

# Human CD4<sup>+</sup> T cell recent thymic emigrants are identified by protein tyrosine kinase 7 and have reduced immune function

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CD4<sup>+</sup> recent thymic emigrants (RTEs) comprise a clinically and immunologically important T cell population that indicates thymic output and that is essential for maintaining a diverse  $\alpha\beta$ -T cell receptor (TCR) repertoire of the naive CD4<sup>+</sup> T cell compartment. However, their frequency and function are poorly understood because no known surface markers distinguish them from older non-RTE naive CD4<sup>+</sup> T cells. We demonstrate that protein tyrosine kinase 7 (PTK7) is a novel marker for human CD4<sup>+</sup> RTEs. Consistent with their recent thymic origin, human PTK7<sup>+</sup> RTEs contained higher levels of signal joint TCR gene excision circles and were more responsive to interleukin (IL)-7 compared with PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells, and rapidly decreased after complete thymectomy. Importantly, CD4<sup>+</sup> RTEs proliferated less and produced less IL-2 and interferon- $\gamma$  than PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells after  $\alpha\beta$ -TCR/CD3 and CD28 engagement. This immaturity in CD4<sup>+</sup> RTE effector function may contribute to the reduced CD4<sup>+</sup> T cell immunity observed in contexts in which CD4<sup>+</sup> RTEs predominate, such as in the fetus and neonate or after immune reconstitution. The ability to identify viable CD4<sup>+</sup> RTEs by PTK7 staining should be useful for monitoring thymic output in both healthy individuals and in patients with genetic or acquired CD4<sup>+</sup> T cell immunodeficiencies.

CD4<sup>+</sup> T cells are essential for adaptive immune responses to pathogens and vaccines. Relatively constant numbers of naive CD4<sup>+</sup> T cells are maintained throughout life by both the thymic output of CD4<sup>+</sup> recent thymic emigrants (RTEs) and by homeostatic proliferation of existing peripheral naive T cells, which replace those cells lost by death or conversion to memory/effector cells (1–3). The relative contribution of thymic output and homeostatic proliferation to the composition of the human naive CD4<sup>+</sup> T cell pool throughout aging has been difficult to determine precisely.

Frequencies of human CD4<sup>+</sup> RTEs have been inferred by PCR assays of populations of cells for their content of signal joint TCR gene

excision circles (sjTRECs), which are circular DNA products of intrathymic V(D)J recombination (4). However, using sjTREC content to study RTE abundance and biology has important disadvantages. First, homeostatic proliferation results in increased sjTREC loss from the naive peripheral T cell compartment (4, 5). Second, sjTREC content is not a highly dynamic measurement of thymic output, as is indicated by its stability for months after complete thymectomy (3). Third, PCR analysis of sjTREC content precludes any type of functional analysis of viable CD4<sup>+</sup> RTEs at the single-cell level. Although other approaches allow direct in vivo labeling of RTEs in humans, such as with <sup>2</sup>H<sub>2</sub>O (6), they also do not allow a facile determination of RTE phenotype and function. Thus, identifying an endogenous surface marker for

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RTEs would overcome the disadvantages of sjTREC assays or of in vivo labeling with stable isotopes.

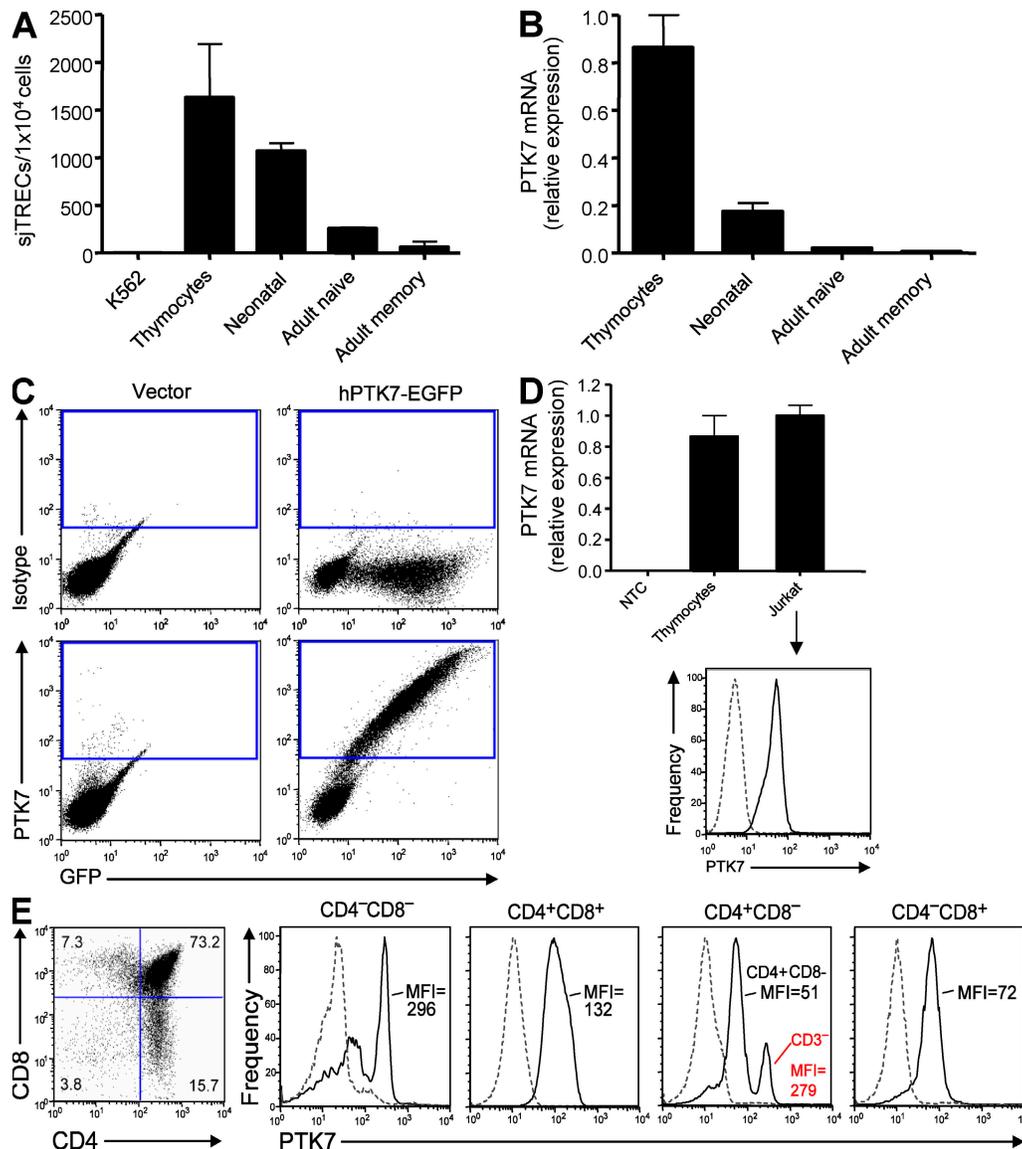
An understanding of RTE biology in humans is also of clinical interest, as studies in rodents have suggested impaired RTE function (7, 8). If this immaturity also applies to human RTEs, this could limit T cell effector function when RTEs likely predominate in the naive T cell compartment, such as in the neonate (9) and in older individuals undergoing immune reconstitution, e.g., after highly active antiretroviral therapy for HIV infection (2). In this paper, we have identi-

fied protein tyrosine kinase 7 (PTK7) as a surface marker for human CD4<sup>+</sup> RTEs that allows enumeration of their frequency and analysis of their function.

