demonstrate the novel finding that human ILC2s highly express the signaling lymphocyte activation molecule family member CD84, although this expression is not selective for ILC2s. Allergen challenge did not increase levels of CD84 expression on ILC2s or CD4 cells.

We next assessed the percentage of CRTH2+ ILC2s in the peripheral blood of cat-allergic subjects before and 4 hours after nasal cat allergen or diluent challenges (Fig 2). The baseline percentage of CRTH2+ cells within the lineage-negative population was 10.7 ± 1.9 and 12.0 ± 1.3 at the diluent and cat allergen challenge visit, respectively (Fig 2, *B*). Four hours after diluent challenge, the percentage of CRTH2+ cells did not change significantly (9.7 ± 1.8) compared with time zero. However, after cat allergen challenge, the percentage of CRTH2+ cells nearly doubled to 19.1 ± 2.6 compared with baseline ($P = .05$) and compared with diluent challenge at 4 hours ($P < .05$) (Fig 2, *B*). Thus, nasal cat allergen challenge induced an increased percentage of peripheral blood CRTH2+ ILC2s when measured 4 hours after challenge.

ILC2s produce large amounts of IL-5 and IL-13 in response to IL-25, IL-33, TSLP, and LTD4 and could initiate and/or propagate allergic airway inflammation. Our studies demonstrate that the percentage of CRTH2+ ILC2s in the peripheral blood is rapidly increased (within 4 hours) after allergen challenge. Potential mechanisms for the increase in ILC2s in the peripheral blood may be due to enhanced recruitment of ILC2s from the bone marrow triggered by either humoral (cytokine, chemokine, or mediator production in the nose) and/or cellular mechanisms (cells released from the nasal mucosa trafficking to the bone marrow). The human ILC2 marker CRTH2 is the receptor for prostaglandin D2 (PGD2), a lipid mediator that has a known role in chemotaxis and activation of immune cells. Importantly, a previous study demonstrated that high levels of serum 9α,11β-PGF_2, the major PGD2 metabolite, are induced within 5 minutes after airway allergen challenge, suggesting that PGD2 is rapidly available systemically for the recruitment of CRTH2+ cells after allergen exposure.[8] We have also recently determined that PGD2 induces chemotaxis of CRTH2+ human blood ILC2s *in vitro*, suggesting that PGD2 may directly regulate the migration of human ILC2s into tissues.[9] The role of increased peripheral blood ILC2 numbers after allergen challenge is unclear. One hypothesis is that greater ILC2 availability in the blood (within 4 hours after challenge) may result in greater numbers of cytokine-producing nasal mucosa ILC2s at later time points, but this would need to be investigated in future studies. Strategies to inhibit the recruitment of ILC2s in allergic individuals may reduce tissue T_H2 cytokine levels that contribute to allergic inflammation.

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Enrichment of the rare CD4$^+$ γδ T-cell subset in patients with atypical CD3δ deficiency

To the Editor:

T lymphocytes detect antigens with the T-cell receptor (TCR), which is composed of a variable heterodimer (either αβ or γδ) and 3 invariant dimers (CD3γε, CD3δε, and ζζ). The invariant proteins participate in TCR assembly and surface expression and in the delivery of intracellular signals required for T-cell

