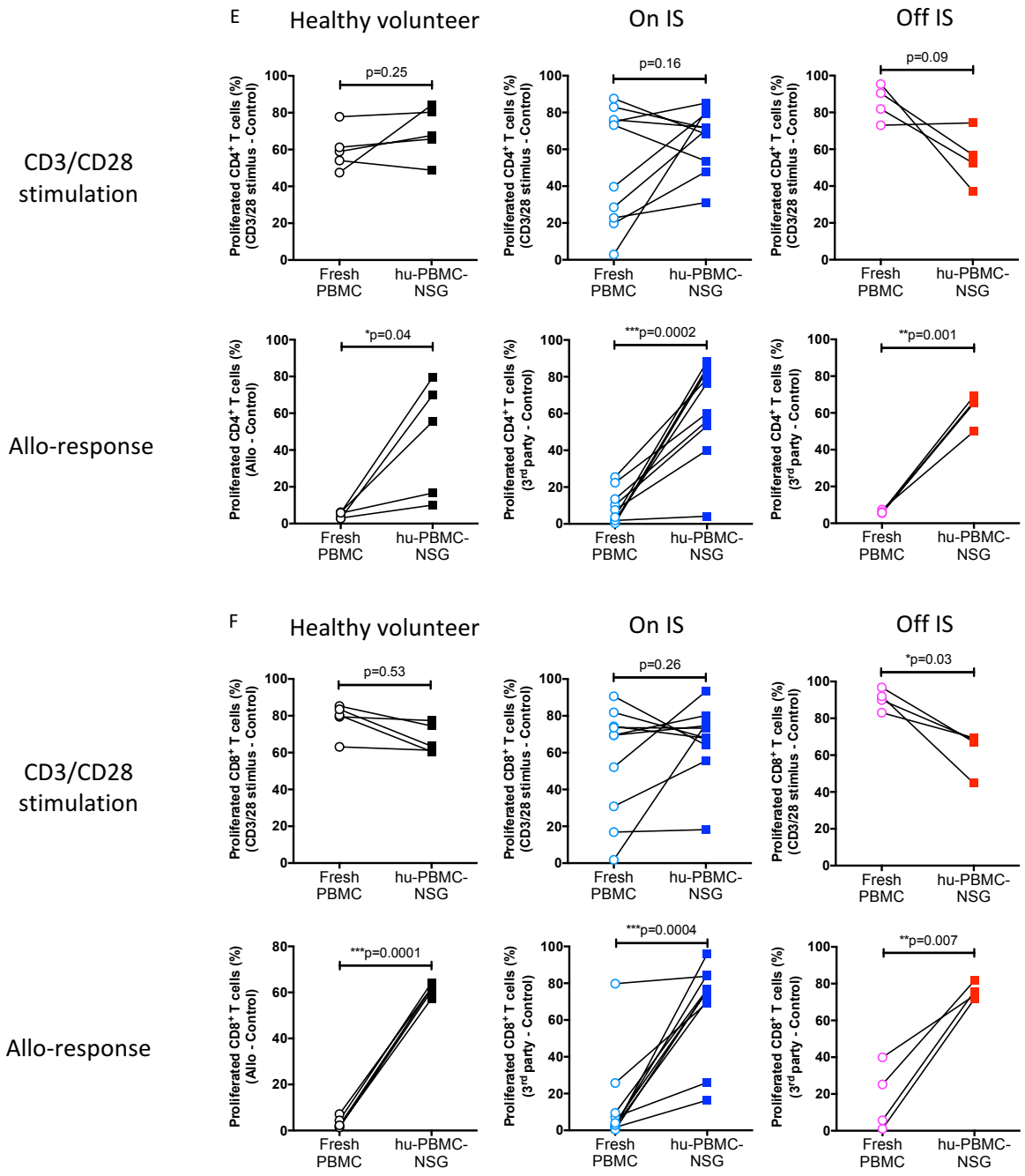

E
Healthy volunteer
On IS
Off IS
CD3/CD28 stimulation
Allo-response
Proliferated CD4+ T cells (%) (CD3/28 stimulus - Control)
Proliferated CD4+ T cells (%) (Allo - Control)
Proliferated CD4+ T cells (%) (3rd party - Control)
p=0.25
p=0.16
p=0.09
*p=0.04
***p=0.0002
**p=0.001
F
CD3/CD28 stimulation
Allo-response
Proliferated CD8+ T cells (%) (CD3/28 stimulus - Control)
Proliferated CD8+ T cells (%) (Allo - Control)
Proliferated CD8+ T cells (%) (3rd party - Control)
p=0.53
p=0.26
*p=0.03
***p=0.0001
***p=0.0004
**p=0.007
Fresh PBMC
hu-PBMC-NSG

