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# IL-10 induces IgG4 production in NOD-*scid* Il2rγ$^{null}$mice humanized by engraftment of peripheral blood mononuclear cells

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To the editor,

IgG4 antibodies are considered to have anti-inflammatory activity and may confer protection against anaphylaxis and allergic inflammation. Strong increases in production of allergen-specific IgG4 antibodies are frequently observed in allergic patients receiving allergen-specific immunotherapy (AIT).[1] The production of IgG4 by human B cells *in vitro* is strongly enhanced by IL-10.[2,3] *In vivo* studies of the regulation of IgG4 have been handicapped by the fact that mice do not express this immunoglobulin isotype. As a result, conventional mouse models and gene-deficient or transgenic mice cannot be used to study aspects of the regulation of IgG4. The use of humanized mice allows the study of human immunoglobulin regulation including IgG4. Immunodeficient NOD.c*g-Prkdc*$^{scid}$ *Il2rg*$^{tm1Wjl}$/SzJ (alternatively named NOD-*scid* *Il2r*$\gamma^{null}$ or NSG) mice lack murine lymphocytes, including T, B and NK cells. This strain enables efficient engraftment of human hematopoietic progenitor cells (HPC) and peripheral blood mononuclear cells (PBMC).[4] While human lymphocytes engraft relatively well in NSG mice, there are certain limitations to this model, such as the low levels of human cytokines and growth factors present in serum and tissues, HLA incompatibility between engrafted lymphocytes and murine host cells, which will eventually culminate in graft-versus-host disease. Moreover, NSG mice have underdeveloped secondary lymphoid tissues. While engrafted human T- and B-cells populated lymph nodes and spleen, these tissues will not fully resemble

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human secondary lymphoid tissues.[5] Nevertheless, this model has been successfully used to study allergen-specific T cell responses.[6] Here, we established a humanized mouse model to optimize B cell and plasma cell engraftment and to study the regulation of human immunoglobulin production *in vivo*.

To determine optimal conditions for B cell engraftment in NSG mice, engraftment of human leucocytes after intraperitoneal (IP) and intravenous (IV) injections of $5x10^6$ and $20x10^6$ PBMC were analyzed after 14 and 21 days in spleen. Engraftment of human cells was determined by flow cytometry. Expression of human (hu)CD45 was used for gating of human leukocytes (Figure 1A). Successful engraftment of human cells was associated with increased spleen size (Supplementary Figure 1). In control mice that did not receive PBMC, no huCD45+ cells were detected (Figure 1A-D). Engraftment was not efficient (<1% huCD45+ splenocytes) for one out of 4 PBMC donors, and mice injected with these PBMC were excluded from the analysis. The percentages of huCD45+ cells ranged from 20% (in the $5x10E^6$ IV group) to 70% (in the $20x10E^6$ IV and $20x10E^6$ IP group) on day 14 and from 50% ($5x10E^6$ IV group) to 90% (in the $20x10E^6$ IV group) on day 21 (Figure 1B. The highest level of engraftment of huCD45+ cells was observed 21 days after IV injection of $20x10^6$ PBMC. No significant differences were found between the IV and IP groups in terms of total splenocyte counts and absolute numbers of huCD45+ cells (Figure 1B). Human B lineage cells (gated as huCD45+CD19+) were detectable at low levels on day 14 in the IV group and in both the IV and IP group on day 21 (Figure 1 E,F). To differentiate between B cells and plasma cells, we gated B cells as CD19+CD138- (Figure 1E,G) and plasma cells as CD138+ (Figure 1E,H). Total CD19+ B lineage cells, CD19+CD138- B cells and CD138+ plasma cells showed a similar pattern in which the highest level of engraftment, both as a percentage of huCD45+ cells and in absolute engrafted cell numbers, was observed with IV injection of $5x10^6$ PBMC. The higher B lineage engraftment observed in the $5x10^6$ IV group coincided with reduced T cell frequencies (Figure 1I,J, Supplementary figure 2A). Interestingly, the IV route of application was associated with a trend towards higher frequencies of CD4+ T cell engraftment, while the IP route was associated with increased CD8+ T cell frequencies (Figure 1J-L and Supplementary figure 2B,C). The small fraction of CD3+CD4-CD8- cells (Figure 1I) may represent natural killer T cells or γδ-T cells. We concluded that the IV route with $5x10^6$ PBMC was optimal for B lineage and CD4+ T helper cell engraftment.