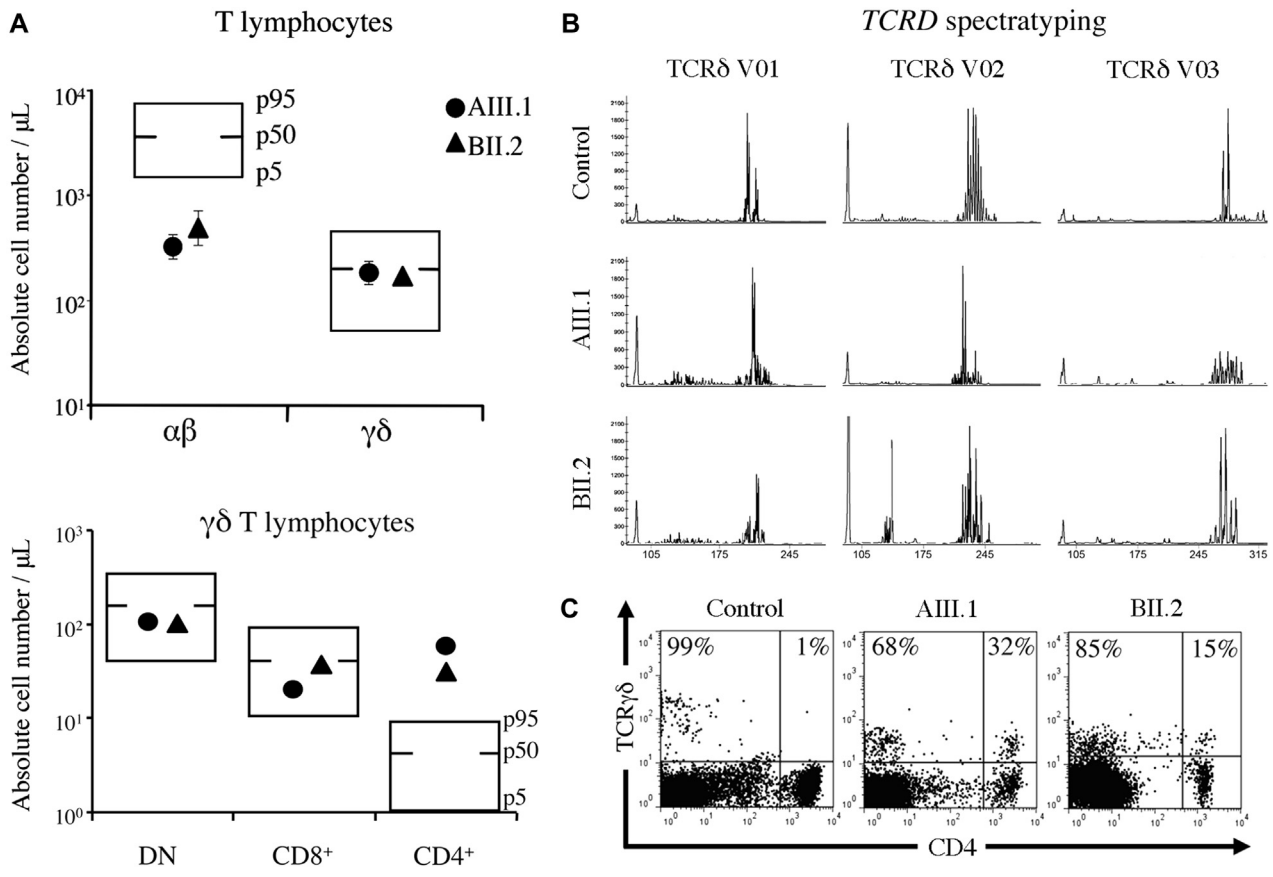

FIG 1. T-lymphocyte analyses in 2 patients with atypical CD3δ deficiency. **A,** Absolute αβ and γδ *(top)* and DN, $CD8^+$, and $CD4^+$ γδ *(bottom)* T-cell numbers (mean ± SD) in comparison with the normal age-matched distribution in percentiles. **B,** γδ T-cell repertoire analysis by Vδ CDR3 length profiling. Abscissae are base pairs as indicated. Ordinates are peak heights. *TCRD* spectratyping is very variable in controls (see Fig E1 for further examples) but can be used to exclude clonal γδ T-cell expansions. **C,** $TCR\gamma\delta^+$ cell distribution between $CD4^-$ and $CD4^+$ subsets *(upper quadrants). DN,* Double negative.

selection and function.[1] αβ and γδ T cells arise from a common progenitor in the thymus, but the developmental requirements to become one or the other remain elusive.[2] Patients with typical (complete) CD3δ deficiency lack both αβ and γδ T cells and suffer early-onset severe combined immunodeficiency (SCID).[3] It was thus unexpected that 2 unrelated patients with SCID with atypical (partial) CD3δ deficiency showed normal numbers of γδ T cells (Fig 1, *A*, top). Their molecular, clinical, and immunologic features have been previously reported.[4,5] Both patients shared a splicing mutation in *CD3D* (c.274+5G>A), which strongly impaired, but did not abrogate, *CD3D* splicing, and allowed for 50% normal CD3δ protein expression despite the presence of a dominant truncated transcript that encoded an unstable aberrant CD3δ chain. As a consequence, they showed some common clinical and immunophenotypic features: early-onset SCID, $T\alpha\beta^-T\gamma\delta^+B^+NK^+$ phenotype, low CD3 expression, impaired proliferative responses to T-cell mitogens, severe lymph-node T-cell depletion, lack of activated germinal centers, and poor induction of specific antibodies. Patient AIII.1, but not patient BII.2, showed hyper-IgE and eosinophilia, although a severe protein-losing enteropathy of patient BII.2 made his immunoglobulin measurements unreliable (see Table E1 in this article's Online Repository at www.jacionline.org).