Figure 4:

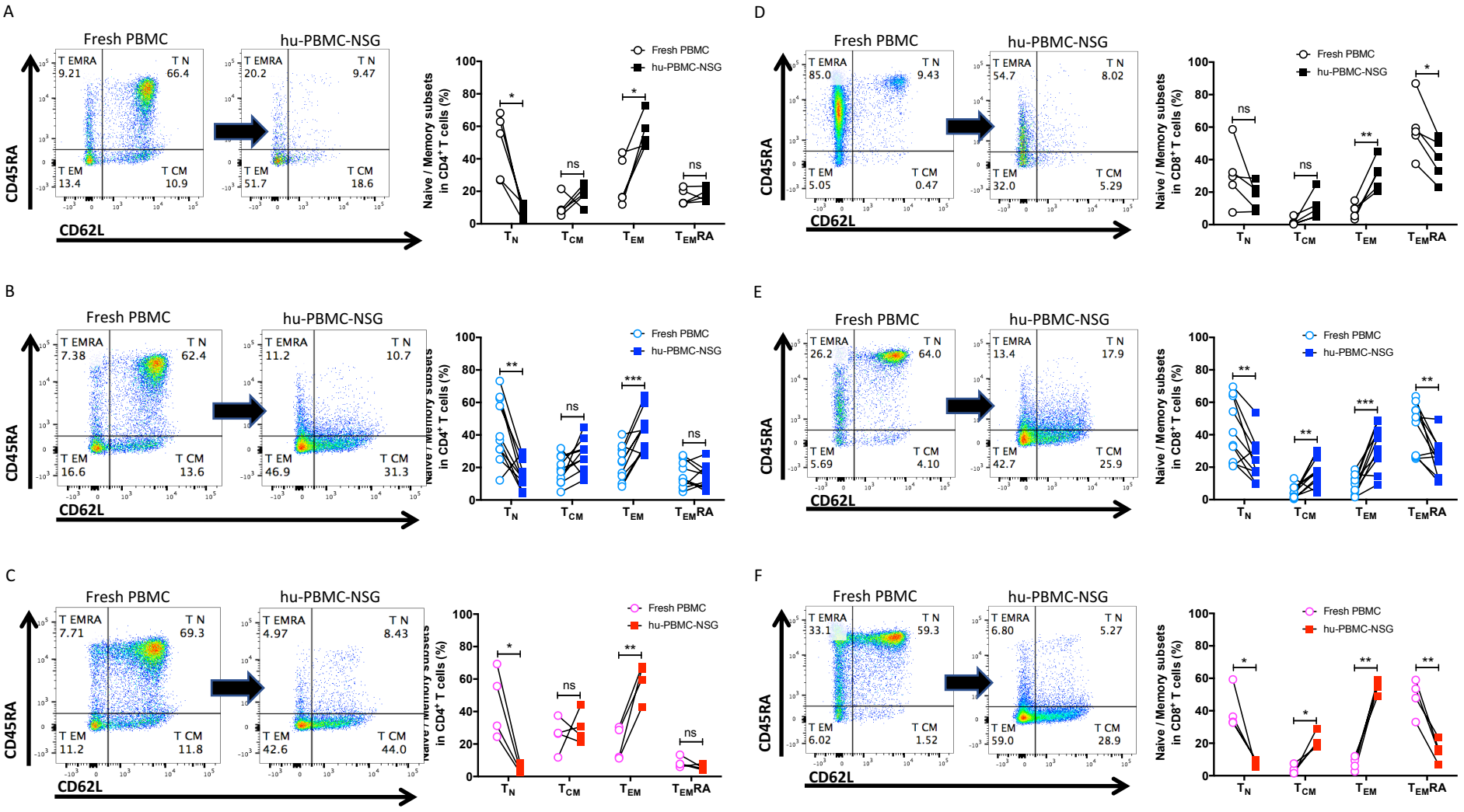

A
Fresh PBMC
hu-PBMC-NSG
T EMRA 9.21
T N 66.4
T EM 13.4
T CM 10.9
T EMRA 20.2
T N 9.47
T EM 51.7
T CM 18.6
CD45RA
CD62L
Naive / Memory subsets in CD4+ T cells (%)
Fresh PBMC
hu-PBMC-NSG
TN
TCM
TEM
TEMRA
B
T EMRA 7.38
T N 62.4
T EM 16.6
T CM 13.6
T EMRA 11.2
T N 10.7
T EM 46.9
T CM 31.3
C
T EMRA 7.71
T N 69.3
T EM 11.2
T CM 11.8
T EMRA 4.97
T N 8.43
T EM 42.6
T CM 44.0
D
T EMRA 85.0
T N 9.43
T EM 5.05
T CM 0.47
T EMRA 54.7
T N 8.02
T EM 32.0
T CM 5.29
Naive / Memory subsets in CD8+ T cells (%)
E
T EMRA 26.2
T N 64.0
T EM 5.69
T CM 4.10
T EMRA 13.4
T N 17.9
T EM 42.7
T CM 25.9
F
T EMRA 33.1
T N 59.3
T EM 6.02
T CM 1.52
T EMRA 6.80
T N 5.27
T EM 59.0
T CM 28.9