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Next, we assessed the effect of IL-10 on the B cell compartment and immunoglobulin production. IP injection of 3 μg IL-10 at day 3 and day 12 after PBMC engraftment did not significantly affect the engraftment of huCD45+ cells or CD3+ T cells (Figure 2A-C). The frequencies of CD19+ cells were significantly reduced in the IL-10 treated group (Figure 2D), while CD19+CD138- B cells (Figure 2E) and CD138+ plasma cell (Figure 2F) frequencies showed trends of reduction, with no statistically significant difference. All human immunoglobulin isotypes were detectable in serum of IL-10-treated or saline-treated control mice (Figure 2G). Interestingly, IL-10 induced a significant increase in the serum level of IgG4, while no significant changes were observed for IgG1, 2, 3, IgM, IgA and IgE. Macrophages are involved in the regulation of acquired immune responses in the spleen. Because IL-10 is a know inhibitor of macrophage activation it could be speculated that IL-10 may affect different features of macrophages.

These data show that NSG mice can be used to efficiently engraft human B cells and plasma cells. However, it should be emphasized that this model has several limitations. Firstly, although human immunoglobulin isotypes are readily detectable in humanized NSG mice, murine Fcγ receptors have different affinities for human IgG subclasses than their human counterparts. Therefore an increase in IgG4 might not be functional in future studies due to human aberrant or abrogated Fc fragment-murine Fc receptor interactions. We observed a relatively large degree of variation in human immunoglobulin levels between individual mice within the same treatment groups (Figure 2G). This may result from differences in the degree of plasma cell differentiation and survival or class switch recombination between individual mice. Such variations may impair the detection of subtle effects in more advanced studies involving antigen-specific responses. Moreover, it remains to be determined whether allergen-specific B cell and antibody responses, can be efficiently induced in this model. Taken together, our findings demonstrate that human PBMC-engrafted NSG mice can be utilized to study human B cell and immunoglobulin responses. $5X10^6$ PBMC and application through IV injection were optimal for B cell and plasma cell engraftment and IL-10 treatment stimulated the *in vivo* upregulation of IgG4 production. In the allergy field, this model may be further developed to study *in vivo* antigen-specific B cell and antibody responses. It may be used to test novel AIT approaches, and combining this model with transfer of allergen-specific

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T cells lines (as reported by Vizzardelli *et al.*)[6] will allow in depth analysis the effect of different T cell subsets on B cell and antibody responses.

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**CONFLICT OF INTEREST**

The authors declare that they have no relevant conflict of interest.

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**Figure legends**

**Figure 1. Optimization of human lymphocyte engraftment in NSG mice.** (A) Representative dot plots showing muCD45 and huCD45 staining in splenocytes of a mouse that did not receive PBMC and a mouse that was engrafted with human PBMCs. (B) Frequencies of huCD45+ cells in splenocytes of NSG mice 14 and 21 days after injection of $5x10^6$ or $20x10^6$ PBMC through IV or IP injection. (C) Total cell counts per spleen. (D) Total number of engrafted huCD45+ cells. (E) Left: Isotype control (i.c.) for CD19 and CD138, right: gating strategy for CD19+, CD19+CD138- and CD138+ cells. Dot plots show huCD45+ splenocytes . (F) Frequencies (left) and absolute numbers per spleen (right) of CD19+, (G) CD19+CD138-, (H) CD138+ cells. (I) Dotted line in histogram: isotype control (i.c.) for CD3. Bottom left dot plot: i.c. for CD4 and CD8. Bottom right: gating strategy for CD4+ and CD8+ T cells. Frequencies of (J) total CD3+ T cells, (K) CD4+ and (L) CD8+ T cells. *P < .05, ** P < .01. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. Graphs show mean ± SEM from three mice per group.

