Supplementary Appendix

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This appendix has been provided by the authors to give readers additional information about the work.

Supplementary Appendix

Idiopathic CD4 Lymphocytopenia at 30: A reappraisal

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1. Supplementary Methods

Study Cohort:

Participants were enrolled in the prospective protocol “Etiology, pathogenesis and natural history of patients with idiopathic CD4 lymphopenia” (NCT00867269) between January 2009 and March 2020. All participants signed informed consent before any study procedures.

The protocol participants had to travel to the NIH Clinical Center for their study visits. Eligible participants were adults with confirmed ICL ($CD4^+$T-cell count <300 cells/µl or <20% of total T lymphocytes at the time of screening and on at least 2 occasions at least 6 weeks apart in the absence of any illness, treatment, or condition accounting for CD4 lymphopenia. Ongoing care by a referring primary care physician and willingness to allow storage of blood and tissue samples for future analysis were additional inclusion criteria. Exclusion criteria were defined as HIV-1/2, HTLV-1/2 infection as per serological/molecular testing; any known acquired immunodeficiency or clinical condition associated with persistent lymphopenia; any inborn error of immunity (IEI) based on the 2022 International Union of Immunological Societies (IUIS) genotypic-phenotypic classification[1]; active malignancy at screening; any medication or biologic/herbal substances known to cause lymphopenia.

The screening visit included a clinical and laboratory assessment with the following study procedures: complete history and physical examination, utilizing standard intake evaluation as specified in the attached clinical protocol; acquisition of any available clinical, radiological, microbiological and cytological/histological records of previous infectious complications; and rheumatologic evaluation by the NIH Rheumatology consult service including a structured physical examination and review of symptoms. Dermatology, pulmonary, ophthalmology, and other consultations (i.e. endocrinology, gynecology) by the NIH consult services were obtained

as medically indicated based on initial history and physical examination. Screening laboratory testing included the following: HIV serology and viral load, hepatitis B and C, and HTLV-I and II serologies, and Hepatitis B antigen; Diphtheria and tetanus antibody titers; Other infectious serologies, including Cytomegalovirus (CMV), Epstein-Barr Virus (EBV), Herpes Simplex Virus I and II, and Varicella Zoster Virus; CMV/EBV blood plasma PCR; Urine histoplasma antigen; Serum cryptococcal antigen; Complete blood count (CBC) and differential; PT/PTT; biochemical profile; quantitative immunoglobulins; TSH, Total Complement, C3/C4.; Erythrocyte sedimentation rate (ESR), d-Dimer, and CRP; Urinalysis, urine pregnancy test (female patients of childbearing age).

Autoantibodies profiling with Anti-ds DNA Antibody, Anti-Cardiolipin Antibody, Anti-Thyroid Panel, Anti-Nuclear Antibody, Rheumatoid Factor, Anti-ENA Screen, Lupus Anticoagulant, Beta 2 Glycoprotein and Anti-CCP Antibody. Lymphocyte immunophenotyping (CD4-T cells, CD8-T cells, B-cells, NK-cells); HLA testing; T-cell receptor gene rearrangement and Immunoglobulin gene rearrangements by PCR; Splenic ultrasounds; Genetic screening by targeted gene panel or whole exome sequencing. Other radiological or laboratory studies as well as invasive procedures (i.e. bone marrow biopsy, skin or core lymph node biopsy) were done as directed by study and clinical findings.

The follow-up visits included a clinical and laboratory assessment with the following study procedures: complete history and physical examination; and evaluations by the NIH sub-specialty consult services as medically indicated based on initial and follow-up history and physical examination. Follow-up laboratory testing included the following: HIV serology and viral load; Urine histoplasma antigen; Serum cryptococcal antigen; CMV/EBV PCR; CBC with differential; PT/PTT; Biochemical profile; TSH; Quantitative immunoglobulins; Urinalysis and

urine pregnancy test (female patients of child-bearing age/ability); Autoantibodies (as listed above in screening visit). Any other radiological or laboratory studies as well as invasive procedures (i.e. bone marrow biopsy, skin or core lymph node biopsy) were done as directed by study and clinical findings.