Figure 5:

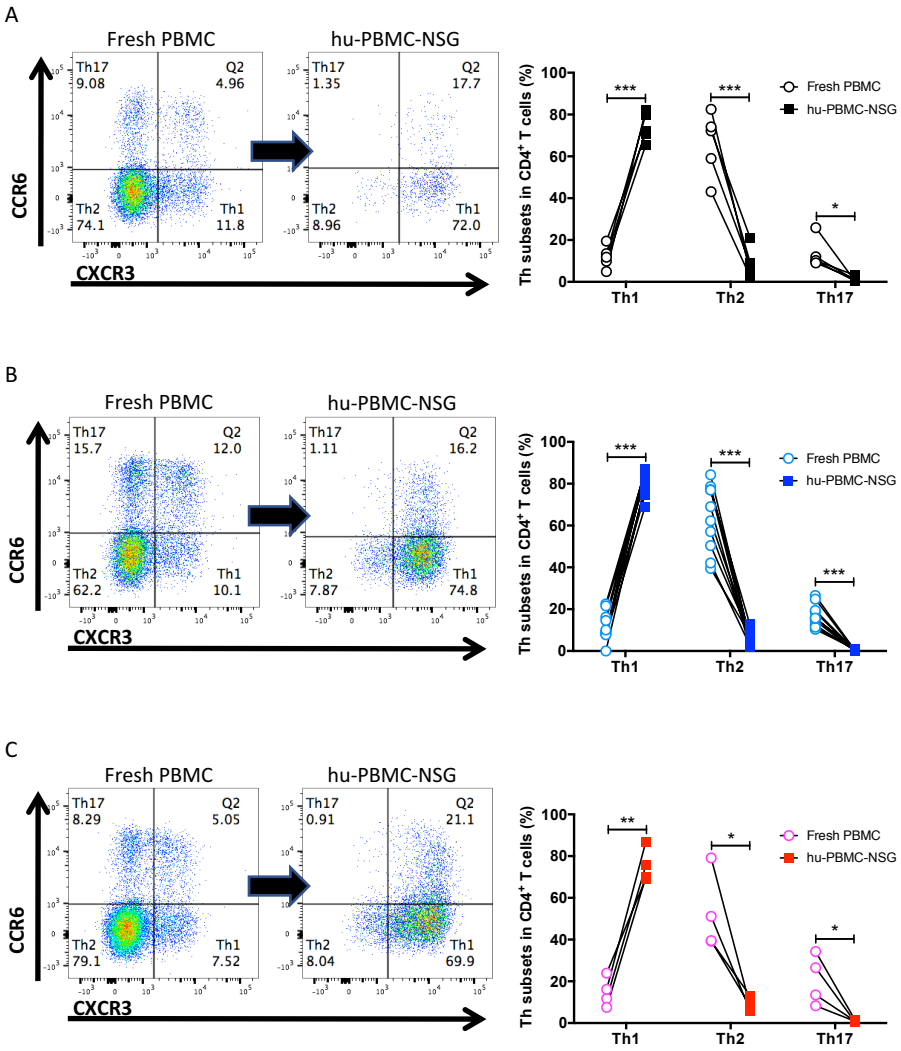
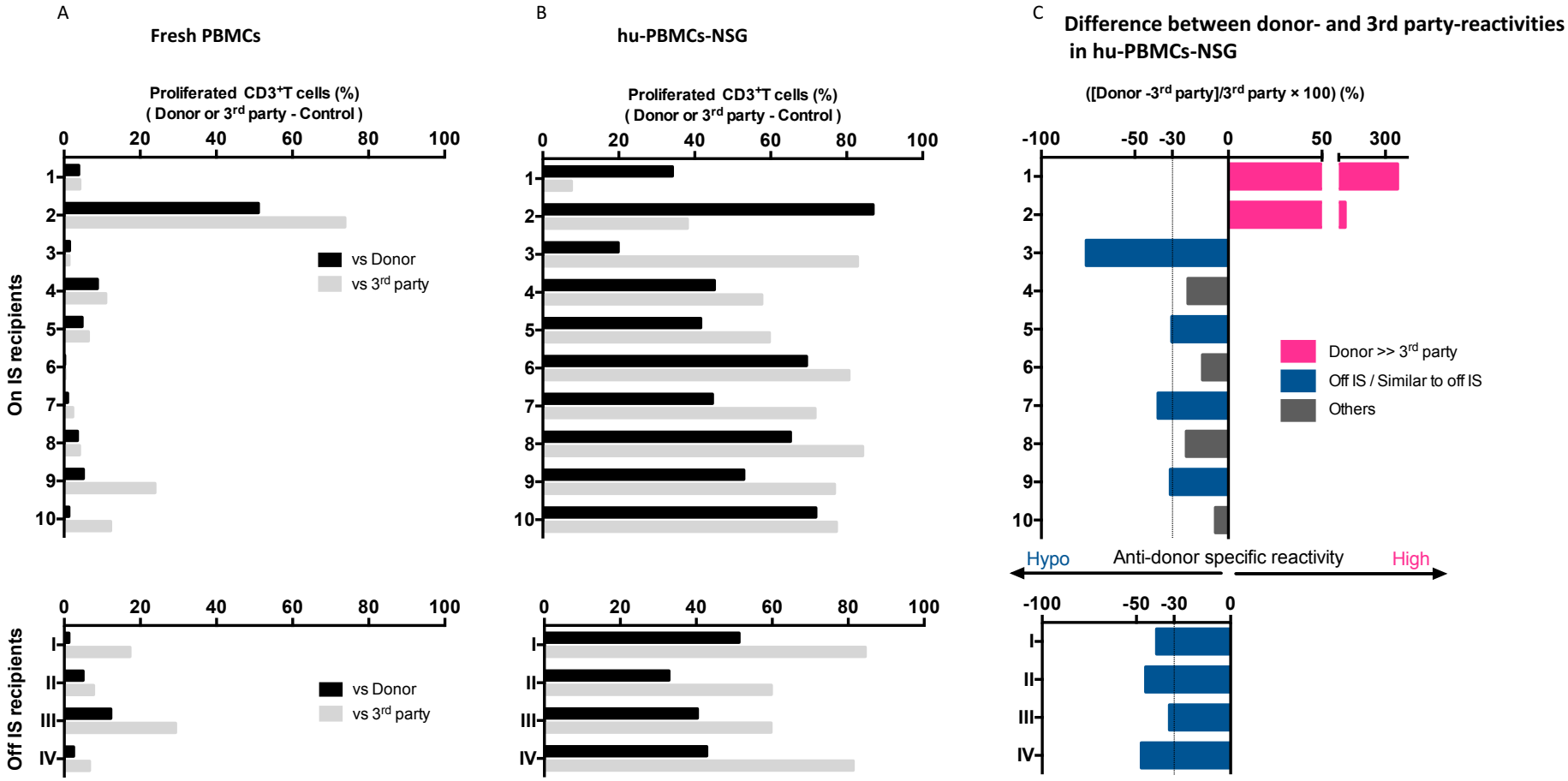