Although one of the patients (BII.2) likely died because of cytomegalovirus infection, virus-induced single clonal expansions of γδ T cells, as reported in certain SCID conditions, were excluded in both patients by Vδ CDR3 spectratyping, which confirmed polyclonal expression of the 3 tested Vδ genes (Fig 1, *B*; additional controls in Fig E1 in this article's Online Repository at www.jacionline.org).

Three subsets of γδ T cells have been identified on the basis of CD4 and CD8 expression, $CD4^-CD8^-$ (double negative), $CD8^+$, and $CD4^+$. While $CD4^+$ γδ T cells are extremely rare in normal individuals (<1% of γδ T cells[6]), they were strongly enriched in the patients (around 10-fold, Fig 1, *A*, bottom; for relative numbers, see Fig 1, *C*, and Fig E2 in this article's Online Repository at www.jacionline.org). Upon long-term culture, they retained surface CD4 (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org), indicating that they were not a result of CD4 upregulation in the $CD4^-$ subset, and this was confirmed by clonal analysis (see Table E2 in this article's Online Repository at www.jacionline.org). $CD4^+$ γδ T cells from the patients were not a clonal expansion because they showed a low but diverse surface TCR, as demonstrated by the detection of $V\delta1^+$, $V\delta2^+$, and $V\gamma9^+$ cells (see Fig E3, *B*). As expected in infants, γδ T cells (whether $CD4^+$ or $CD4^-$) were enriched for non-$V\delta2^+$ cells.