**RESULTS AND DISCUSSION**

**Identification of PTK7 as a putative CD4<sup>+</sup> RTE marker**

To identify surface markers on human CD4<sup>+</sup> RTEs, we compared the gene expression profiles of postnatal mature CD4<sup>+</sup> CD8<sup>-</sup> thymocytes and circulating neonatal and adult naive CD4<sup>+</sup> T cells. We reasoned that neonatal naive CD4<sup>+</sup> T cells



**Figure 1. sjTREC levels and PTK7 expression decrease with T cell maturation.** (A and B) sjTREC content (A) and PTK7 mRNA expression (B) of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes, neonatal CD45RA<sup>high</sup> CD4<sup>+</sup> T cells, and adult naive (CD45RA<sup>high</sup>) and memory (CD45RA<sup>low</sup>) CD4<sup>+</sup> T cells. K562 cells, which lack sjTRECs, were a negative control (sjTREC content and PTK7 mRNA are indicated as mean ± SD of two to three donors for each cell type per experiment, with results representative of two independent experiments). (C) PTK7 staining of a human PTK7-EGFP-transfected CHO cell line (representative result of four experiments). (D and E) Jurkat cells (D; representative result of three independent experiments) and human CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> thymocyte subsets (E, representative result from one of six donors) after PTK7 surface staining (solid line) and isotype control antibody staining (dashed line). Error bars show the mean ± SD.

were enriched in RTEs compared with adult naive CD4<sup>+</sup> T cells, as they contained higher levels of sjTREC than adult naive CD4<sup>+</sup> T cells (Fig. 1 A). We also hypothesized that peripheral CD4<sup>+</sup> RTEs, but not adult naive CD4<sup>+</sup> T cells, continued to express a subset of genes expressed by mature CD4<sup>+</sup>CD8<sup>-</sup> thymocytes because CD4<sup>+</sup> RTEs have recently completed thymocyte differentiation (1). To test this hypothesis, we generated a complementary DNA (cDNA) microarray of ~2,500 targets in which the transcript abundance of these three T cell populations was compared (unpublished data). Using quantitative real-time PCR, we confirmed that transcripts for PTK7, a member of the receptor tyrosine kinase family (10–12), were expressed at higher levels by CD4<sup>+</sup>CD8<sup>-</sup> thymocytes and neonatal CD4<sup>+</sup> T cells compared with adult naive CD4<sup>+</sup> T cells (Fig. 1 B).

To confirm these findings, we first established that a rabbit anti-mouse PTK7 antibody (13) specifically stained human PTK7 on the cell surface using flow cytometry. This specificity was verified by analysis of a human PTK7-EGFP cDNA-transfected Chinese hamster ovary (CHO) cell line and Jurkat CD4<sup>+</sup>CD8<sup>-</sup> T-lineage tumor cells, which express high levels of PTK7 messenger RNA (mRNA; Fig. 1, C and D). The specificity of the antibody was also validated using a mouse PTK7-EGFP cDNA-transfected CHO cell line, mouse fetal (E17) *PTK7*<sup>+/+</sup> and *PTK7*<sup>-/-</sup> thymocytes (13), and mouse EL-4 thymoma cells, which lack PTK7 mRNA (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20080996/DC1>).

Next, we tested whether human thymocytes also express endogenous surface PTK7. The highest expression was observed on the least mature triple-negative (CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>) thymocyte subset (14) and their immediate derivatives, the immature single-positive (CD3<sup>-</sup>CD4<sup>+</sup>CD8<sup>-</sup>) subset (15) (Fig. 1 E). PTK7 surface expression progressively decreased as thymocytes subsequently transitioned from the double-positive (CD4<sup>+</sup>CD8<sup>+</sup>) to the most mature single-positive (CD3<sup>high</sup>CD4<sup>+</sup>CD8<sup>-</sup> or CD3<sup>high</sup>CD4<sup>-</sup>CD8<sup>+</sup>) stage of development (1). Mouse thymocytes, like human thymocytes, also exhibited a progressive decrease in PTK7 expression with maturation (Fig. S1).

### Age-dependent frequency of PTK7-expressing peripheral naive CD4<sup>+</sup> T cells

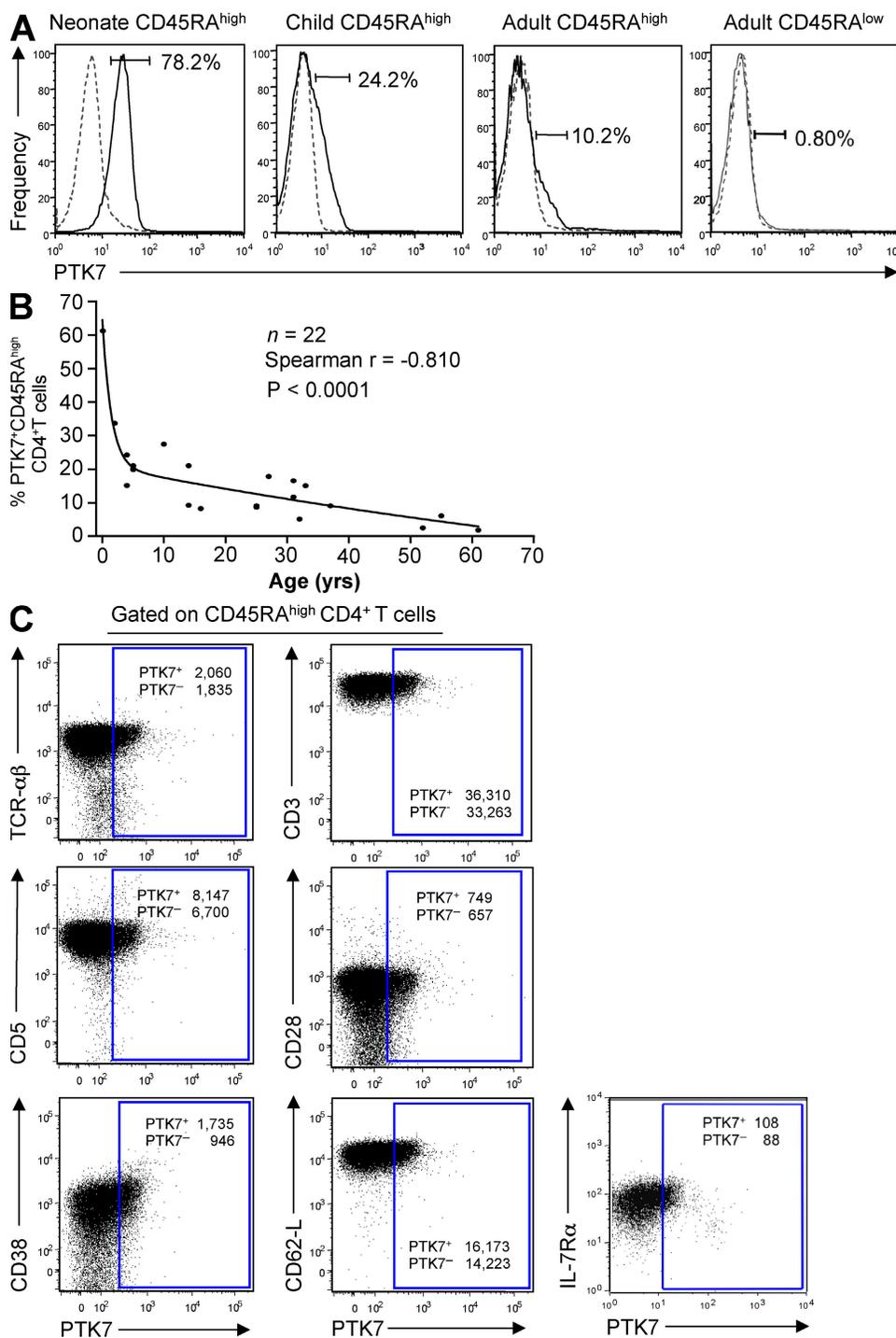
If PTK7 represents a marker for human CD4<sup>+</sup> RTEs, then a fraction of peripheral CD4<sup>+</sup> T cells should continue to express it after their export from the thymus. Indeed, we observed a distinct PTK7<sup>+</sup> subset of ~10% of antigenically naive CD4<sup>+</sup> T cells from adult peripheral blood, as defined by their CD45RA<sup>high</sup>CD45RO<sup>low</sup> surface phenotype (16) (Fig. 2 A). As predicted by earlier results of PTK7 mRNA expression (Fig. 1 B), the neonatal naive CD4<sup>+</sup> T cell population consistently contained a higher percentage of PTK7<sup>+</sup> cells than the analogous adult population (Fig. 2 A) and a higher amount of protein per cell, based on mean fluorescence intensity values (not depicted). An examination of 22 different healthy donors ranging in age between 1 mo and 61 yr demonstrated that the frequency of PTK7<sup>+</sup> cells among

