# Supplementary Materials for

**Defects in mucosal immunity and nasopharyngeal dysbiosis in HSC transplanted SCID patients with IL2RG/JAK3 deficiency**

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## Supplementary Materials and Methods

### Patient cohorts

The healthy donors were recruited originally as part of the Milieu Intérieur cohort 1 (BioTrial, Rennes, France). The clinical study was approved by the Comité de Protection des Personnes - Ouest 6 (Committee for the protection of persons) on 13 June 2012 and by the French Agence Nationale de Sécurité du Médicament (ANSM) on 22 June 2012. The study is sponsored by the Institut Pasteur (Pasteur ID-RCB Number: 2012-A00238-35) and the original protocol was registered under ClinicalTrials.gov (study# NCT01699893). The samples and data used in this study were formally established as the Milieu Interieur biocollection (NCT03905993), with approvals by the Comité de Protection des Personnes – Sud Méditerranée and the Commission nationale de l'informatique et des libertés (CNIL) on April 11, 2018.

HSCT-treated SCID patients were recruited at Hôpital Necker-Enfants Malades (French National Reference Center for Primary Immunodeficiencies). Pathogenic mutations were identified in all cases (Supplementary Table 1). Some patients were receiving regular IgG replacement therapy (IgRT) by subcutaneous Ig injection with a trough level above 800 mg/dl (800 to 1385). No patients were receiving antibiotic therapy. At time of study, patients were aged 12 to 48 years (median 23). The age and gender is not significantly different between patients and healthy. Written informed consent was obtained from all patients and/or

parents. The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

**Human blood and nasopharyngeal swab collection**

Healthy donors were randomly selected (age and sex) and peripheral blood obtained from Establissement Francais du Sang (EFS) under protocol HS 2105-24405. Human peripheral blood mononuclear cells (PBMC) from healthy and SCID patients were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation. Nasopharynx specimens were obtained with sterile dry swabs (COPAN LQ Stuart Transport Swab; COPAN Italia S.p.A, Brescia, Italy), which are rotated five times around the inside of each nostril while applying constant pressure. Left and right nasopharynx swabs were collected from each patient in the office under strict aseptic conditions with sterile gloves and instrumentation, and snap frozen (-80°C). Laboratory protocols were standardized and staff members were trained in sample preparation protocols [1].

**PBMCs FACS analysis**

Flow cytometry analysis of T cells and ILCs was performed using the following antibodies: biotinylated anti-human CD1a (HI149; BioLegend), CD14 (61D3; Thermo Fisher Scientific), CD19 (HIB19; Thermo Fisher Scientific), CD34 (4H11; BioLegend), CD123 (6H6; BioLegend), CD203c (FR3-16A11; Miltenyi Biotec), CD303 (AC144; BioLegend), TCRγδ (B1; Thermo Fisher Scientific) and FcεRIα

(AER-37; BioLegend) Abs in combination with the streptavidin BUV661 (BD Biosciences); and conjugated anti-human CD3 BV650 (UCHT1, BD Biosciences), CD4 BV750 (SK3, BD Biosciences), CD8a BV785 (RPA-T8, BioLegend), CD45 BUV805 (HI30, BD Biosciences), CD45RA BV570 (HI100, BioLegend), CD7 BV711 (M-T701; BD Biosciences), CD16 BUV496 (3G8, BD Biosciences), CD56 BUV737 (NCAM16.2; BD Biosciences), CD127 PE (hIL-7R-M21; BD Biosciences), CD161 Alexa Fluor 700 (HP-3G10; BioLegend), CD25 BUV563 (2A3; BD Biosciences), CRTh2 Alexa Fluor 647 (BM16, BD Biosciences), CD117 BV605 (104D2, BioLegend), CXCR3 PE-CF594 (1C6/CXCR3; BD Biosciences), CXCR5 BUV395 (RF8B2, BD Biosciences), CCR6 BB515 (11A9, BD Biosciences), CCR4 PE-Cy7 (1G1, BD Biosciences). Fc receptors were blocked using IgG from human serum (Millipore Sigma). Surface membrane staining was performed in Brilliant Stain Buffer (BD Biosciences). The fixable viability dye eFluor 506 (Thermo Fisher Scientific) was used to exclude dead cells. Samples were fixed in 2% PFA, resuspended in PBS 2% FCS and acquired on a Symphony A5 (BD Biosciences) using FACSDiva 8 and analyzed with FlowJo 10 (BD Biosciences).

**Nasopharynx swabs processing**

Left and right nasopharynx swabs were thawed and vortexed for 1 minute at 2500 rpm to insure complete sample recovery. Both swabs were mixed and 1 ml media (in duplicate) were processed independently. Samples (1 ml media) was transferred in a 96 well deep-well plate and centrifuged at 16.000g for 10 minutes at 4°C to pellet the cells and accompanying microbes for 16S rRNA sequencing

analysis. Supernatants were recovered using a TECAN Evo 75 robot for antibodies and cytokine analysis.