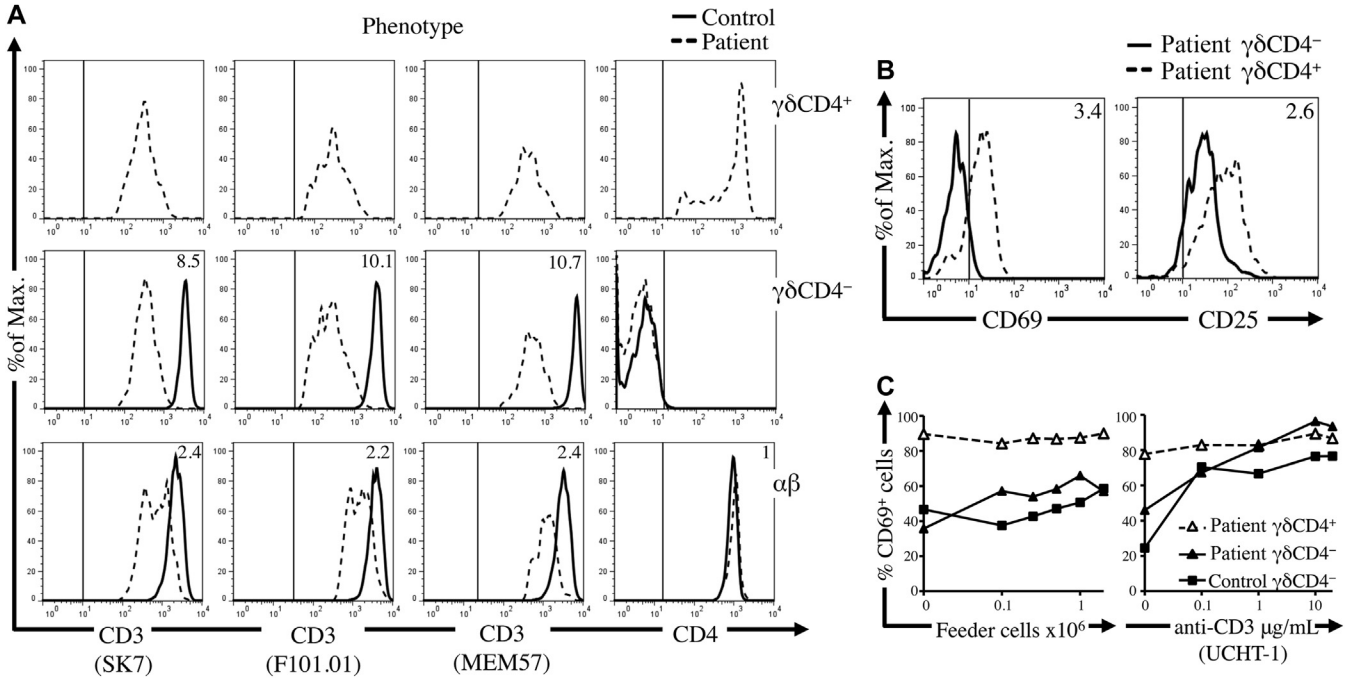

FIG 2. Comparative phenotypic and functional characteristics of γδ $CD4^+$ versus $CD4^-$ T cells. **A,** Surface CD3 (and CD4) expression. αβ T cells are shown for reference. Similar results for AIII.1 (not shown) and with TCR-specific mAb.[4] Numbers are control/patient MFI ratios. *Vertical lines* indicate background staining. **B,** Expression of activation markers. **C,** Cultured γδ T-cell activation (% $CD69^+$ cells 24 hours after stimulation). *MFI*, Mean fluorescence intensity.

Normal $CD4^+$ γδ T cells show a T_H2 phenotype[6] and have been reported to be enriched in bronchial asthma lungs.[7] Mutant mice with expanded $CD4^+$ γδ T cells showed abnormally high IgE levels.[8] However, T_H2-type features such as hyper-IgE, eosinophilia, and dermatitis as observed only in patient AIII.1 are characteristic of Omenn syndrome, which is not characterized by high γδ T cells.[9]

Enrichment of $CD4^+$ γδ T cells was not a consequence of αβ T-cell lymphopenia in the patients because it was not observed in other patients with mutations in TCRα.[10] It was, however, specific for atypical CD3δ deficiency because it was not found in patients with typical CD3δ deficiency[11] or with deficiency in the highly homologous CD3γ chain (see Fig E4 in this article's Online Repository at www.jacionline.org). These results may be of some diagnostic value to identify the molecular basis of primary immunodeficiencies: patients with high $CD4^+$ γδ T cells should be tested for atypical *CD3D* mutations.

$CD4^+$ γδ T cells in atypical CD3δ deficiency, and also their $CD4^-$ counterparts, showed very poor surface TCR expression (almost 10-fold less than did controls, Fig 2, *A*). Surprisingly, αβ T cells showed only 2- to 3-fold less surface TCR than did controls. A stronger impact on γδ TCR than on αβ TCR has also been observed in CD3γ and CD3δ haploinsufficiencies (to a lesser extent because CD3 chain reductions were milder), which unexpectedly also showed that γδ TCR expression is more dependent on CD3γ than on CD3δ.[12] Compared with $CD4^-$ γδ T cells, $CD4^+$ γδ T cells displayed close to 3-fold higher expression of CD69 and CD25 activation markers (Fig 2, *B*). Similar findings have been reported in mice with expanded $CD4^+$ γδ T cells.[8] In contrast to patient or control $CD4^-$ γδ T cells, patient $CD4^+$ γδ T cells could not upregulate CD69 expression further in response to anti-CD3 or allogeneic feeder cells (Fig 2, *C*). Thus $CD4^+$, but not $CD4^-$, γδ T cells from the patients displayed an activated phenotype and could not be induced further through their TCR.

The factors that regulate intrathymic γδ T-cell development in humans are poorly understood.[2] It is unclear whether functional γδ TCR expression plays a role in positive and negative selection, whereas this is undisputed for the αβ TCR. Our findings suggest that $CD4^+$ and $CD4^-$ γδ T-cell development is less TCR-dependent for positive selection than is αβ T-cell development. In contrast, $CD4^+$, but not $CD4^-$, γδ T cells could be targets of TCR-mediated negative selection, hence their scarcity in normal individuals. Impaired γδ TCR expression in $CD4^+$ γδ T cells, as observed in the patients, might weaken the TCR signals required for negative selection, thus causing their enhanced development and enrichment.

The striking discrepancy in surface TCR levels between αβ and γδ T cells from the patients did not correlate with intracellular CD3δ levels, which were similar for both lineages (around 2-fold less than controls; see Fig E3, *C*). It has been reported that CD3δ affinity may be weaker for the γδ TCR than for the αβ TCR.[13] This could contribute to a stronger impairment of γδ versus αβ TCR assembly and/or stability when CD3δ is limiting, as observed in atypical CD3δ deficiency. To test whether this differential effect on TCR expression was a consequence of intrathymic development, or, rather, could take place in mature primary T cells, CD3δ was knocked down in PBMCs of healthy controls by using *CD3D*-specific shRNA. The results showed a similar reduction of the surface TCR in both T-cell lineages

(see Fig E5 in this article's Online Repository at www.jacionline.org), supporting the former hypothesis. Our findings thus suggest that most γδ T cells are less dependent than αβ T cells on surface TCR expression for development in the thymus, with CD3δ playing a critical role in establishing the threshold of TCR expression for proper thymic selection of each lineage. A higher heterogeneity of surface TCR levels in normal γδ T cells as compared with αβ T cells further supports this notion (see Fig E6 in this article's Online Repository at www.jacionline.org).