naive CD4<sup>+</sup> T cells (Fig. 2 B) and the absolute number of PTK7<sup>+</sup> naive CD4<sup>+</sup> T cells (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20080996/DC1>) progressively and significantly decreased with age. This decline appeared to be biphasic, with a more rapid phase in early childhood, followed by a subsequent slower but progressive decrease with aging, and is similar to the pattern of exponential rather than linear decay of RTEs with aging reported in nonhuman vertebrates, including rodents (8, 17) and birds (18). These findings are consistent with the possibility that PTK7<sup>+</sup> naive CD4<sup>+</sup> T cells represent RTEs, as thymic cellularity and, likely, RTE production also peak in early childhood followed by a progressive decline with aging (19).

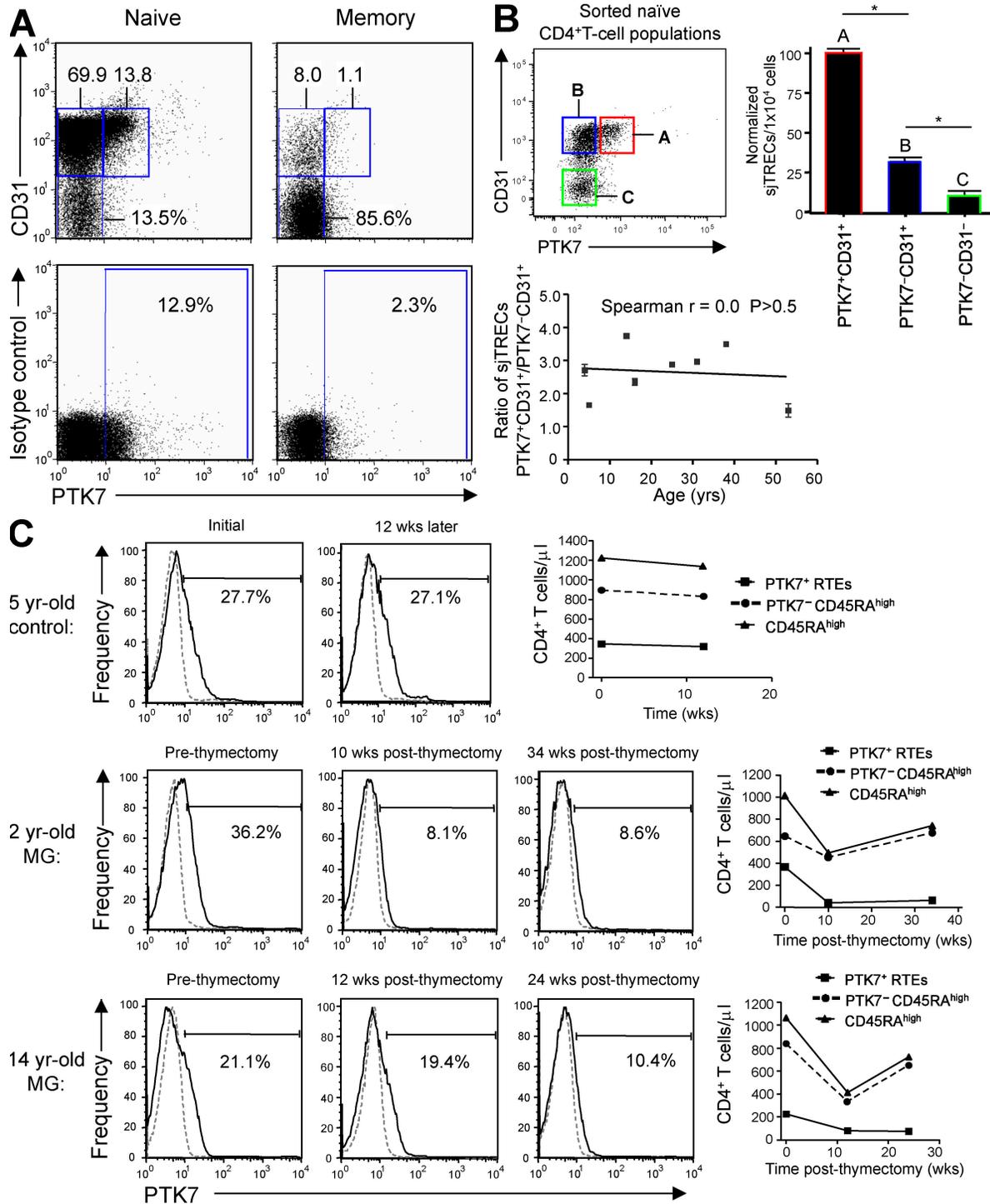
In contrast to the results for thymocytes and naive CD4<sup>+</sup> T cells, neither PTK7 transcripts (Fig. 1 B) nor surface PTK7 (Fig. 2 A) were detectable in adult memory CD45RA<sup>low</sup> (CD45RO<sup>high</sup>) CD4<sup>+</sup> T cells (16). This suggests that PTK7 surface protein was lost during postthymic conversion of naive CD4<sup>+</sup> T cells into memory cells. We did not detect inducible expression of PTK7 by PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells after in vitro stimulation with CD3 and CD28 mAbs (unpublished data), indicating that this protein was not up-regulated by T cell activation. PTK7<sup>+</sup> and PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells had similar surface levels of  $\alpha\beta$ -TCR, CD3, CD5, CD28, CD38, CD62-L, and IL-7R $\alpha$  (Fig. 2 C) and diversity of the  $\alpha\beta$ -TCR repertoire (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20080996/DC1>). These findings are also consistent with the PTK7<sup>+</sup> subset representing an RTE precursor of PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells. PTK7 expression was not detected on circulating human adult CD8<sup>+</sup> T cells, including the reported CD103<sup>+</sup>CD8<sup>+</sup> RTE subset (20), CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells,  $\gamma\delta$ -TCR<sup>+</sup> T cells, B cells, or NK cells (Fig. S4 and not depicted).