Some enrolled study participants were subsequently excluded if a specific diagnosis of the CD4 lymphopenia was confirmed during follow-up. Except for the qualitative serological response to SARS-COv2 immunizations, all other clinical and immunological data were censored in March 2020, since thereafter regular protocol visits to the NIH Clinical Center were interrupted by the COVID-19 pandemic and have not yet been fully restored since. Follow-up data on the qualitative serological response to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) immunizations were censored in October 2021.

Immunological Evaluations:

Immunological evaluation. Immunophenotyping was performed in a Clinical Laboratory Improvement-certified laboratory to enumerate CD4, CD8 T, B lymphocytes, and NK cells in peripheral blood. Immunophenotyping of peripheral blood mononuclear cells was performed staining cells with monoclonal antibodies from BD Biosciences (San Jose, CA) and analyzed immediately on a Becton Dickinson FACSCanto flow cytometer (BD Biosciences, San Jose, CA). SARS-CoV2 serological response in 22 ICL patients was evaluated in patients either seen at the NIH Clinical Center for clinical indications or who provided the results of commercial serological testing by the Elecsys® (Roche Diagnostics) assay.

Genetic work up:

1 84 of the 108 study participants underwent genetic analysis by whole exome sequencing (80
2 patients) or targeted gene panel sequencing (4 patients) under the NIH internal Review Board
3 approved protocols "Etiology, pathogenesis and natural history of patients with idiopathic CD4
4 lymphopenia" (NCT00867269) and/or the "NIAID Centralized Sequencing Protocol
5 (NCT03206099).
6
7 **Statistical Analysis:**
8 For descriptive statistics the median with 25%-75% interquartile ranges are reported.
9 Two-tailed nonparametric tests were used to compare ranks or differences between groups. To
10 assess the potential prognostic value of T cell counts, we used the T cell tertiles[2], for CD4 the
11 cutoff points were 43 and 128, for CD8 the cutoff points were 91 and 234, and for NK the cutoff
12 points were 120 and 167, using the highest tertile as reference.
13 The tertiles were calculated from the baseline (screening visit) measurement of all
14 participants with the goal of evaluating whether being in a particular tertile was associated with
15 higher or lower odds of presenting a condition (OIs, cancer, autoimmunity) via logistic
16 regression with Firth's bias reduction. Because logistic regression is meant for independent data,
17 we used multiple outputation which allowed us to apply a method designed for independent data,
18 in a setting where we have multiple cell counts per participant (longitudinal data). Here, cell
19 counts represent either CD4-, CD8-, or NK-cells, as we evaluated them separately. Briefly, in
20 multiple outputation, an observation (cell count at a particular visit) from each participant is
21 randomly selected and Firth's logistic regression is implemented. This procedure is repeated
22 multiple times. During each iteration for the selected observation, the binary condition is the
23 response, the covariate of interest is a variable determining the observed tertile (lowest – tertile