In conclusion, atypical CD3δ deficiency leading to reduced surface TCR expression showed preserved γδ, but not αβ, T-cell numbers with overrepresentation of $CD4^+$ γδ T cells, supporting low TCR-dependence for positive selection of $CD4^-$ γδ T cells and disrupted negative selection of $CD4^+$ γδ T cells.

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Impact of Down syndrome on the performance of neonatal screening assays for severe primary immunodeficiency diseases

To the Editor:

Neonatal screening programs for severe combined immunodeficiency (SCID) and X-linked agammaglobulinemia (XLA) have recently been established on the basis of molecular quantitation of levels of T-cell receptor excision circles (TRECs) for SCID and kappa-deleting recombination excision circles (KRECs) for XLA in dried blood spot samples (DBSS) obtained from regular Guthrie cards.[1-4] This technique features a remarkable sensitivity for the identification of newborns characterized by severe T lymphocytopenia and/or B lymphocytopenia at birth. However, after testing a second punch from the primary Guthrie card ("retest rate"), approximately 1 in 500 samples require a second Guthrie card ("rerun rate"), which suggests that diseases or conditions other than SCID or XLA yield abnormal test results in the combined TREC/KREC assay.[2] For example, it has previously been shown that immaturity of the immune system in preterm neonates, inflammatory conditions (eg, sepsis and lymphocyte extravasation), and 22q11 deletion syndromes (velocardiofacial and DiGeorge syndrome, OMIM 192430 and 188400, respectively) have a negative impact on target-disease recall rates.[3]

Throughout life, patients with Down syndrome (trisomy 21; OMIM 190685) have decreased numbers of T lymphocytes and B lymphocytes,[5,6] and are thus likely to have lower TREC and KREC copy numbers. Clinicians identify the vast majority of these newborns by clinical examination, yet this information is often not made available to the screening laboratory. Given a Down syndrome birth rate of about 1 in 600 to 900 newborns, we hypothesized that this condition may thus contribute to the