### Immunological phenotype of circulating PTK7<sup>+</sup> CD4<sup>+</sup> RTEs and their postthymic maturation

Next, we determined whether these putative PTK7<sup>+</sup> CD4<sup>+</sup> RTEs exhibited the expected pattern of expression of markers characteristic for naive CD4<sup>+</sup> T cells. For example, most adult circulating naive CD4<sup>+</sup> T cells are CD31<sup>+</sup>, but a CD31<sup>-</sup> subset has lower sjTREC content and less  $\alpha\beta$ -TCR diversity than the CD31<sup>+</sup> fraction and may be generated by foreign antigen-independent homeostatic proliferation (21, 22). We found that adult PTK7<sup>+</sup> naive CD4<sup>+</sup> T cells were uniformly CD31<sup>+</sup>, which is consistent with this population representing a precursor of the CD31<sup>-</sup> cell subset (Fig. 3 A). As sjTRECs are diluted in peripheral T cells by postthymic mitosis, such as homeostatic or activation-induced proliferation (3), we predicted that if PTK7<sup>+</sup> CD4<sup>+</sup> T cells are bona fide RTEs, then they should, like CD31<sup>+</sup> naive CD4<sup>+</sup> T cells, have a higher sjTREC content compared with the CD31<sup>-</sup> naive CD4<sup>+</sup> T cells that have undergone homeostatic proliferation (21). We found that the PTK7<sup>+</sup>CD31<sup>+</sup> putative RTEs contained high levels of sjTRECs (Fig. 3 B), and the PTK7<sup>-</sup>CD31<sup>-</sup> cells had extremely low levels of sjTRECs, which is similar to those of memory CD4<sup>+</sup> T cells (Fig. 1 A). Interestingly,



**Figure 2. PTK7 is expressed on a subset of circulating naive CD4<sup>+</sup> T cells that decreases with age and that has a surface phenotype similar to PTK7<sup>-</sup> naive cells.** (A) PTK7 staining (solid line) and isotype staining (dashed line) of circulating naive CD4<sup>+</sup> T cells from a term gestation neonate, a 4-yr-old child, and an adult, or of adult memory CD4<sup>+</sup> T cells. (B) Frequency of circulating PTK7<sup>+</sup> naive CD4<sup>+</sup> T cells from donors of various ages (1 mo–61 yr;  $n = 22$ ). A two-phase exponential decay curve (plotted using Prism software [GraphPad Software, Inc.]) was fitted and is presented for graphical purposes only. The correlation between age and the frequency of PTK7<sup>+</sup> naive CD4<sup>+</sup> T cells was significant (Spearman's  $r$ -value =  $-0.810$ ;  $P < 0.0001$ ; 95% CI of  $-0.920$  to  $-0.581$ ). (C) Surface expression of PTK7 versus the indicated T cell markers by CD4<sup>+</sup> T cells (representative result of three donors).



**Figure 3. Circulating PTK7<sup>+</sup> naive CD4<sup>+</sup> T cells are uniformly CD31<sup>+</sup>, contain higher sjTREC levels than CD31<sup>+</sup> or CD31<sup>-</sup> naive CD4<sup>+</sup> T cells lacking PTK7, and persistently decline after thymectomy.** (A) CD31 and PTK7 surface expression by circulating adult CD4<sup>+</sup> T cells of the CD45RA<sup>+</sup> (naive) and CD45RA<sup>-</sup> (memory) subsets (representative result of four donors). (B) sjTREC content of the PTK7<sup>+</sup>CD31<sup>+</sup>, PTK7<sup>-</sup>CD31<sup>+</sup>, and PTK7<sup>-</sup>CD31<sup>-</sup> subsets of adult naive CD4<sup>+</sup> T cells purified by FACS (mean ± SD of *n* = 3 donors per experiment, with results representative of three independent experiments). The sjTREC content of PTK7<sup>+</sup>CD31<sup>+</sup> naive CD4<sup>+</sup> T cells was arbitrarily set as 100 for each donor. \*, *P* < 0.0002 by the two-tailed paired Student's *t* test after Bonferroni correction for two comparisons. The bottom graph shows the ratio of sjTREC content of PTK7<sup>+</sup>CD31<sup>+</sup> naive CD4<sup>+</sup> T cells to that of PTK7<sup>-</sup>CD31<sup>+</sup> naive CD4<sup>+</sup> T cells for eight individuals varying in age [Spearman's *r*-value = 0.0; *P* > 0.05]. (C) Left panels show circulating PTK7<sup>+</sup>CD45RA<sup>+</sup> naive CD4<sup>+</sup> T cells obtained before and at two time points after thymectomy from myasthenia gravis (MG) patients who were 2 and 14 yr of age (isotype staining, dashed line). A 5-yr-old healthy control donor was analyzed twice at a 12-wk interval. Right panels show the concentration of PTK7<sup>+</sup> CD4<sup>+</sup> RTEs, PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells, and total naive CD4<sup>+</sup> T cells.

the sjTREC content of PTK7<sup>+</sup>CD31<sup>+</sup> naive CD4<sup>+</sup> T cells was also substantially and consistently higher than that of PTK7<sup>-</sup>CD31<sup>+</sup> naive cells (Fig. 3 B). For eight individuals ranging in age between 4 and 55 yr, the mean ratio  $\pm$  SD of the sjTREC content of PTK7<sup>+</sup>CD31<sup>+</sup> cells to that of PTK7<sup>-</sup>CD31<sup>+</sup> naive CD4<sup>+</sup> T cells was  $2.66 \pm 0.80$ , and this ratio did not significantly change with age (Fig. 3 B). These findings were consistent with PTK7 representing a marker for CD4<sup>+</sup> RTEs at all ages tested, and this consistency of higher sjTREC content in PTK7<sup>+</sup> naive CD4<sup>+</sup> T cells contrasts with the sjTREC content of CD31<sup>+</sup> naive CD4<sup>+</sup> T cells overall, which has been reported to decline with age (23). This suggests that PTK7<sup>+</sup> naive CD4<sup>+</sup> T cells, which are hereafter referred to as PTK7<sup>+</sup> CD4<sup>+</sup> RTEs, have undergone fewer cell divisions in the periphery than their PTK7<sup>-</sup> counterparts, including CD31<sup>+</sup> naive CD4<sup>+</sup> T cells. Assuming that the PTK7<sup>+</sup> CD4<sup>+</sup> RTEs are an obligatory precursor of PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells, this indicates that normal postthymic CD4<sup>+</sup> RTE maturation includes mitosis that, in contrast with CD31<sup>-</sup> cells that have undergone homeostatic proliferation, maintains CD31 expression and a diverse  $\alpha\beta$ -TCR repertoire.