**Figure 2. Administration of IL-10 to humanized NSG mice results in elevated IgG4 production *in vivo*.** (A) Gating strategy for gating of huCD45+, CD3+, CD19+ ,CD19+CD138- and CD138+ cells. (B-F) Frequencies at 21 days after PBMC engraftment in spleens of human huCD45+ (B), CD3+ T cells (C), CD19+ B lineage cells (D), CD19+CD138- B cells (E) and CD19+CD138+ plasma cells (F). (G) Serum immunoglobulin levels measured 21 days after PBMC engraftment in mice treated with saline or IL-10. Statistics were calculated using Mann-Whitney test, *P < .05. Graphs show mean ± SEM from 9 mice (saline treated) and 10 mice (IL-10 treated).

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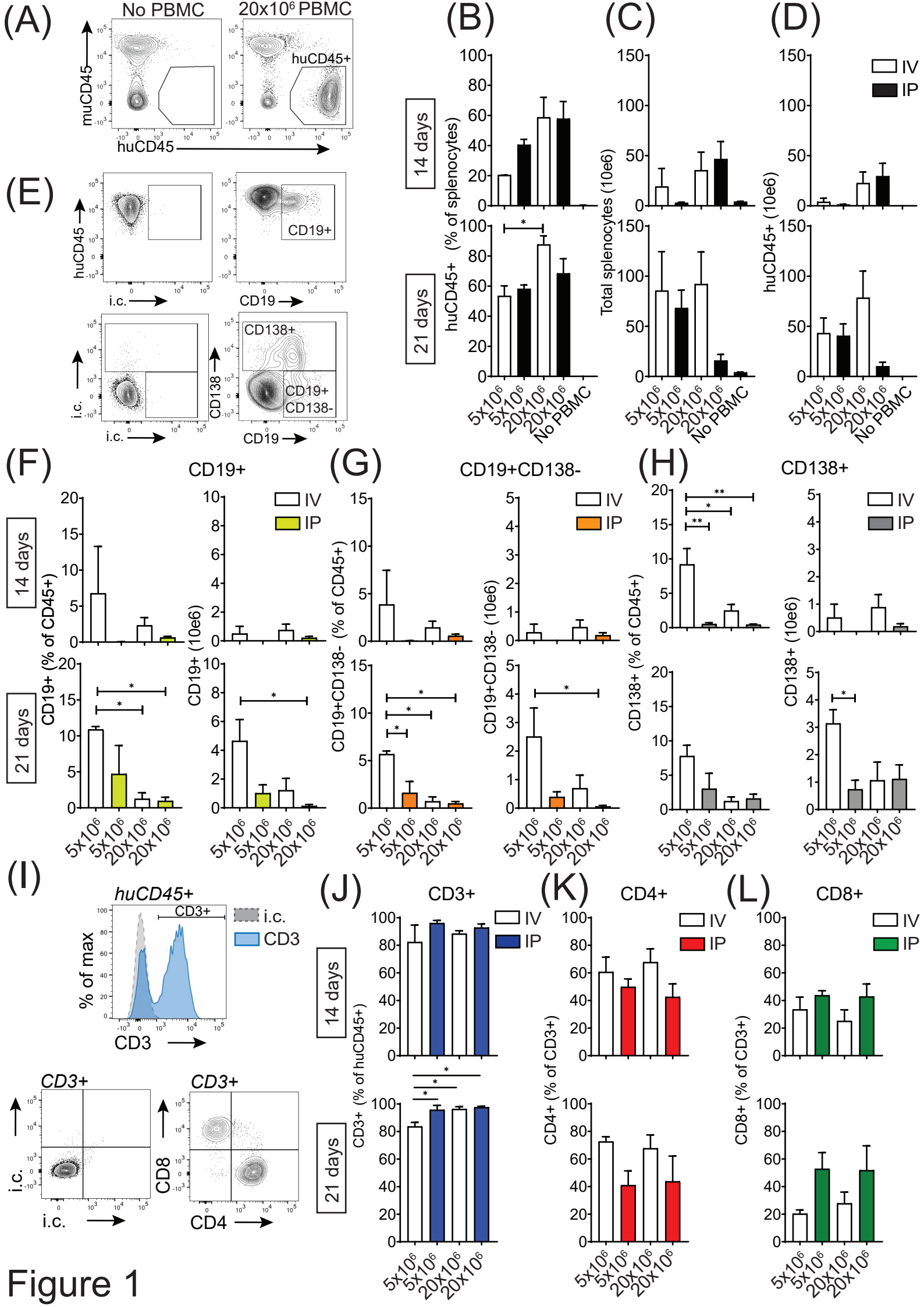