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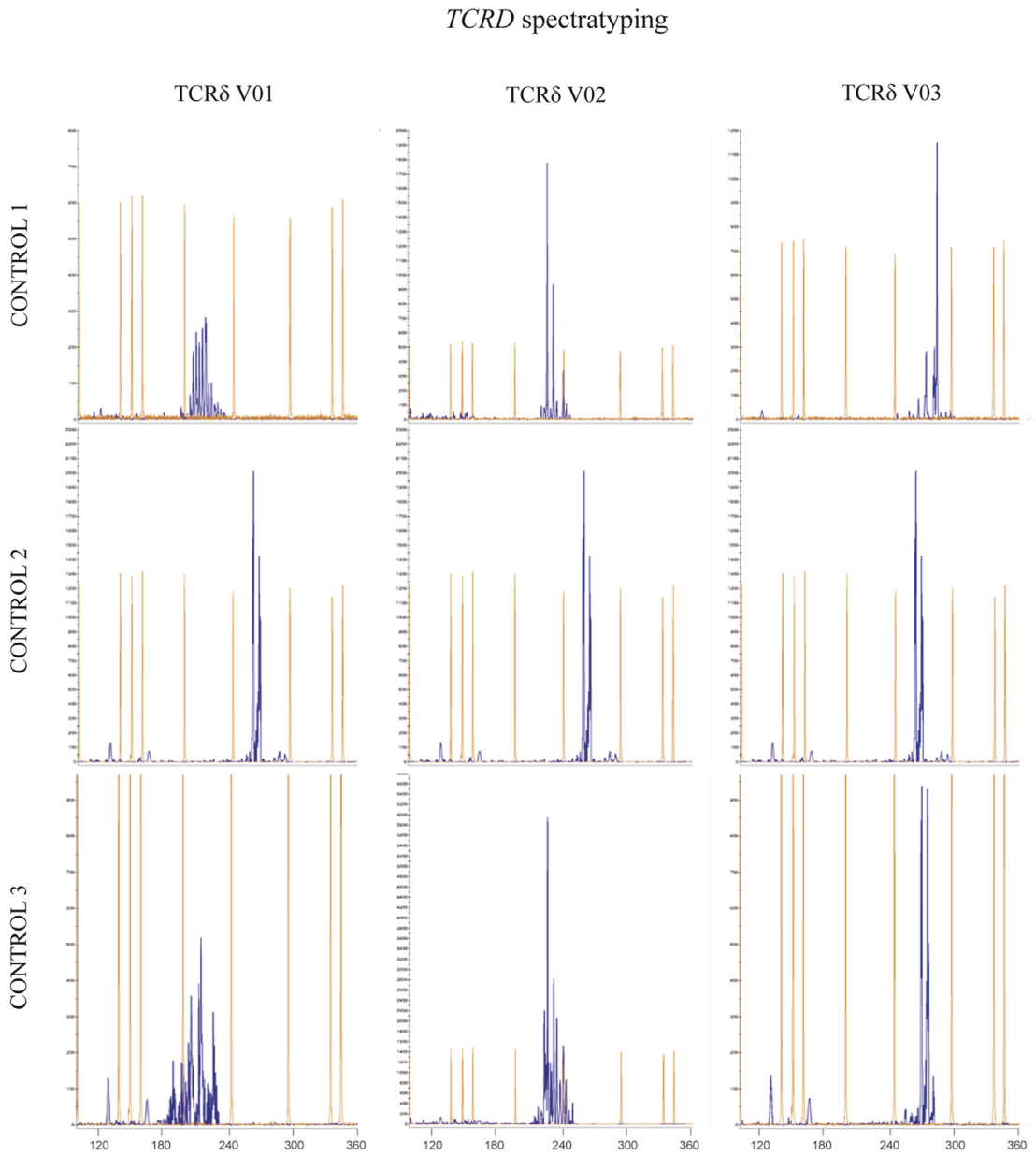

FIG E1. γδ T-cell repertoire analysis by Vδ CDR3 length profiling in 3 unrelated normal controls, to illustrate the variability of *TCRD* spectratyping. Abscissae are base pairs as indicated. Ordinates are peak heights. The *orange* peaks along abscissae are molecular weight standards.

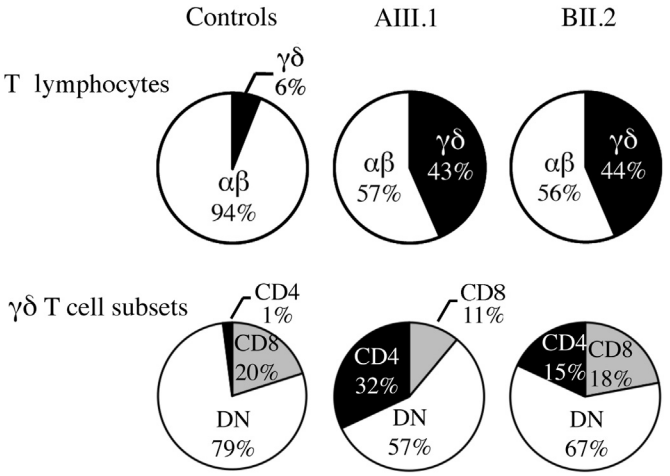

FIG E2. Relative αβ and γδ T-cell numbers *(top)* and DN, $CD8^+$, and $CD4^+$ γδ T-cell numbers *(bottom)*. *DN*, Double negative.