To confirm that PTK7<sup>+</sup> CD4<sup>+</sup> RTEs are recent and direct products of the thymus, we examined the impact of complete thymectomy for the treatment of myasthenia gravis on this cell population. In a 2-yr-old and a 14-yr-old patient, there was a marked decline in both the frequency and circulating numbers of PTK7<sup>+</sup> CD4<sup>+</sup> RTEs at 10 or 12 wk after surgery, respectively, and these remained low at the second postsurgical time point, 34 or 24 wk, respectively (Fig. 3 C). Although the circulating numbers of both PTK7<sup>-</sup> and total naive CD4<sup>+</sup> T cells also declined at the first postthymectomy time point, these rebounded to near presurgical levels by the second postsurgical time point, whereas, in contrast, PTK7<sup>+</sup> CD4<sup>+</sup> RTEs did not. Analysis of a healthy 5-yr-old individual showed that the PTK7<sup>+</sup> CD4<sup>+</sup> RTE population remained stable over a 12-wk period, indicating that the alterations observed after thymectomy were not attributable to random biological variation (Fig. 3 C).

Collectively, these findings are consistent with the following: (a) the direct and recent production of PTK7<sup>+</sup> CD4<sup>+</sup> RTEs by the thymus; (b) an inability of extrathymic sources to replenish this cell population; and (c) a relatively short half-life of PTK7<sup>+</sup> CD4<sup>+</sup> RTEs as a result of either their conversion to PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells and/or their loss by cell death. The fraction of PTK7<sup>+</sup> CD4<sup>+</sup> RTEs that are converted into PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells versus those that die in the periphery while expressing surface PTK7 remains uncertain. Future *in vivo* labeling studies might be informative in better quantifying the precursor-product relationship between circulating PTK7<sup>+</sup> CD4<sup>+</sup> RTEs and PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells.

To determine if PTK7<sup>+</sup> CD4<sup>+</sup> RTEs can proliferate in an antigen-independent manner to become PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells, sorted PTK7<sup>+</sup> CD4<sup>+</sup> RTEs were CFSE labeled and cultured *in vitro* with a combination of cytokines to induce proliferation independent of  $\alpha\beta$ -TCR/CD3 engagement.

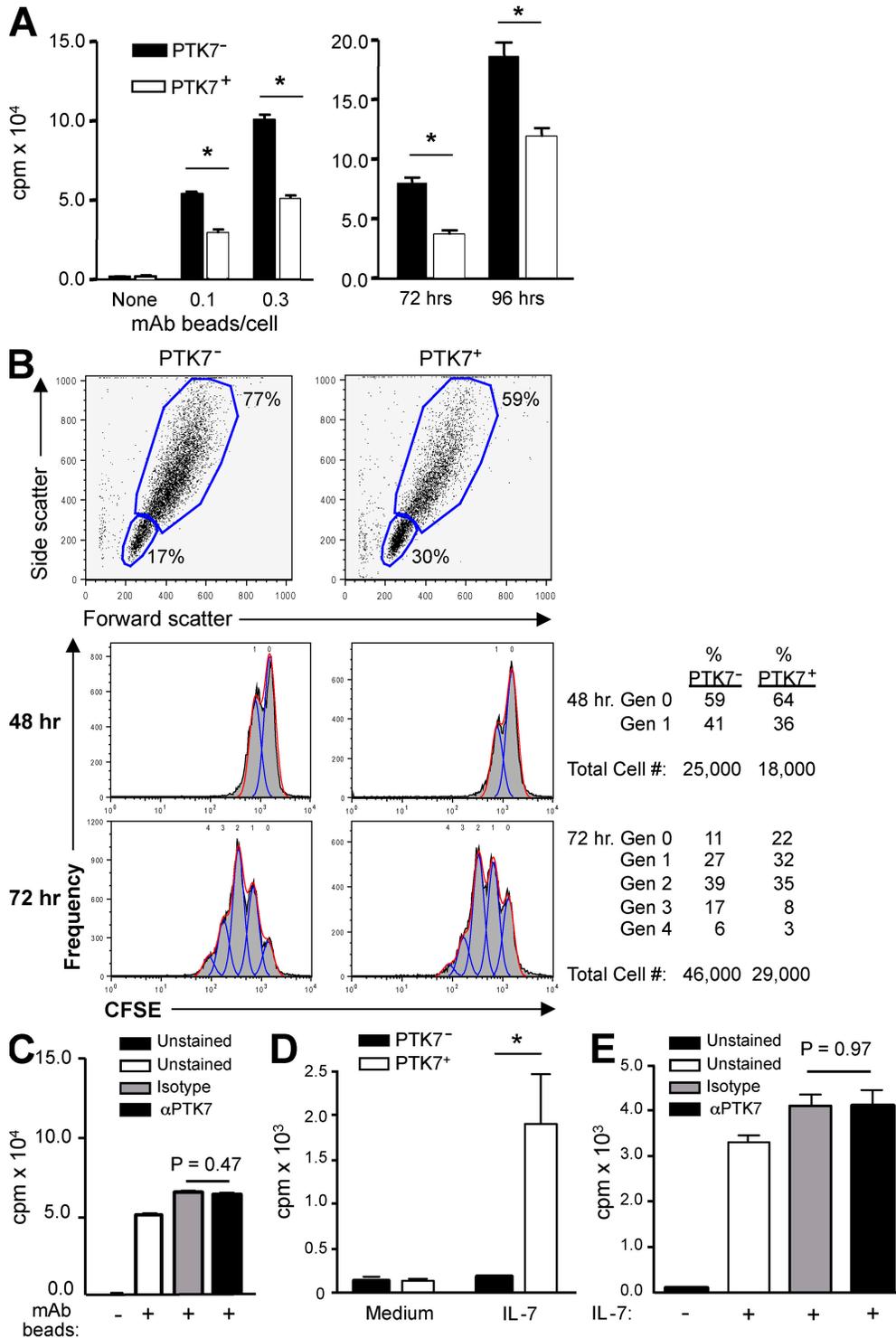
After 7 d of culture, surface CD31 levels were either unchanged or only slightly diminished, which is similar to the pattern previously described for naive CD4<sup>+</sup> T cells overall (22). In contrast, surface PTK7 levels expressed by the RTEs substantially and progressively declined with each round of mitosis (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20080996/DC1>). These results support a precursor-product relationship in which PTK7<sup>+</sup> CD4<sup>+</sup> RTEs undergo proliferation by a mechanism independent of  $\alpha\beta$ -TCR/CD3 engagement and convert to a PTK7<sup>-</sup> cell population with lower sjTREC content while retaining a CD31<sup>high</sup> naive surface phenotype.