1 1, middle – tertile 2, highest – tertile 3) based on where the selected observed cell count lies in
2 comparison with the baseline tertiles, adjusted for age and gender at baseline. To calculate the
3 effect estimate the average of all estimates is used, and to calculate the effect estimate variance,
4 the average of the estimated variances minus the sample variance of the effect estimate is used.
5 The opportunistic infections of clinical significance were: cryptococcosis, histoplasmosis,
6 disseminated or pulmonary non-tubercular mycobacterial infections, severe HPV-related
7 diseases (anogenital dysplasia requiring surgical management, recurrent skin warts refractory to
8 therapeutic interventions, combined mucosal and skin HPV-related diseases irrespective of
9 presence of dysplasia), complicated or disseminated VZV infections, complicated or
10 disseminated oral or anogenital herpetic infections, malignancies associated with oncogenic viral
11 infections (i.e. HPV, HHV-8, EBV), molluscum contagiosum, CMV end-organ disease,
12 *Pneumocystis Jirovecii* pneumonia, coccidioidomycosis, Progressive Multifocal Encephalitis
13 (PML). General infectious complications were categorized as the above-mentioned conditions as
14 well as dermatomal and uncomplicated zoster, localized and uncomplicated oral or anogenital
15 herpetic infections, HPV-related anogenital lesions without evidence of dysplasia on
16 biopsy/colposcopy spontaneously resolving or not recurring after 1 medical or surgical treatment,
17 as well as skin warts spontaneously resolving or responsive to therapy. Longitudinal linear-
18 mixed models were used to evaluate the longitudinal trajectories of CD4 and CD8 T cell counts
19 over time.

20 The expected death rate was calculated from the CDC national death rate broken down by
21 age, sex, race and ethnicity[3]. To get the probability of each person dying during the study, we
22 determined the national death rate matching their gender, age, race, and ethnicity for each year
23 they were in the study. To determine the expected number of patients who would have died during

1 follow-up had they been healthy, we summed all subjects' probability of death during the study.
2 We used a one-sided Poisson test to determine if the number of deaths in our cohort is significantly
3 larger than the expected count.
4 Using the SEER Cancer Statistics Review (CSR)[4,5], we obtained the prevalence percent of the
5 different invasive cancer types in the ICL cohort by gender (all races). Prevalence percent not
6 shown in SEER due to there being fewer than 5 cases were assumed to be 0. Only invasive cancers
7 diagnosed within the previous 25 years were included in the analysis, and all invasive cancers were
8 assumed to be prevalent cancers, regardless of when they developed. We assumed the SEER
9 prevalence as of January 1, 2017, was the same as at the end of the study follow-up period. For
10 each invasive cancer type and for each subject, we determined whether that subject was diagnosed
11 with that cancer, as well as the SEER prevalent percent using the gender and age for that subject
12 at the end of the follow-up period. For each invasive cancer type, we summed the total number of
13 subjects with that cancer and the total prevalent percent for that cancer. We compared them using
14 a one-sided Poisson test to determine the probability of observing as many or greater than the
15 number of cases in our cohort, assuming that the true rate is the sum of prevalent percentages. *p-*
16 *values* less than 0.05 was considered significant.
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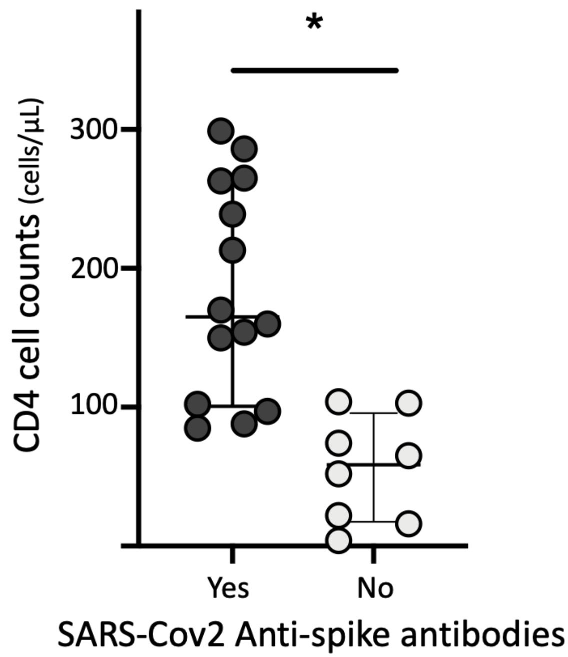
20

1 **Figure S1: Immune response to SARS-Cov2 vaccines in ICL.**

2



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5 Absolute CD4 cell counts in ICL patients with or without detectable anti-Spike antibodies after 2

6 doses of mRNA vaccines. * Denotes a statistically significant difference.