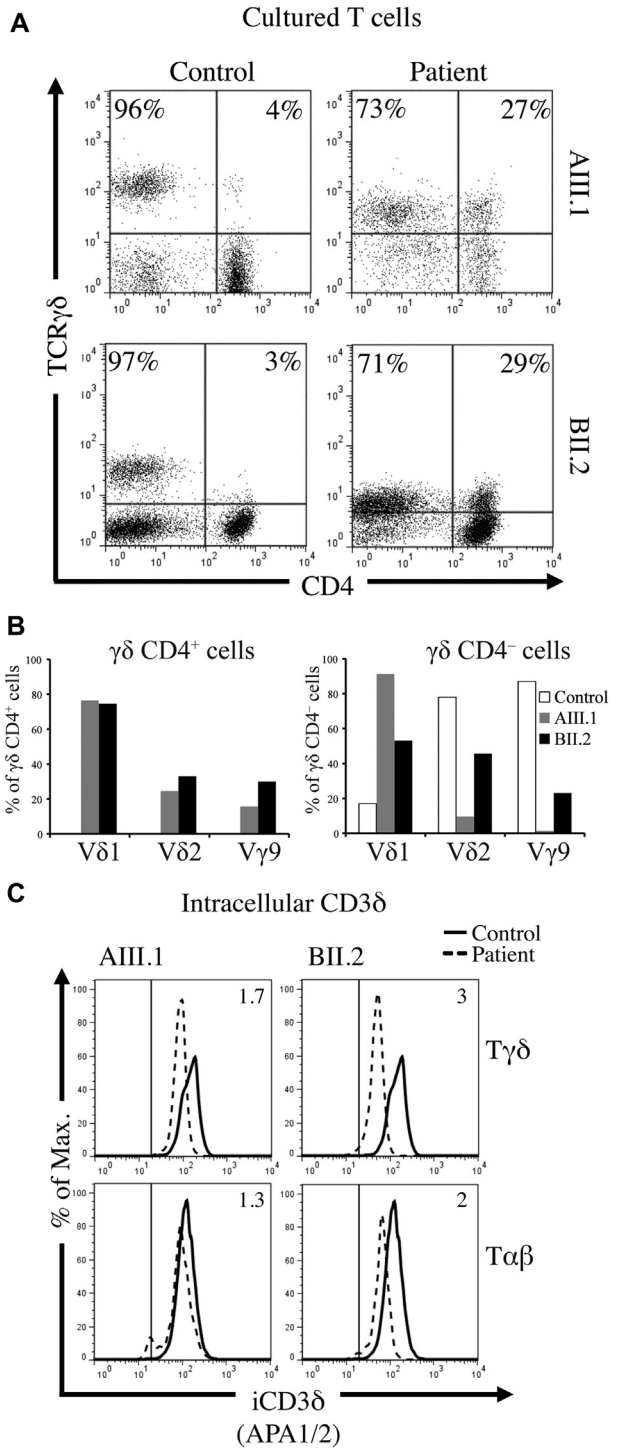

FIG E3. Phenotypical characterization of cultured γδ T cells. **A,** TCRγδ$^+$ cell distribution between CD4$^-$ and CD4$^+$ subsets *(upper quadrants)*. **B,** Vδ1, Vδ2, and Vγ9 usage within CD4$^+$ or CD4$^-$ γδ T-cell subsets. **C,** Intracellular CD3δ levels detected in permeabilized T cells by using APA1/2 after gating for TCRαβ or TCRγδ, respectively. The *vertical lines* indicate the upper limit staining of the isotype control. The numbers in each histogram indicate control/patient MFI ratios. *MFI*, Mean fluorescence intensity.

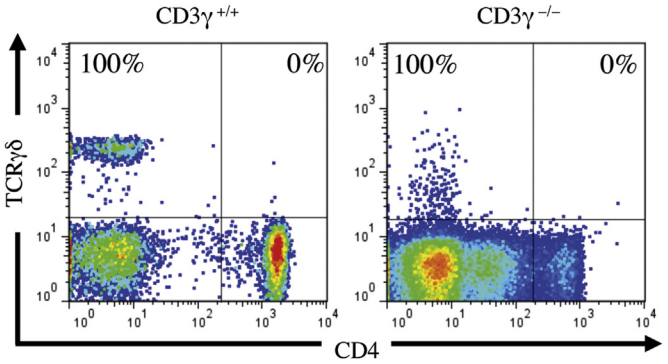

FIG E4. γδ T-cell subsets in human CD3γ deficiency.[E1] The numbers indicate the TCRγδ$^+$ cell distribution between the CD4$^-$ and CD4$^+$ subsets *(upper quadrants)* in fresh PBMCs compared with a control.