### Immunological function of PTK7<sup>+</sup> CD4<sup>+</sup> RTEs

As previous studies have shown that mouse CD4<sup>+</sup> RTEs do not fully respond to  $\alpha\beta$ -TCR/CD3 stimulation (8, 24), we next determined if human CD4<sup>+</sup> RTEs also had functional limitations. FACS-purified human PTK7<sup>+</sup> CD4<sup>+</sup> RTEs activated using a combination of CD3 and CD28 mAbs coupled to beads ( $\alpha$ CD3/ $\alpha$ CD28 beads) had substantially less incorporation of <sup>3</sup>H-thymidine, reduced mitosis in a CFSE assay (25), and decreased blastogenesis compared with their PTK7<sup>-</sup> naive CD4<sup>+</sup> T cell counterparts (Fig. 4, A and B). Reduced mitosis using the CFSE assay was also observed with 0.03 and 0.01 beads/cell at both 48 and 72 h (unpublished data). To rule out the possibility that the PTK7 antibody staining used to purify the cells might inhibit their function, we isolated neonatal naive CD4<sup>+</sup> T cells, the majority of which express high levels of PTK7 (Fig. 2 A). We compared their proliferative responses after either staining with PTK7 antibody or an isotype-matched control antibody followed by staining with a secondary fluorochrome antibody before  $\alpha$ CD3/ $\alpha$ CD28 bead stimulation and found no inhibition of their proliferation (Fig. 4 C). In addition, no significant differences in apoptosis were observed between activated PTK7<sup>+</sup> CD4<sup>+</sup> RTEs and PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells that could account for the reduced proliferative response of the PTK7<sup>+</sup> CD4<sup>+</sup> RTEs (unpublished data).

Because PTK7<sup>+</sup> CD4<sup>+</sup> RTEs are immediate peripheral descendants of thymocytes, we investigated whether they possessed any residual biological characteristics of thymocytes. Previous work has shown that IL-7, which plays a critical role in thymocyte development (26), induces greater proliferation of mature single-positive thymocytes than peripheral T cells (27). Here, IL-7 treatment induced higher levels of proliferation of PTK7<sup>+</sup> CD4<sup>+</sup> RTEs compared with PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells (Fig. 4 D). This greater proliferation to IL-7 was unlikely to be an artifact of PTK7 antibody staining, as neonatal naive CD4<sup>+</sup> T cells proliferated similarly to IL-7 after staining with PTK7 antibody or isotype control antibody or no staining (Fig. 4 E). These results are consistent with PTK7<sup>+</sup> CD4<sup>+</sup> RTEs having a thymocyte-like functional phenotype of increased IL-7 responsiveness. The mechanism for this increased IL-7 responsiveness remains unclear, but is not accounted for by higher surface levels of IL-7R $\alpha$  (Fig. 2 C).

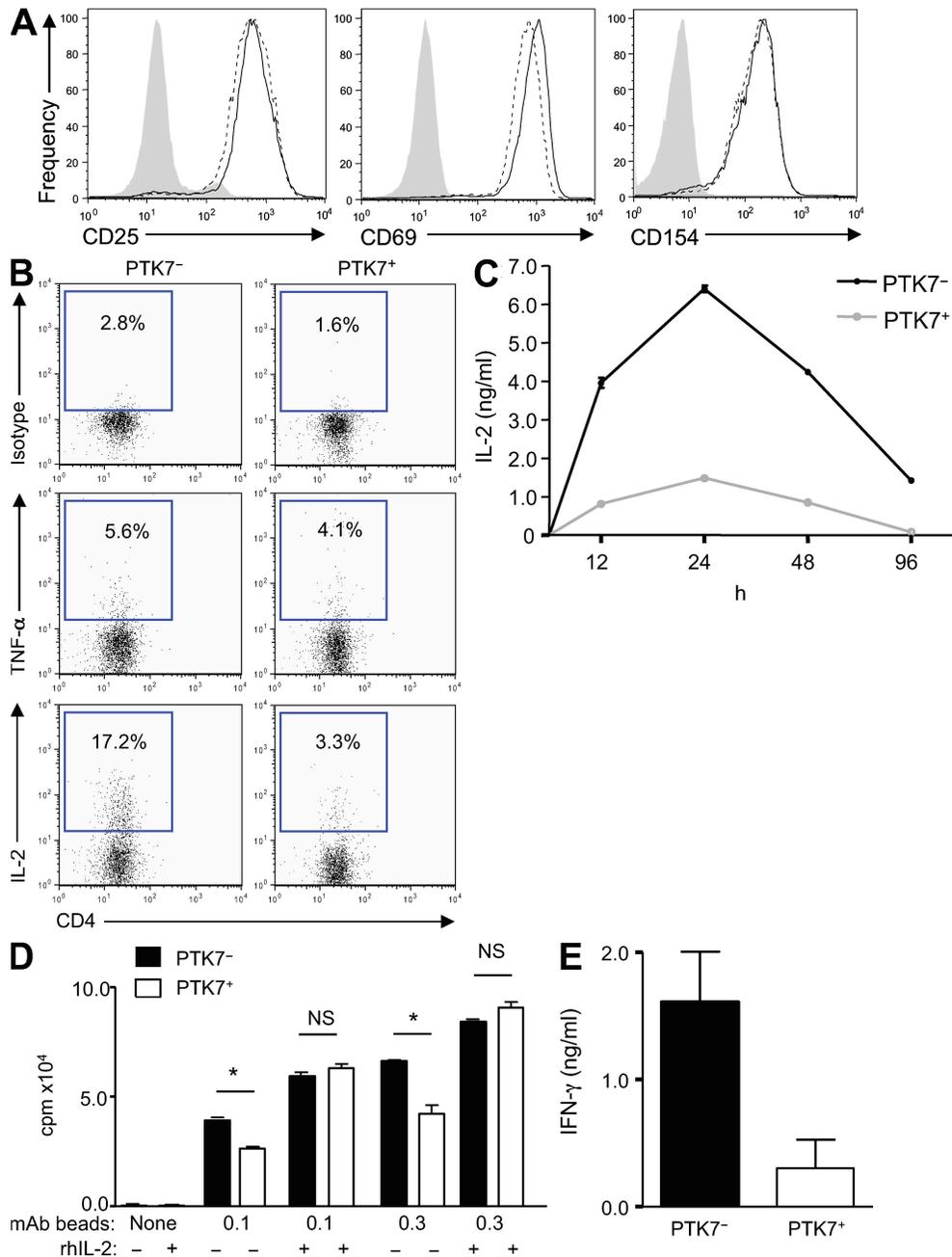
To determine whether decreased proliferation by PTK7<sup>+</sup> CD4<sup>+</sup> RTEs reflected a generalized limitation in T cell