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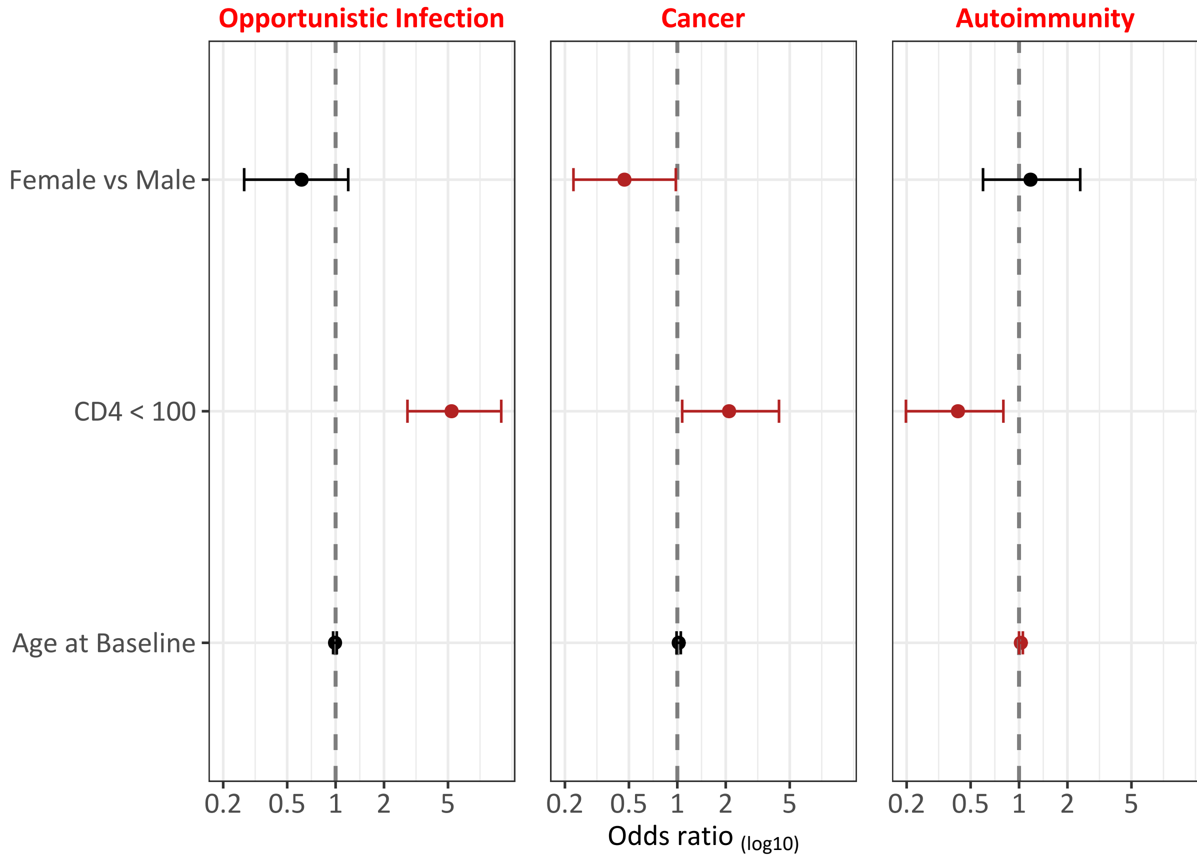
8

1 **Figure S2: Sensitivity analysis for the association between CD4 cell counts and**

2 **opportunistic infections of clinical significance (OIs), cancers and autoimmune diseases.**



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6 Odds ratios (ORs) and 95% confidence intervals for OI, cancer, or autoimmune disease of CD4

7 cell count <100 vs. ≥100 cells/μL (a pre-established cutoff), adjusted for age and sex. In red, the

8 ORs for which the 95% confidence intervals exclude the null value of 1.

9

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