**Digital enzyme-linked immunosorbent assay**

The Single Molecule Array (Simoa) Analyzer (Quanterix©, Lexington, MA 02421, USA) is automated equipment and enables detection of lower concentrations, compared to conventional ELISA and radioimmunoassay technology, by providing the capability of detection of proteins by a so-called digital ELISA principle based on counting individual enzyme-labeled immunocomplexes of proteins captured on paramagnetic beads in single-molecule arrays [2,3]. The combination of this technology with high-affinity antibodies enabled the direct quantification of proteins at attomolar (femtograms per milliliter) concentrations [4]. The Simoa HD-1 Analyzer consumables including wash buffers, Simoa discs, 96 well plates, sealing oil, pipette tips, enzyme substrate kit and cuvettes were purchased from Quanterix Corporation (Quanterix©). A Simoa IgA assay was developed using a Quanterix homebrew development kit assay according to the manufacturer's instructions. Two antibodies specific for human IgA1 (B3506B4, Southern Biotech) and IgA2 (14AS, Novus Biologicals) are used to biotinylation in paramagnetic beads. The anti-human IgA (AD3, Thermofisher) capable to recognizing both human IgA1 and IgA2 are used to the detection. Capture antibody and coupled to the paramagnetic beads at a concentration of 0.5 mg/ml. The natural human IgA1 (ab91020) and IgA2 protein (ab91021) (Abcam Cambridge, UK), claimed to be > 95% purity on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by the

supplier, was used as a standard curve after cross-reactivity testing. A quantification range of 0.01 to 10.000.000 pg/ml was utilized in the present study. IL-6, IL-17A and TNFα was quantified by Simoa® Cytokine 3-Plex B (101319), IL-22 by Simoa® Total IL-22 Discovery Kit (103071), IL-1β by Simoa® IL-1β Advantage Kit (101605), IL-33 by Simoa® IL-33 Discovery Kit (103093), IL-4 by Simoa® IL-4 Advantage Kit (100196), IL-13 by Simoa® IL-13 Advantage Kit (102732), IL-5 by Simoa® IL-5 Advantage Kit (102860), according to the manufacturer's instructions. IFN-γ and IL-17F (both single plex) protein were quantified by Simoa assays developed with Quanterix Homebrew kits. For the IFNγ assay, the MD-1 antibody clone (BioLegend) was used as a capture antibody after coating on paramagnetic beads (0.3 mg/mL), the 25718 antibody clone (R&D Systems) was biotinylated (biotin/antibody ratio = 40/1) and used as the detector antibody at a concentration of 0.3ug/mL. The SBG revelation enzyme concentration was 150pM. Recombinant protein (PBL Assay Science) was used to quantify IFNγ concentrations.

**Total protein and Ig determination**

Total IgA, IgM, IgG1, IgG2, IgG3 and IgG4 were determined for swabs medium using the Bio-Plex Pro Human Isotyping Assay Panel (Biorad, Hercule, CA, USA) according to the manufacturers' instructions. Samples were diluted 1/200 and run in duplicate using Bio-Plex 200 in two independent experiments. The total protein content of the supernatants of nasopharynx swabs was determined by the

Bradford method [5] using the Bio-Rad Protein Assay and serum albumin as standard.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISAs were performed according to the manufacturers' instructions and samples were run in duplicate in two independent experiments. Absorption was measured at 450 nm using the Multiskan Spectrum microplate reader (Thermo Scientific, USA). Total IgD were determined for swabs medium using a kit (MBS564048, Mybiosource, San Diego, USA, detection range: 6,25-400 ng/ml, diluted 1/100). Total IgE were determined for swabs medium using a kit (88-50610, Invitrogen, Massachusetts, USA, detection range: 7.8-500 ng/mL, diluted 1/3). Eosinophil cationic protein (ECP) were determined for swabs medium using a kit (MBS2602477, Mybiosource, San Diego, USA, detection range: 0.312-20 ng/ml, diluted 1/2). Nasopharyngeal mucin levels were analyzed using a MUC5AC ELISA Kit (NBP2-76703, Novus Biologicals, diluted 1/50).

**Bacterial Flow Cytometry**

Bacterial species-specific antibody against microbiota were assessed by a flow cytometry assay as previously described [6]. Briefly, the bacterial pellet was washed and resuspended in flow cytometry buffer (1x-PBS, 2% BSA, 0.02% w/v sodium azide) with SYBR Green (1/10000 (v/v) dilution, Invitrogen) incubated 30 min on ice. After, bacterial suspension was washed twice and pelleted by centrifugation at 16.000g for 10 min. Supernatant was removed and pellets were resuspended in

100 µL flow cytometry buffer with mouse anti-human IgA-PE (clone IS11-8E10), anti-human IgD-PerCP-Cy5.5 (clone IAG-2), anti-human IgM-APC (clone SA-DA4) and anti-human IgG-BUV395 (clone G18-145) at a final IgG concentration of 10 µg/mL in a 96-well V-bottom plate and incubated for 20 min on ice. Suspensions were washed once with 100 uL of flow cytometry buffer and cells pelleted by centrifugation, then resuspended in flow cytometry buffer with DAPI (Life Technologies) prior to flow cytometry. Acquisition of cells events was performed using a FACS LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA) and analysis was performed with Flow-Jo software software (TreeStar, Ashland, OR). Staining of live bacteria was visualized by gating on SYTO BC$^{+}$FSC$^{+}$SSC$^{+}$ DAPI$^{-}$ cells. The gating strategies used to analyze different bacterial populations are shown in [7].

**Bacterial DNA isolation and 16S rRNA sequencing**

Extraction of total genomic DNA from nasal swabs samples used the TECAN Freedom EVOware workstation and NucleoSpin® 96 Genomic DNA kit (Macherey-Nagel ®). For DNA extraction, a "negative" control was included containing buffers. Briefly, the pellets that remain in the deep well plate were incubated with Ready-Lyse™ Lysozyme Solution (250 U/µl) (Epicentre, Hessisch Oldendorf, Germany) for 30 minutes at 37°C followed by Proteinase K/buffer T1 treatment overnight (55°C). 20 µg of glycogen carrier was added prior to DNA extraction and elution in 25 µl dH20. DNA concentration was determined using TECAN (QuantiFluor® ONE dsDNA System, Promega), and DNA integrity and

size were also confirmed with the Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