(A)
No PBMC
20x10⁶ PBMC
muCD45
huCD45
huCD45+
(B)
(C)
(D)
14 days
21 days
huCD45+ (% of splenocytes)
Total splenocytes (10e6)
huCD45+ (10e6)
IV
IP
5x10⁶
5x10⁶
20x10⁶
20x10⁶
No PBMC
(E)
huCD45
i.c.
CD19+
CD19
CD138+
CD138
CD19+
CD138-
(F)
CD19+
CD19+ (% of CD45+)
CD19+ (10e6)
(G)
CD19+CD138-
CD19+CD138- (% of CD45+)
CD19+CD138- (10e6)
(H)
CD138+
CD138+ (% of CD45+)
CD138+ (10e6)
(I)
huCD45+
CD3+
i.c.
CD3
% of max
CD3
CD8
CD4
(J)
CD3+
CD3+ (% of huCD45+)
(K)
CD4+
CD4+ (% of CD3+)
(L)
CD8+
CD8+ (% of CD3+)

Figure 1

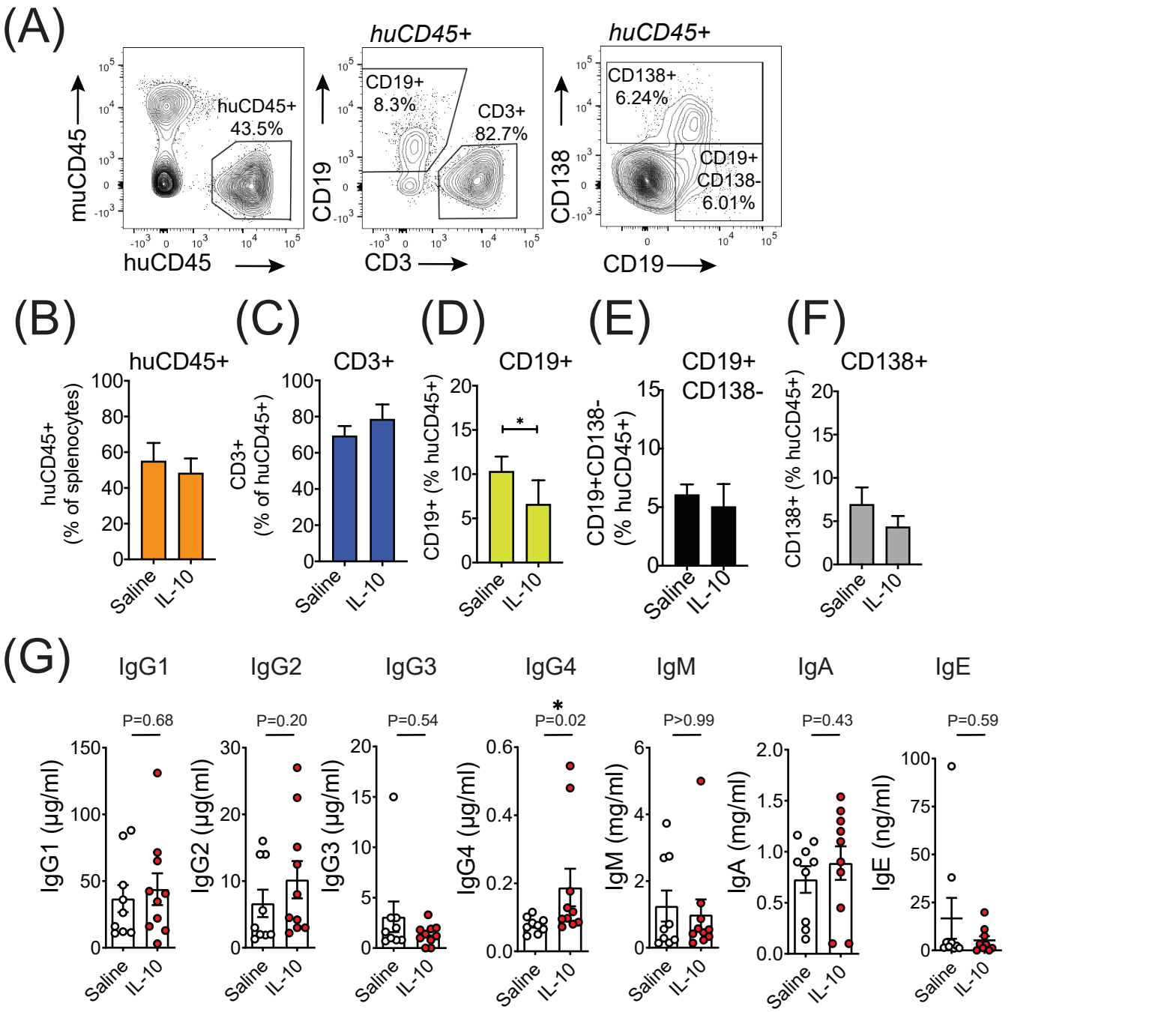

(A)
huCD45+
muCD45
huCD45
huCD45+
43.5%
huCD45+
CD19
CD3
CD19+
8.3%
CD3+
82.7%
huCD45+
CD138
CD19
CD138+
6.24%
CD19+
CD138-
6.01%
(B)
huCD45+
huCD45+
(% of splenocytes)
Saline
IL-10
(C)
CD3+
CD3+
(% of huCD45+)
Saline
IL-10
(D)
CD19+
CD19+ (% huCD45+)
*
Saline
IL-10
(E)
CD19+
CD138-
CD19+CD138-
(% huCD45+)
Saline
IL-10
(F)
CD138+
CD138+ (% huCD45+)
Saline
IL-10
(G)
IgG1
IgG2
IgG3
IgG4
IgM
IgA
IgE
P=0.68
P=0.20
P=0.54
*
P=0.02
P>0.99
P=0.43
P=0.59
IgG1 (µg/ml)
IgG2 (µg/ml)
IgG3 (µg/ml)
IgG4 (µg/ml)
IgM (mg/ml)
IgA (mg/ml)
IgE (ng/ml)
Saline
IL-10

Figure 2

**Supplementary figure 1. Spleen size is increased in NSG mice with engrafted human PBMC.** Photos showing spleens from NSG mice that were not injected with human PBMC (left) and from mice that were injected with $20x10^6$ PBMC IV.

**Supplementary figure 2. Absolute numbers of T cells in spleens of human PBMC engrafted NSG mice.** Absolute number per spleen of (A) CD3+, (B) CD4+, (C) CD8+ T cells in spleens of NSG mice. Graphs show mean ± SEM from three mice per group.