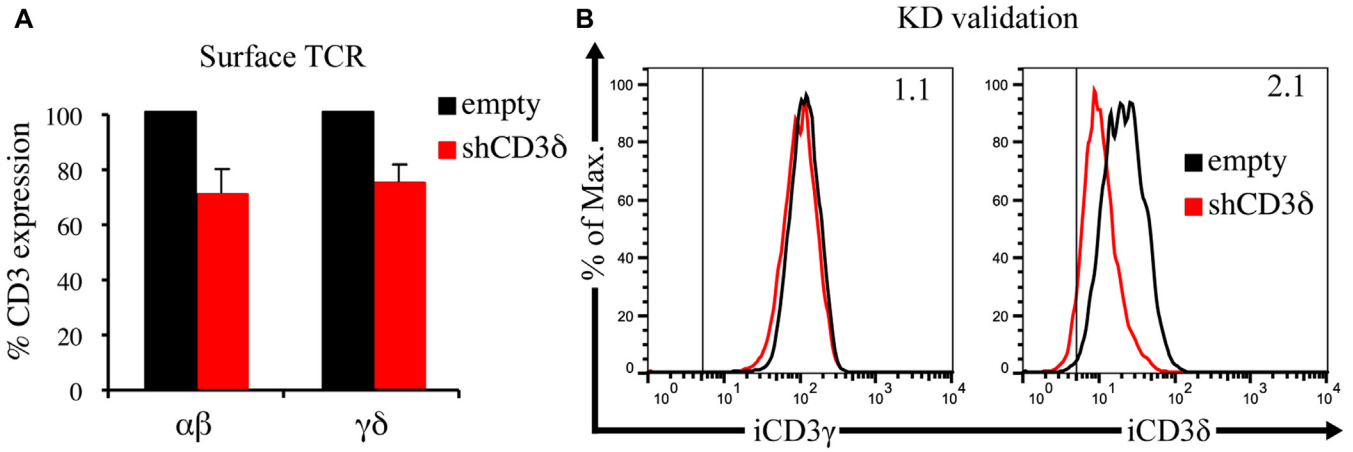

FIG E5. CD3δ KD in primary T cells. **A,** PBMCs from healthy donors were infected with lentiviruses carrying shRNA for CD3δ (GAGGACAGAGTGTTTGTGAAT) or no shRNA (empty) cloned in pLKO.1. After selection with puromycin, GFP^+ cells were analyzed for surface TCR expression by using anti-CD3 mAb (SK7) and normalized to the empty vector (n = 2, mean ± SD). αβ T cells were gated as $TCR\alpha\beta^+$ (IP26) and γδ T cells as $TCR\gamma\delta^+$ (IMMU510). **B,** KD specificity was ascertained in permeabilized samples by flow cytometry using CD3δ-specific or, as a negative control, CD3γ-specific mAb to probe for intracellular CD3 expression (iCD3δ [EPR4426] and iCD3γ [EPR4517] from Abcam). The numbers in each histogram indicate empty/shCD3δ MFI ratios. *KD*, Knock down; *MFI*, mean fluorescence intensity.

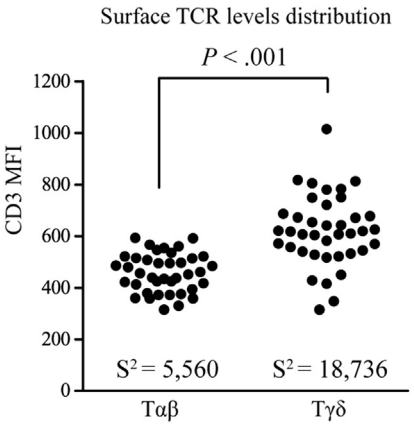

FIG E6. Heterogeneous surface TCR levels in γδ *versus* αβ T cells. Anti-CD3 mAb (SK7) MFI is represented within gated αβ ($IP26^+$) and γδ ($IMMU510^+$) T cells from 39 different healthy donors. In addition to the reported higher surface TCR MFI in γδ T cells,[E2] variance (S^2) homogeneity was compared by using the Snedecor F distribution test with 38 and 38 degrees of freedom. *MFI,* Mean fluorescence intensity.

TABLE E1. IgE levels and eosinophil counts (pretransplant values)

	AIII.1	BII.2	Normal range
IgE (IU/mL)			
Minimum	2141	<2	0-120
Mean	3314	14	
Maximum	4525	51	
Eosinophils (cells/μL)			
Minimum	1200	0	70-550
Mean	3043	103	
Maximum	5900	500	

Data updated from Marcus et al[E3] (erratum in J Allergy Clin Immunol 2013 ;132:1259).

TABLE E2. T-cell clones phenotypes

	Control	AIII.1	BII.2
$CD4^+$ αβ	8	22	1
$CD8^+$ αβ	—	—	—
DN γδ	—	—	2
$CD4^+$ γδ	—	3	7
$CD8^+$ γδ	—	—	—
Total	8	25	10

DN, Double negative.