The V3-V4 region of bacterial 16S rRNA was amplified using V3-340F (CCTACGGRAGGCAGCAG) and V4-805R (GGACTACHVGGGTWTCTAAT) primers (Chakravorty, Helb et al. 2007, Castelino, Eyre et al. 2015). The primers have a primer linker, primer pad, unique 8-mer Golay barcode which was used to tag PCR products from respective samples and negative control. PCR reactions consisted of 18 µl of AccuPrime Pfx Super Mix (12344-040; Invitrogen), 0.5 µl of each primer and 1 µl of DNA (10 ng). PCR was carried out as follows: 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 55 °C for 15 s and 72 °C for 1 min, and a final extension step at 72 °C for 10 min on a Biorad thermocycler. PCR products were cleaned using Nucleo Mag magnetic purification beads (MACHEREY-NAGEL Kit) following the protocol, quantified with the Quanti Fluor® ONE dsDNA kit (Promega), and pooled in equal amounts of each PCR product. Library pools were loaded at 12pM with a 15% PhiX spike for diversity and sequencing control, onto a v3 300-bp paired end reads cartridge for sequencing on the Illumina MiSeq NGS platform. The raw sequence data for each sample were deposited in the NCBI Sequence Read Archive (SRA) (submission ID: PRJNA772582).

**Sequence processing and statistical analysis**

After removing reads containing incorrect primer or barcode sequences and sequences with more than one ambiguous base, we recovered from 32 samples

a total of 2.974.329 reads (90.131 reads on average per sample). Less than five hundred reads were found in negative controls, suggesting background noise did not have a significant impact on the generation of data. However, to minimize the effect of background noise on a low bacterial load sample data analysis, we additionally removed taxa that are potentially contamination from downstream analysis. These taxa include *Escherichia coli*, a common DNA contamination found in DNA polymerase enzyme. The bioinformatics analysis was performed as previously described [8]. Briefly, amplicons were clustered into operational taxonomic units (OTU) with VSEARCH (v1.4) and aligned against the SILVA database. The input amplicons were then mapped against the OTU set to get an OTU-abundance table containing the number of reads associated with each OTU. The normalization, statistical analyses and multiple visualization were performed with SHAMAN (SHiny application for Metagenomic Analysis (shaman.c3bi.pasteur.fr) based on R software.

**Bacterial quantification by quantitative RT-PCR assays**

To gain further insight into microbiota counts, a qPCR was applied, using universal 16S rRNA primers to measure total bacteria (16S_F: 5'-ATTACCGCGGCTGCTGG-3' and 16S and 16S_R: 5'-ATTACCGCGGCTGCTGG-3') [9]. *Streptococcus pneumoniae* (*LytA* gene, F: 5'-ACGCAATCTAGCAGATGAAGC-3' and R: 5'- TGTTTGGTTGGTTATTCGTGC -3') [10] load was also measured by qPCR. PCR reactions consisting of 10 µL SYBR Green PCR master mix (Roche), 1 µL (10 nM) each primer, 200 ng template cDNA

in 20 µL of reaction carried out on an ABI StepOne Plus Sequence Detection System (Applied Biosystems). Thermocycling reactions consisted of 1 min at 95 °C followed by 35 cycles of 15 s at 95 °C, 15 s at 56 °C, and 45 s at 72 °C.

**Statistical analysis**

Statistics were performed using GraphPad Prism (San Diego, USA). P values were determined by a Kruskal-Wallis test, followed by Dunn's post-test for multiple group comparisons with median reported; *P < 0.05; **P < 0.01; ***P < 0.001. Correlations between the different assays were calculated using Spearman test. Heatmaps were generated with Qlucore OMICS explore Version 3.5(26). Correlation matrices were built using the Spearman correlation, and computed using R (v4.0.3).

**Supplemental References**


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**List of the Milieu Intérieur Consortium members**

The Milieu Intérieur Consortium¶ is composed of the following team leaders: Laurent Abel (Hôpital Necker), Andres Alcover, Hugues Aschard, Philippe Bousso, Nollaig Bourke (Trinity College Dublin), Petter Brodin (Karolinska Institutet), Pierre Bruhns, Nadine Cerf-Bensussan (INSERM UMR 1163 – Institut Imagine), Ana Cumano, Christophe D'Enfert, Ludovic Deriano, Marie-Agnès Dillies, James Di Santo, Françoise Dromer, Gérard Eberl, Jost Enninga, Jacques Fellay (EPFL, Lausanne), Ivo Gomperts-Boneca, Milena Hasan, Gunilla Karlsson Hedestam (Karolinska Institutet), Serge Hercberg (Université Paris 13), Molly A Ingersoll, Olivier Lantz (Institut Curie), Rose Anne Kenny (Trinity College Dublin), Mickaël Ménager (INSERM UMR 1163 – Institut Imagine), Frédérique Michel, Hugo Mouquet, Cliona O'Farrelly (Trinity College Dublin), Etienne Patin, Sandra Pellegrini, Antonio Rausell (INSERM UMR 1163 – Institut Imagine), Frédéric Rieux-Laucat (INSERM UMR 1163 – Institut Imagine), Lars Rogge, Magnus Fontes, (Institut Roche), Anavaj Sakuntabhai, Olivier Schwartz, Benno Schwikowski, Spencer Shorte, Frédéric Tangy, Antoine Toubert (Hôpital Saint-Louis), Mathilde Touvier (Université Paris 13), Marie-Noëlle Ungeheuer, Christophe Zimmer, Matthew L. Albert (In Sitro)§, Darragh Duffy§, Lluis Quintana-Murci§,