## Methods

### Isolation of PBMC

PBMCs were isolated from healthy non-allergic donors by Ficoll (Biochrom) density gradient centrifugation of heparinized peripheral venous blood, and cells were washed three times with PBS and resuspended in 0.9% saline to be used for injection to NSG mice.

**Engraftment of NSG mice with human immune cells.** NOD.Cg-*Prkdc*$^{scid}$ *Il2rg*$^{tm1Wjl}$/SzJ (NSG) mice were obtained from the Jackson Laboratory and raised under specific pathogen-free conditions. For the experiment shown in figure 1, PBMC from three donors were used to engraft NSG mice. PBMC from each donor were used to engraft 1 mouse per condition ($5x10^6$ IV, $20x10^6$ IV $5x10^6$ IP, or $20x10^6$ IP). For the engraftment of human PBMC, cells were suspended in 200 µl 0.9% saline and $5x10^6$, or $20x10^6$ PBMC were administered to 5 weeks old female NSG mice by a single intravenous injection in the lateral tail vein or intraperitoneally. To assess the effect of IL-10, mice engrafted with $5x10^6$ PBMC through a single intravenous injection in the lateral tail vein were injected intraperitoneally with 3 µg recombinant human IL-10 (Peprotech, London, UK), or saline three days and 12 days after PBMC administration. Mice were sacrificed on day 14 or 21 after PBMC injection. Blood samples were obtained through puncture of the retro orbital sinus and collected in plain 1.5 ml eppendorf tubes for serum collection. Blood specimens were rested at room temperature for 1 hour to allow clotting, followed by 15 minutes centrifugation at 1500 x g at 4°C. Splenocytes were prepared as a single cell suspensions by grinding and washing the spleens through a 70 µm nylon mesh (BD Biosciences). Cells were washed twice with PBS and erythrocytes were lysed using Red Blood Cell Lysing Buffer Hybri-Max® (Sigma-Aldrich) and cells were resuspended in RPMI 1640 medium supplemented with MEM Vitamin Solution, penicillin (100 U/ml), streptomycin (100 lg/ml), kanamycin, MEM Non-essential Amino Acid Solution, Sodium pyruvate solution (Sigma-Aldrich Chemie GmbH), and 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich Chemie GmbH).

Mice were considered as successfully engrafted when the frequency of huCD45+ cells in the spleen was >1.0%. When the frequency of huCD45$^+$ cells was <1.0% mice were excluded from analysis. Mice were housed at the AO Research Institute

Davos in individually ventilated cages for the duration of the study and all experimental procedures were carried out in accordance with Swiss law. Animal protocols were approved by the Cantonal Veterinary Office Graubünden. The use of human PBMC was approved by the ethical commission of the canton of Grisons, Switzerland and informed consent was obtained from all blood donors.

**Flow cytometry**

1 million splenocytes were incubated with antibodies listed in table 1. Cells were washed once with PBS pH 7.4 + 0.5% BSA (Sigma-Aldrich) + 2mM EDTA (Sigma-Aldrich) and fixed with 1% paraformaldehyde in PBS. Samples were measured with a FACSAria II instrument (Beckton Dickinson) or Galios (Beckman Coulter) and analyzed using FlowJo V10.4 software. For analysis of human engrafted cells, splenocytes were gated on huCD45+muCD45- cells.