A
Fresh PBMC
hu-PBMC-NSG
Th17 9.08
Q2 4.96
Th2 74.1
Th1 11.8
Th17 1.35
Q2 17.7
Th2 8.96
Th1 72.0
CCR6
CXCR3
Th subsets in CD4+ T cells (%)
Fresh PBMC
hu-PBMC-NSG
Th1
Th2
Th17
B
Th17 15.7
Q2 12.0
Th2 62.2
Th1 10.1
Th17 1.11
Q2 16.2
Th2 7.87
Th1 74.8
C
Th17 8.29
Q2 5.05
Th2 79.1
Th1 7.52
Th17 0.91
Q2 21.1
Th2 8.04
Th1 69.9
D
Healthy volunteer
On IS
Off IS
TREG in CD4+ T cells (%)
ns
Fresh PBMC
hu-PBMC-NSG

Figure 6:


A
Fresh PBMCs
Proliferated CD3+T cells (%)
(Donor or 3rd party - Control)
0 20 40 60 80 100
On IS recipients
1 2 3 4 5 6 7 8 9 10
vs Donor
vs 3rd party
Off IS recipients
I II III IV
B
hu-PBMCs-NSG
Proliferated CD3+T cells (%)
(Donor or 3rd party - Control)
C
Difference between donor- and 3rd party-reactivities in hu-PBMCs-NSG
([Donor -3rd party]/3rd party × 100) (%)
-100 -50 -30 0 50 300
Donor >> 3rd party
Off IS / Similar to off IS
Others
Hypo
Anti-donor specific reactivity
High