¶ unless otherwise indicated, partners are located at Institut Pasteur, Paris

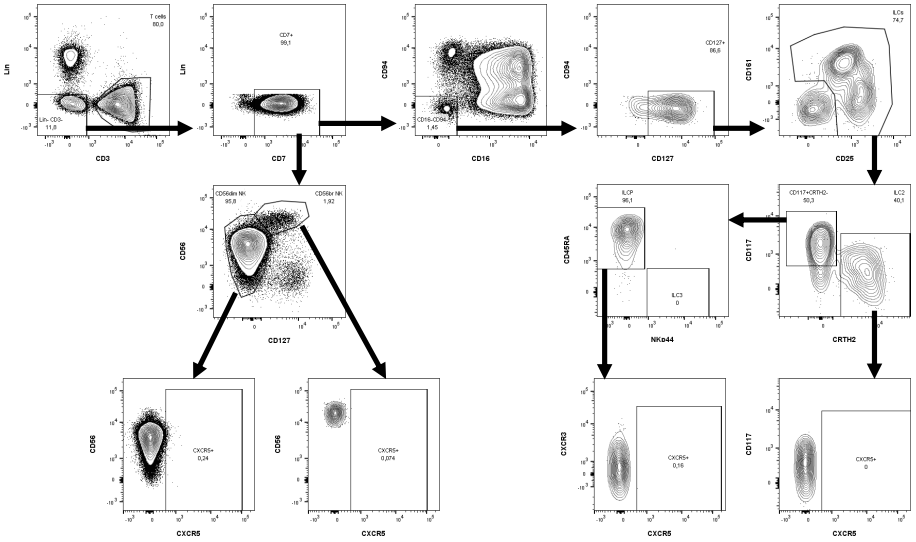
§ co-coordinators of the Milieu Intérieur Consortium