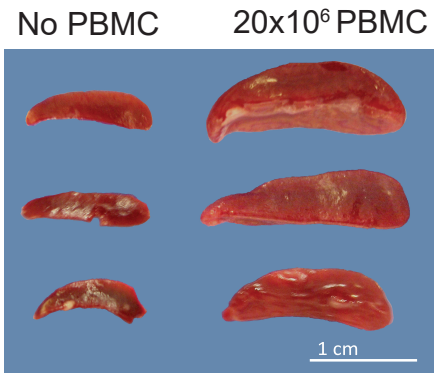
**Immunoglobulin measurement**

Human IgG1, IgG2, IgG3, IgG4, IgA, IgM and IgE in serum samples and cell culture supernatants were measured using a Bio-Plex Pro™ Human Isotyping Panel (Biorad, Hercules CA, USA) according to the manufacturer's instructions and analyzed using a Bioplex 200 system (Biorad). Serum samples were diluted 125000-fold (for IgG1, IgG2, IgG3, IgG4, IgA and IgM) or 10000-fold (for IgE) in serum diluent (provided in the Bio-Plex Pro™ Human Isotyping Panel kit).

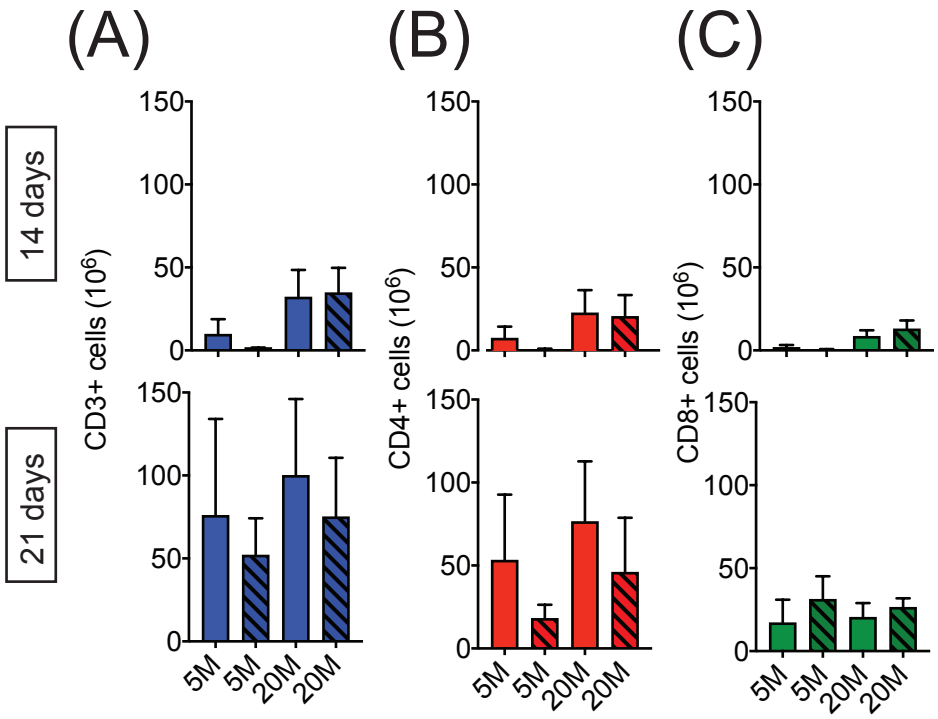
Supplementary table 1

**Antibodies used for flow cytometry**

| Target | Label | Clone | Isotype | Company |
|---|---|---|---|---|
| muCD45 | APC/Cy7 | 30-F11 | Rat IgG2b, κ | Biolegend |
| huCD45 | APC | HI30 | mIgG1, κ | Biolegend |
| huCD3 | RPE | UCHT1 | mIgG1, κ | DAKO |
| huCD4 | FITC | MT310 | mIgG1, κ | DAKO |
| huCD8 | ECD | SFCI21Thy2D3 | mIgG1 | Beckman Coulter |
| huCD4 | ECD | SFCI12T4D11 | mIgG1 | Beckman Coulter |
| huCD19 | ECD | J3-119 | mIgG1 | Beckman Coulter |
| huCD138 | PC5 | B-B4 | mIgG1, κ | Beckman Coulter |




Supplementary Figure 1




Supplementary Figure 2