**Figure 4. Circulating PTK7<sup>+</sup> CD4<sup>+</sup> RTEs exhibit decreased proliferation after αβ-TCR and CD28 engagement and greater IL-7 responsiveness compared with PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells.** (A) [<sup>3</sup>H]thymidine incorporation (mean ± SD of *n* = 4 donors) after culture with the indicated ratio of α-CD3/α-CD28 beads/cell for 72 h (left) and for the indicated times with 0.3 beads/cell (right). \*, *P* < 0.01 by the two-tailed unpaired Student's *t* test. (B) CFSE mitosis assay and lymphocyte blastogenesis (forward vs. side scatter) after 48 and 72 h of stimulation with 0.1 α-CD3/α-CD28 mAb beads/cell (representative results from one of five donors). (C) PTK7 antibody binding to neonatal naive CD4<sup>+</sup> T cells does not influence their proliferative response to α-CD3/α-CD28 beads (representative results from one of three donors). (D) [<sup>3</sup>H]thymidine incorporation (mean ± SD of *n* = 3 donors) by PTK7<sup>+</sup> and PTK7<sup>-</sup> adult naive CD4<sup>+</sup> T cells after 5 d of culture in complete medium with or without 10 ng/ml of rhIL-7. \*, *P* < 0.001 by the two-tailed paired Student's *t*-test. (E) PTK7 antibody binding to neonatal naive CD4<sup>+</sup> T cells does not influence their proliferative response to IL-7 (representative results from one of two donors). Error bars show the mean ± SD.

activation, we compared this cell type and PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells for surface expression of activation-dependent proteins and the production of cytokines. Activated PTK7<sup>+</sup> CD4<sup>+</sup> RTEs expressed similar surface levels of CD25, CD69, and CD154 (Fig. 5 A) and had similar frequencies of TNF- $\alpha$

secreting cells as PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells (Fig. 5 B). In contrast, IL-2 expression at the single cell level (Fig. 5 B) and in terms of overall secretion (Fig. 5 C) was markedly reduced for these cells compared with PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells. Furthermore, the addition of exogenous recombinant IL-2 to



**Figure 5. PTK7<sup>+</sup> CD4<sup>+</sup> RTEs exhibit a selective impairment of activation-induced function after engagement of the  $\alpha\beta$ -TCR/CD3 complex and CD28 compared with PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells.** (A) Activation-dependent surface expression by PTK7<sup>+</sup> CD4<sup>+</sup> RTEs (dashed line) and PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells (solid line) after stimulation with  $\alpha$ -CD3/ $\alpha$ -CD28 mAb beads for 12 (CD154) or 24 (CD25 and CD69) h (representative results from one of three donors). (B) Intracellular TNF- $\alpha$  and IL-2 expression after  $\alpha$ -CD3/ $\alpha$ -CD28 bead stimulation for 12 h (representative results from one of three donors). (C) IL-2 content (mean  $\pm$  SD of duplicate wells, with the results representative of one of four donors) in cell culture supernatants. (D) [<sup>3</sup>H]thymidine incorporation (mean  $\pm$  SD) after culture with the indicated ratio of  $\alpha$ -CD3/ $\alpha$ -CD28 beads/cell for 96 h in the presence and absence of rhIL-2 (representative results from one of three donors). (E) IFN- $\gamma$  content (mean  $\pm$  SD of  $n = 3$  donors) in cell culture supernatants after Th1 differentiation for 72 h. \*,  $P < 0.05$  by the two-tailed unpaired Student's  $t$  test.

PTK7<sup>+</sup> CD4<sup>+</sup> RTEs completely increased their proliferation to levels comparable to those of PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells, suggesting that RTEs have the capacity to fully respond to IL-2 stimulation but lack the ability to produce IL-2 in sufficient quantities for a full response (Fig. 5 D). In contrast, both reduced IL-2 and IL-2 receptor expression may limit the activation of mouse CD4<sup>+</sup> RTEs (8). Activated PTK7<sup>+</sup> CD4<sup>+</sup> RTEs also secreted markedly less IFN- $\gamma$  than PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells using an in vitro assay for Th1 cell generation (Fig. 5 E). These last findings suggest a mechanism for the observed vulnerability of the young infant to infections requiring Th1 immunity for control, such as mycobacterial infections, as PTK7<sup>+</sup> CD4<sup>+</sup> RTEs likely comprise a large fraction of the naive CD4<sup>+</sup> T cell compartment at this age (9).

In contrast to humans, PTK7 was not expressed at detectable levels on mouse peripheral naive CD4<sup>+</sup> T cells at any postnatal age, indicating its lack of utility as a mouse RTE marker (unpublished data). Thus, despite the conserved pattern of PTK7 expression in thymocytes, there may be substantial species-related differences in its peripheral T cell expression. Although PTK7 has been implicated in planar cell polarity during central nervous system embryonic development (13), its function in T cell development remains unclear.

In summary, PTK7 is a novel marker for the measurement of circulating human CD4<sup>+</sup> RTEs, which have impaired immune function compared with more mature naive CD4<sup>+</sup> T cells. The findings of a more pronounced and persistent loss of PTK7<sup>+</sup> CD4<sup>+</sup> than PTK7<sup>-</sup>CD31<sup>+</sup> naive CD4<sup>+</sup> T cells after complete thymectomy, and the higher sjTREC content of PTK7<sup>+</sup> CD4<sup>+</sup> compared with PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells, also suggest that PTK7 may identify RTEs that are more recently produced by the thymus than does CD31. Clinical contexts in which measurement of thymic output based on PTK7 surface staining might be useful include treatment with cytotoxic agents, HIV infection, hematopoietic stem cell transplantation, and primary immunodeficiency (5, 28, 29). Furthermore, the ability to isolate a circulating naive CD4<sup>+</sup> T cell population enriched for RTEs using PTK7 will allow better mechanistic studies of the function and phenotype of CD4<sup>+</sup> RTEs in health and disease.

## MATERIALS AND METHODS

**Cell sources and preparation.** Human thymus tissue was obtained from surgical pathology specimens of children undergoing cardiac surgery who did not have the 22q11.2 deletion syndrome. Human umbilical vein cord blood was obtained from the placentas of uncomplicated term pregnancies after routine Caesarian section delivery. Peripheral blood was obtained from healthy adult volunteer donors, children undergoing evaluation for immunodeficiency who had normal immune function tests, and two patients undergoing complete thymectomy for the treatment of myasthenia gravis. All human samples were obtained with provision of informed consent and in a manner in accordance with approval by the Institutional Review Board of Stanford University. Circulating mononuclear cells were isolated from blood by Ficoll-Hypaque density gradient centrifugation. CD4<sup>+</sup> T cells were purified from circulating mononuclear cells using a negative selection kit and a magnetic-activated cell sorting system (Miltenyi Biotec). Where indicated, CD4<sup>+</sup> T cells or thymocytes were stained for surface markers and subjected to purification by FACS. All mouse studies complied with federal guidelines

and were approved by the Stanford University Administrative Panel on Laboratory Care Committee.

**PTK7-expressing CHO cells.** PCR was used for amplification and subcloning of a human PTK7 cDNA (OriGene) into an hPTK7-pEGFP-N3 construct. 10<sup>6</sup> CHO cells were transfected by electroporation with 20.0  $\mu$ g of hPTK7-EGFP and analyzed by flow cytometry 3 d later to verify EGFP fluorescence and PTK7 expression.