Additional information can be found at: http://www.milieuinterieur.fr

# Supplemental Fig 1

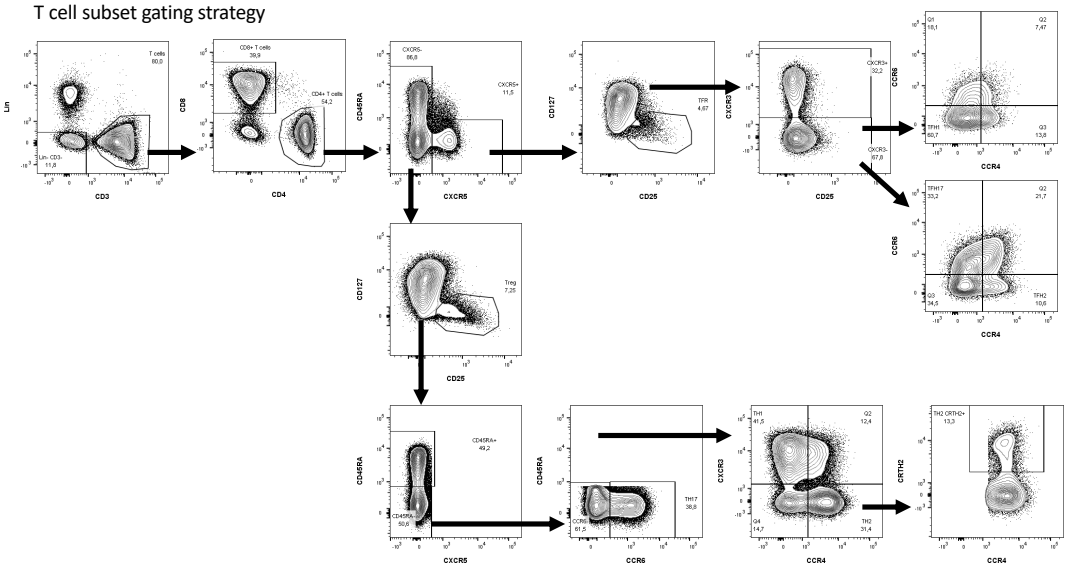
**A**

NK, ILCP, ILC2 and ILC3 gating strategy



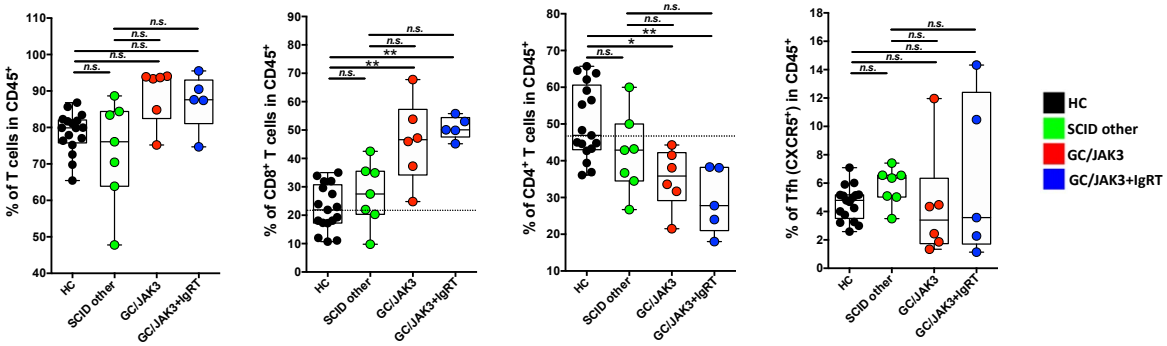
**B**

T cell subset gating strategy

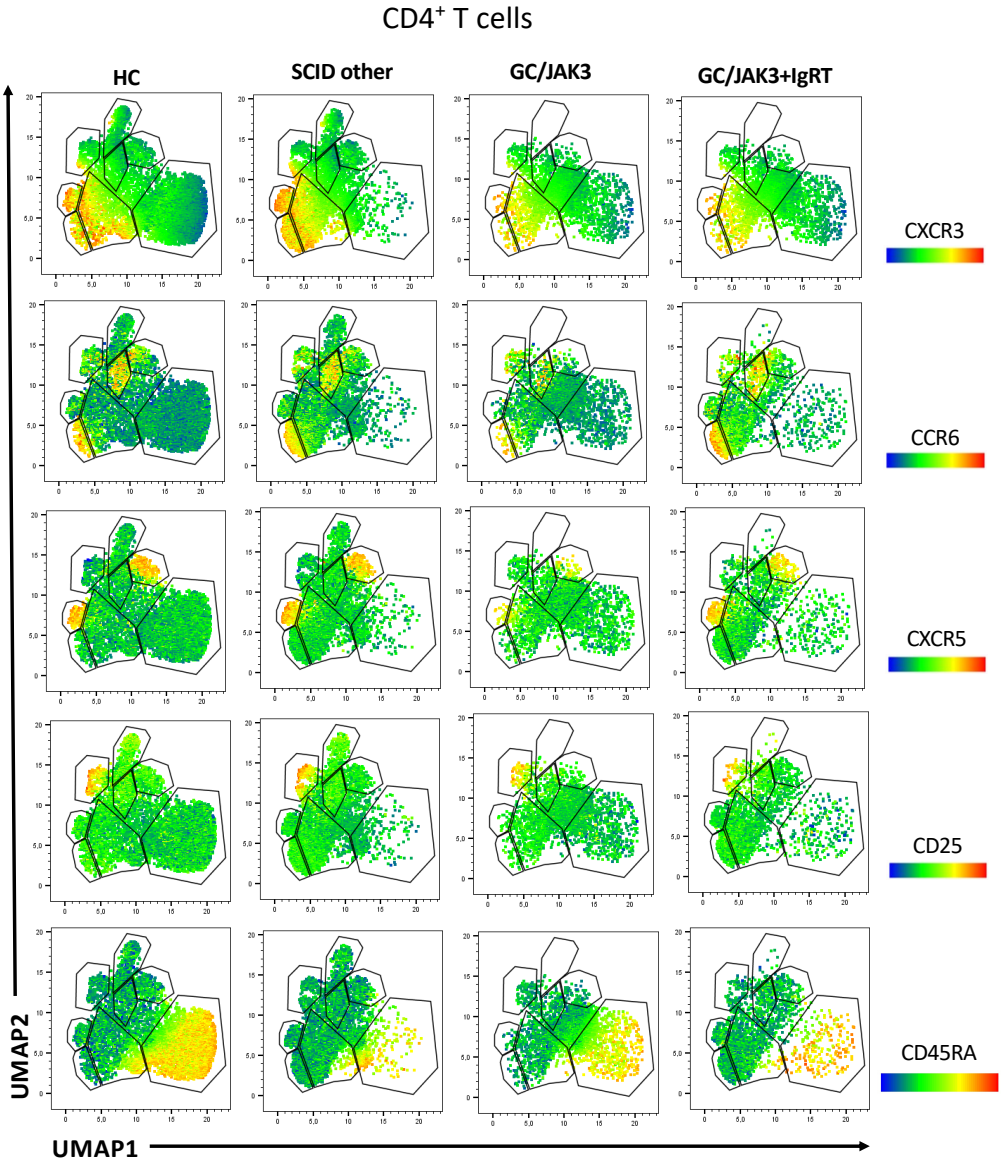


# Supplemental Fig 2

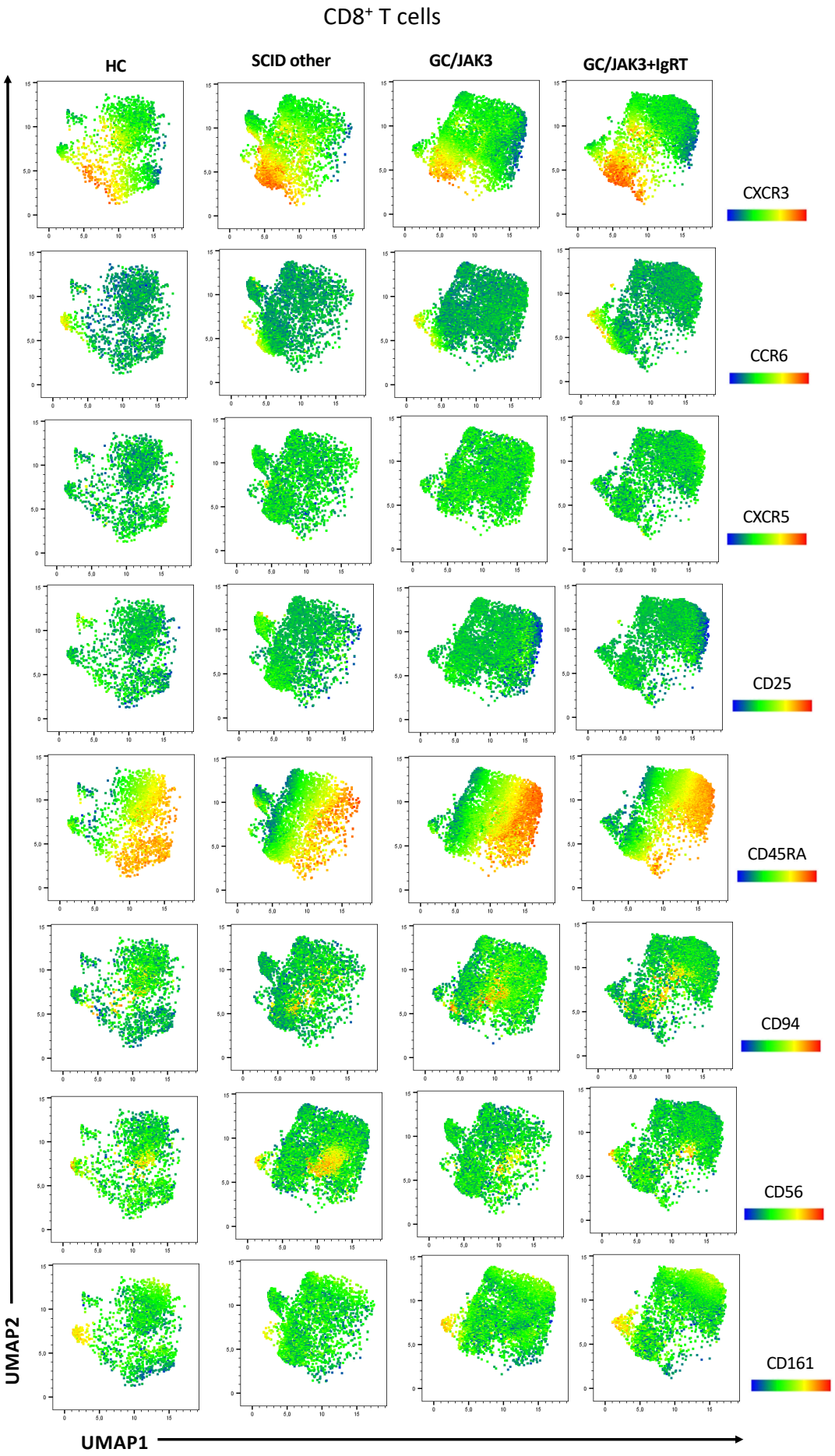
**A**




**B**



# Supplemental Fig 3



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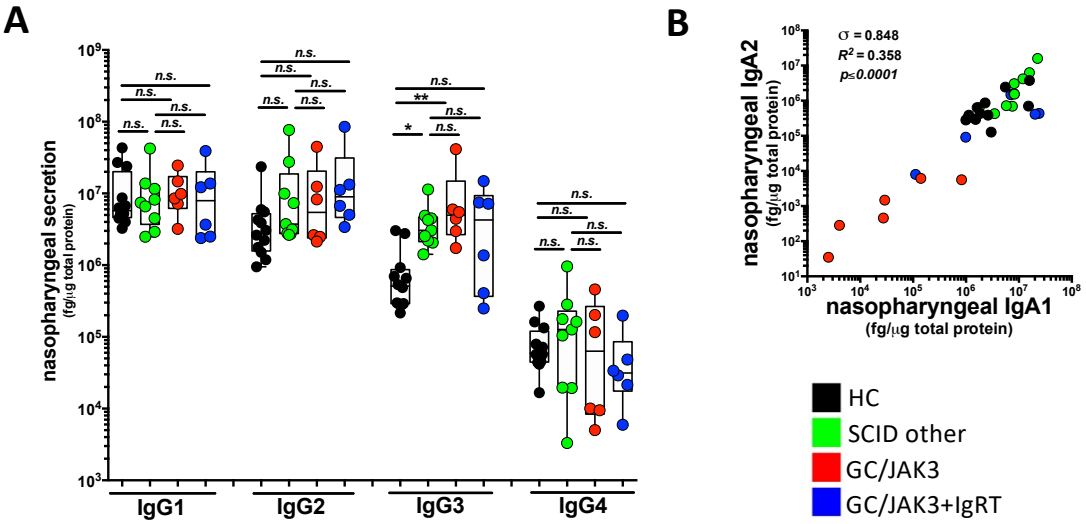