**Cell surface staining with antibodies.** Cells were incubated at room temperature in PBS, pH 7.4, with 0.5% wt/vol BSA with fluorochrome-conjugated mAbs for CD3, CD4, CD8, CD31, CD45RA, and CD45RO (Invitrogen or BD) or isotype-matched control mAbs of irrelevant specificity (Invitrogen). For surface PTK7 staining, cells were first sequentially incubated with purified rabbit IgG (blocking step), affinity-purified rabbit IgG anti-mouse PTK7 (13) or affinity-purified IgG from unimmunized rabbits (Invitrogen) for 20 min, and either FITC- or PE-conjugated F(ab')<sub>2</sub> goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 20 min, with washing between each step. This was followed by the addition of the fluorochrome-conjugated mAbs described at the beginning of this section. Cells were fixed for 5 min at room temperature in 2% paraformaldehyde except for experiments in which viable cells were purified using a FACS. Flow cytometry was performed using a FACSCalibur instrument (BD).

**Calculation of the circulating number of PTK7<sup>+</sup> naive CD4<sup>+</sup> T cells.** Absolute lymphocyte counts were obtained from contemporaneous complete blood count data and were multiplied by the frequency of PTK7<sup>+</sup> naive (CD45RA<sup>high</sup>) CD4<sup>+</sup> T cells among the lymphocytes of the blood sample, which were identified by forward-scatter versus side-scatter properties.

**sjTREC content.** Total cellular DNA was isolated using a QIAamp DNA Mini kit (QIAGEN) and used in 20.0- $\mu$ l real-time PCR reactions in 384-well plates analyzed in a 7900HT Fast Real Time PCR instrument (Applied Biosystems). Reactions included oligonucleotide primers and a TAMRA-FAM labeled internal probe for the  $\delta$ Rec- $\Psi$ J $\alpha$  signal joint (5). Internal standards that were cloned from human thymocyte DNA by PCR included a 381-bp segment containing the  $\delta$ Rec- $\Psi$ J $\alpha$  signal joint (primers 5'-GAAAA-CAGCCTTTGGGACAC-3' and 5'-GTGACATGGAGGGCTGAACT-3', which were designed based on the homology of a rhesus macaque sjTREC sequence with human genomic sequences) and a 496-bp segment of the  $\alpha$  region that is not affected by V(D)J recombination (primers 5'-ATCAC-GAGCAGCTGGTTTCT-3' and 5'-CCATTCCTGAAGCAAGGAAA-3'). These products were used in standard curves ranging from 1.0  $\times$  10<sup>1</sup> to 1.0  $\times$  10<sup>5</sup> copies per reaction. A TAMRA-FAM labeled internal probe for the  $\alpha$  region of the TCR- $\alpha$  gene locus was used for detection of  $\alpha$  copy number (5).

**Real-time PCR.** Total cellular RNA was purified (Trizol; MRC) and 1.0  $\mu$ g was reverse transcribed into cDNA using Superscript II enzyme (Invitrogen) and random hexamer primers. Real-time PCR was performed using SYBR Green (Roche) and primers 5'-CCACCTACCAATGGTTCCGA-3' and 5'-TGCTCTGACCATCAGAAAGGG-3' (Primer Express software; Applied Biosystems) that amplify within exon 4 of PTK7, which is not subject to alternative RNA splicing (11). Samples were normalized for total cDNA based on 18S rRNA levels (30). Transcript levels were calculated using the double delta Ct equation (Applied Biosystems).

**T cell proliferation and mitosis.** FACS-purified PTK7<sup>+</sup> and PTK7<sup>-</sup> naive (CD45RA<sup>high</sup>) CD4<sup>+</sup> T cells were incubated in 200  $\mu$ l of complete medium (RPMI 1640 with 10% heat-inactivated human AB serum, 50.0 U/ml penicillin G, 50.0  $\mu$ g/ml streptomycin, and 2.0 mM L-glutamine) in round-bottom 96-well plates. For [<sup>3</sup>H]thymidine incorporation assays, cells were incubated in complete medium with or without  $\alpha$ -CD3/ $\alpha$ -CD28 mAb-coated paramagnetic beads (Invitrogen) at concentrations of 0.1 and 0.3 beads/cell. Wells were pulsed with 1.0  $\mu$ Ci [<sup>3</sup>H]thymidine during the final 16 h of culture, and

cells were collected after 72 or 96 h of incubation using a cell harvester (Tomtec) and counted by liquid scintillation (Betaplate; PerkinElmer). In some experiments, purified cells were cultured with 0.1 or 0.3  $\alpha$ -CD3/ $\alpha$ -CD28 beads/cells with or without 100 U/ml of rIL-2 (Proleukin) for 96 h, pulsed with [ $^3$ H]thymidine, and assayed for tritium incorporation 18 h later. IL-7-dependent proliferation was assessed by similar methods after purified cells were incubated with 10 ng/ml of recombinant human IL-7 (R&D Systems) or complete medium alone and harvested after 120 h of incubation. For mitotic analysis, cells were stained with 5.0  $\mu$ M CFSE (Invitrogen) in 1 ml of 5% FCS in PBS for 5 min at room temperature, washed, and incubated with 0.1, 0.03, or 0.01  $\alpha$ -CD3/ $\alpha$ -CD28 beads/cell in complete medium. After 48 and 72 h, cells were collected and analyzed by flow cytometry.

**Intracellular cytokine staining.** Purified CD4<sup>+</sup> T cell subsets were incubated with 0.3  $\alpha$ -CD3/ $\alpha$ -CD28 beads/cell in complete medium for 12 h, with 10.0  $\mu$ g/ml brefeldin A added during the last 3 h of culture. Cells were fixed, permeabilized, and stained for CD4 and intracellular IFN- $\gamma$  and TNF- $\alpha$  (BD) and analyzed flow cytometry.

**Short-term Th1 cell generation for IFN- $\gamma$  content.** CD4<sup>+</sup> T cell subsets were cultured at a 10:1 ratio with autologous CD14<sup>+</sup> monocytes and 0.3  $\alpha$ -CD3/ $\alpha$ -CD28 beads/T cell. Cell supernatants were collected after 72 h and IFN- $\gamma$  content was determined by ELISA (BD).

**Online supplemental material.** Fig. S1 shows that PTK7 expression progressively decreases with mouse thymocyte maturation. Fig. S2 shows a decrease of the circulating concentration of PTK7<sup>+</sup> cells with aging. Fig. S3 shows spectratyping of the TCR- $\beta$  CDR3 region for three V $\beta$  family members, indicating that  $\alpha\beta$ -TCR repertoire for human PTK7<sup>+</sup> and PTK7<sup>-</sup> naive CD4<sup>+</sup> T cells are similar and highly diverse. Fig. S4 shows a lack of PTK7 expression by CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells. Fig. S5 shows a loss of surface expression of PTK7 but retention of CD31 with cytokine-mediated in vitro maturation. Supplemental materials and methods was used to generate the results of Figs. S1–S5. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20080996/DC1>.

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