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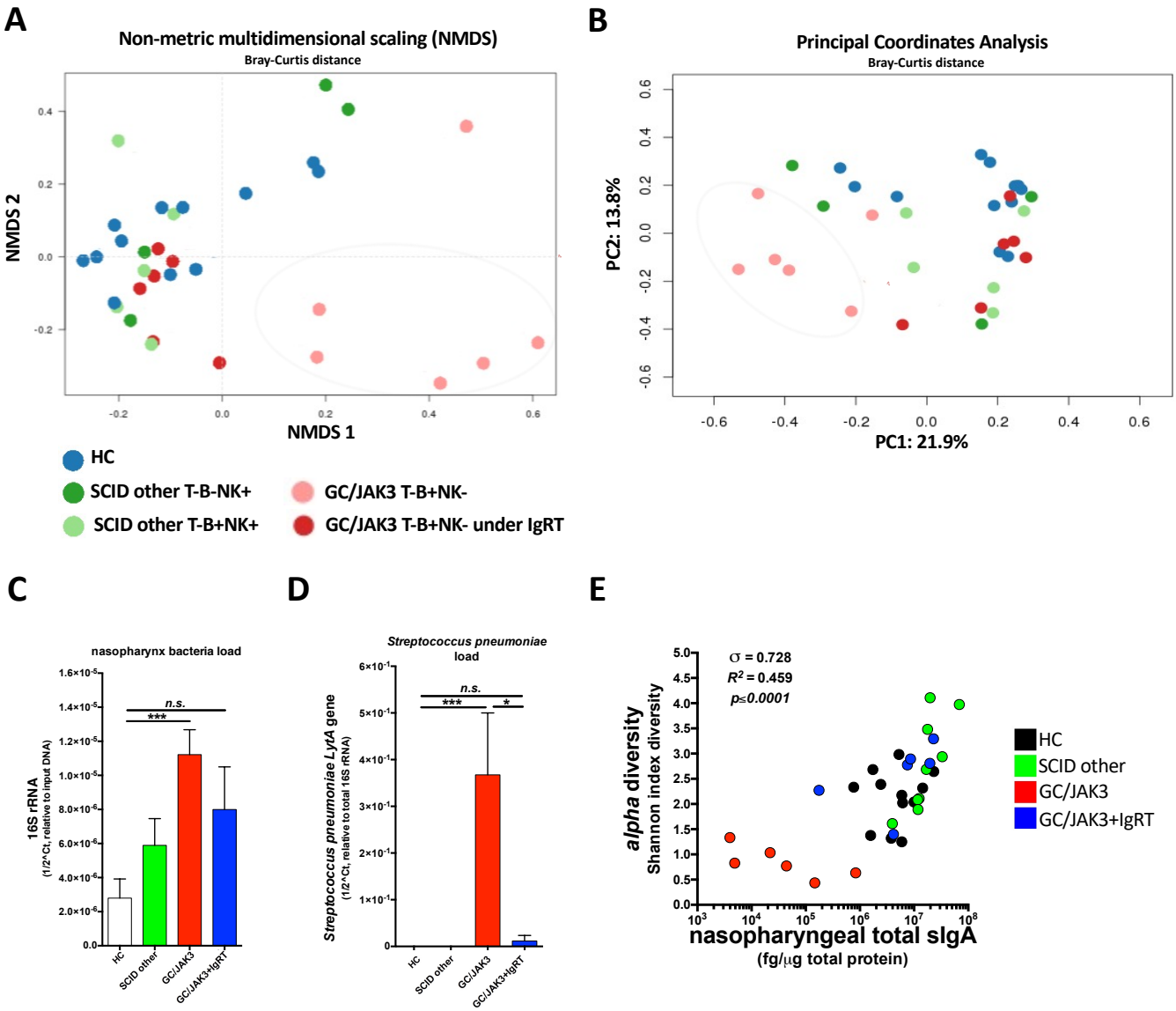
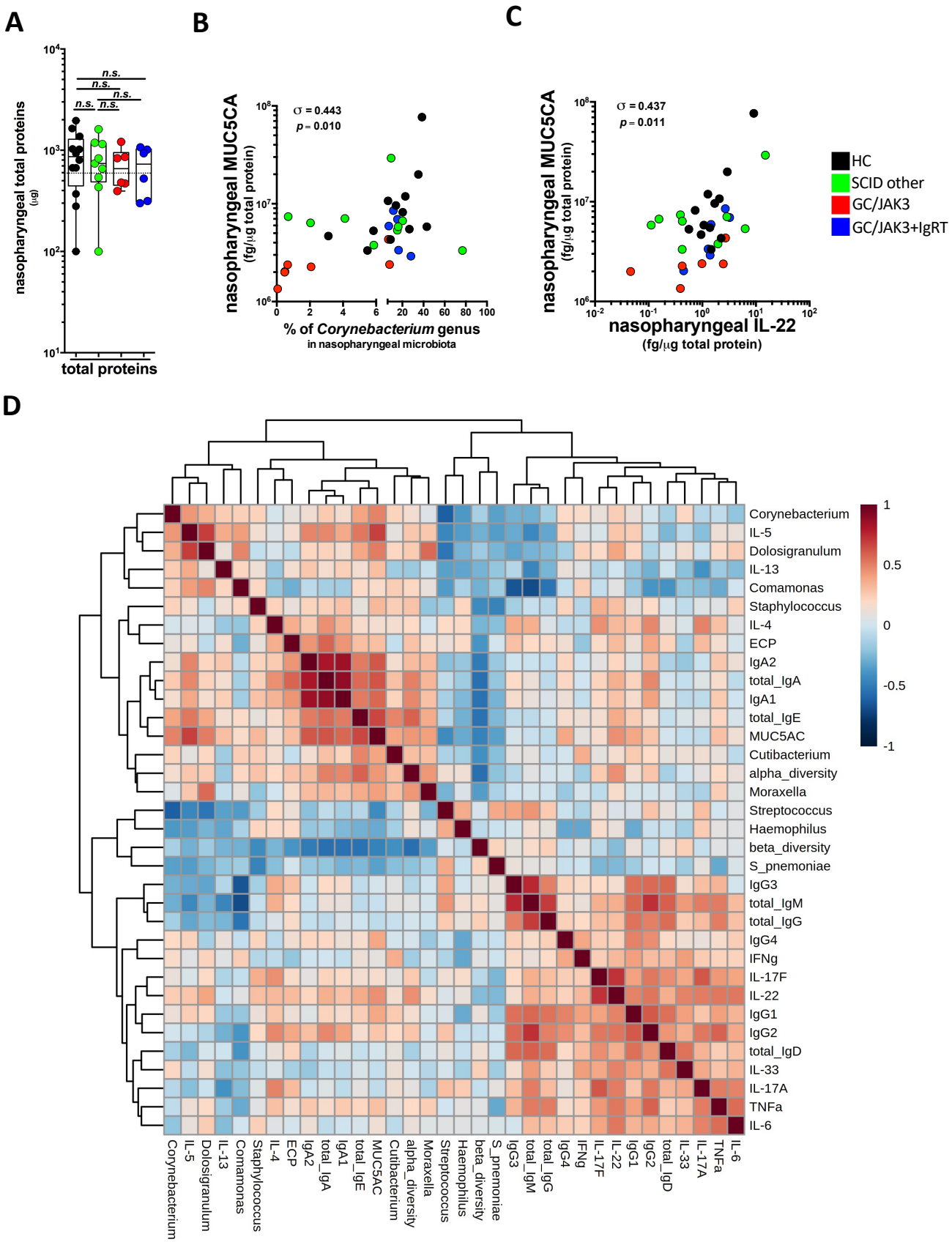


# Supplemental Fig 6



**Supplemental Figure legends**

**Supplementary Figure 1 – PBMC gating strategies.** Related to Figure 1. Identification of NK and Innate Lymphoid Cells (**A**) and T cell subsets (**B**).

**Supplementary Figure 2 – CD4+ T cell analysis in HSCT-treated SCID patients.** Related to Figure 1. **A**, Supervised analysis of circulating T cell subpopulations. Box plots with median ± minimum to maximum are shown. P values were determined with the Kruskal Wallis test followed by with Dunn's post-test for multiple group comparisons; *P < 0.05, **P < 0.005. **B**, UMAP analysis on CD4+ T cells for CXCR3, CCR6, CXCR5, CD25 and CD45RA.

**Supplementary Figure 3. – CD8+ T cell analysis in HSCT-treated SCID patients.** Related to Figure 1. UMAP analysis on CD8+ T cells including CXCR3, CCR6, CXCR5, CD25, CD45RA, CD94, CD56 and CD161.

**Supplementary Figure 4. – Analysis of nasopharyngeal IgG subclass levels.** Related to Figure 3. **A,** Nasopharyngeal concentrations of different IgG subclass levels in healthy and HSCT-treated SCID patients. **B,** Individual correlation plot between nasopharyngeal and IgA1 and IgA2 concentration. In (**A**), box plots with median ± minimum to maximum. P values were determined with the Kruskal Wallis test followed by with Dunn's post-test for multiple group comparisons; *P < 0.05. In (**B**) σ represents Spearman coefficient and p the p value.

**Supplementary Figure 5. – Analysis of nasopharyngeal microbial communities.** Related to Figure 5. Microbiota operational taxonomic units (OTUs) analysis using non-metric multidimensional scaling (NMDS) (**A**) or classical unsupervised Principal Coordinates Analysis (PCoA) (**B**). Nasopharynx bacterial load (**C**) and *Streptococcus pneumoniae* load (**D**) measured by qPCR. (**E**) Individual correlation plot between nasopharyngeal MUC5AC concentration and $\alpha$-diversity. Mean and standard error of mean (SEM) values are indicated. P values

were determined with the Kruskal Wallis test followed by with Dunn's post-test for multiple group comparisons; *P < 0.05, **P < 0.005. In **E,** σ represents Spearman coefficient and p the p value.

**Supplementary Figure 6. – Analysis of nasopharyngeal microbiota, mucus and cytokines.** Related to Figure 6. **A,** Nasopharyngeal total proteins. **B,** Individual correlation plot between nasopharyngeal MUC5AC concentration and *Corynebacterium* genus abundance. **C,** Individual correlation plot between nasopharyngeal MUC5AC concentration and nasopharyngeal IL-22 concentration. **D,** Spearman correlation matrix of nasopharyngeal compartment (cytokines, antibodies, microbiome). P values were determined with the Kruskal Wallis test followed by with Dunn's post-test for multiple group comparisons. In (**B, C**) σ represents Spearman coefficient and p the p value.

**Supplementary Table 1.** Characteristics of HSCT-treated SCID patients

| Group | Sex | Gene | Busulfan | Chimerism | T cell % | B cell % | NK % | ILC2 % | IgRT | PBMCs | Nasal swab |
|---|---|---|---|---|---|---|---|---|---|---|---|
| T-B+NK+ | M | HLA cl II | + | Full | - | - | - | - | - | No | Yes |
| T-B+NK+ | M | HLA cl II | + | Full | 69.2 | 21.4 | 2.9 | 0.04 | - | Yes | Yes |
| T-B+NK+ | M | IL7R | - | T | 46.5 | 25.6 | 21.7 | 0.0001 | - | Yes | Yes |
| T-B+NK+ | M | IL7R | - | 45% donor | 80.3 | 6.5 | 7.0 | 0.0051 | - | Yes | Yes |
| T-B+NK+ | F | IL7R | + | Full | 64.9 | 17.1 | 8.2 | 0.0073 | - | Yes | Yes |
| | | | | | | | | | | | |
| T-B-NK+ | F | Artemis | + | Full | 62.2 | 4.3 | 27.5 | 0.0280 | - | Yes | Yes |
| T-B-NK+ | F | RAG1 | - | Full | - | - | - | - | - | No | Yes |
| T-B-NK+ | M | RAG1 | +/- | Full | 78.7 | 4.1 | 11.0 | 0.0140 | - | Yes | Yes |
| T-B-NK+ | F | RAG1 | +/- | 39% donor | 84.9 | 6.0 | 4.4 | 0.0160 | - | Yes | Yes |
| | | | | | | | | | | | |
| T-B+NK- | M | JAK3 | - | T | 85.5 | 7.1 | 0,060 | 0.0021 | - | Yes | Yes |
| T-B+NK- | M | IL2RG | - | T | 80.8 | 3.4 | 0,078 | 0.0074 | - | Yes | Yes |
| T-B+NK- | F | JAK3 | - | T | 89.3 | 4.4 | 0,070 | 0.0026 | - | Yes | Yes |
| T-B+NK- | F | JAK3 | - | T | 69.1 | 13.2 | 11.6 | 0.0110 | - | Yes | Yes |
| T-B+NK- | F | JAK3 | - | T | 75.4 | 18.0 | 0.4 | 0.0036 | - | Yes | Yes |
| T-B+NK- | M | IL2RG | - | 52% donor | 87.5 | 2.7 | 3.6 | 0.0044 | - | Yes | Yes |
| | | | | | | | | | | | |
| T-B+NK- | M | IL2RG | - | T | 71.0 | 25.6 | 0.47 | 0.0087 | + | Yes | Yes |
| T-B+NK- | M | IL2RG | - | T | 79.8 | 16.2 | 0.015 | 0.0017 | + | Yes | Yes |
| T-B+NK- | M | IL2RG | - | T | 88.4 | 5.2 | 0.2 | 0.0053 | + | Yes | Yes |
| T-B+NK- | M | IL2RG | +/- | T | - | - | - | - | + | No | Yes |
| T-B+NK- | M | IL2RG | - | T | 83.3 | 9.8 | 0.06 | 0.0042 | + | Yes | Yes |
| T-B+NK- | F | JAK3 | - | T | 77.8 | 16.0 | 0.11 | 0.0037 | + | Yes | Yes |

Busulfan conditioning dosage: “+” = 16 mg/Kg, “+/-“ = 8 mg/Kg. Chimerism: “Full” = complete (100%) donor B, T and NK reconstitution, otherwise partial donor chimerism levels are indicated. “T” = selective donor T cell reconstitution. Percentages of T, B, NK and ILC2 within CD45+ gated lymphocytes are indicated. IgRT, Ig replacement therapy.