CD31 Expression on CD4⁺ Cells: A Simple Method for Quantitation of Recent Thymus Emigrant CD4 Cells

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Abstract. Measurements of CD4⁺CD31⁺ cells gave results consistent with those expected for recent thymus emigrant (RTE) CD4⁺ cells. The method was markedly simpler than established procedures for measurement of CD4⁺ RTE cells and is usable in locations with limited facilities and budgets.

For human immunodeficiency virus (HIV)–infected individuals, thymus production of naive T-cells is central to maintenance and recovery of robust immune capacities. As combined antiretroviral therapy (cART) has become more widely available, it has become clear that while a majority of patients on cART have reduction in viral load, recover, and maintain improved immune capacities, a subset either fails to rapidly recover or maintain robust T-cell capacities.^{1–3} Direct evidence for thymus failure in these patients can be provided through measurements of naive T-cells that have recently emigrated from the thymus (recent thymus emigrant [RTE]). The reference procedure for measuring RTE cells is quantitation in peripheral blood mononuclear cells of T-cell receptor excision circles (TRECs).⁴ This multistep procedure requires advanced instrumentation and makes the TREC test unsuitable for use in the majority of clinics.

In 2002, Kimmig and others^{5–7} demonstrated that the expression of CD31 on naive CD4⁺ T-cells identifies a population that overlaps with TREC-high CD4⁺ T-cells, RTE. Subsequent studies showed similar results, including HIV-positive individuals.^{6,7} Findings by Kimmig and others have been adopted, and CD31 on naive CD4⁺ T-cells has since been used as a marker of RTE, using procedures comprising identification of CD4⁺ cells, and then further steps to enable identification of naive subsets of CD4⁺ (using CD45RA⁺,⁸ CD45RA⁺CCR7⁺,⁹ CD45RA⁺CD62L⁺¹ or CD45RA⁺CD27⁺¹⁰) before quantitation of the CD31⁺ fraction.

In the reports of Kimmig and others,⁵ CD4⁺ cells were segregated into naive (CD45RA⁺) and not naive (CD45RA[−]), and each subset was tested for TREC and CD31 expression. Their critical demonstration was that the CD4⁺CD45RA⁺ subset rich in TREC expresses CD31, whereas the CD4⁺CD45RA⁺ subset with low TREC content had almost no expression of CD31. It was thus concluded that measuring CD31 on this subset was a surrogate measurement of CD4⁺ RTE. Prior studies showed that CD31 expression on CD4⁺ cells was mostly restricted to the naive subtype in healthy children and adults as well as in HIV-infected individuals.^{6,10} Expressed differently, these data show that CD31 expression on CD4⁺ cells was essentially limited to naive CD4⁺ cells and correlated highly with TREC expression in these cells. This suggested that direct measurement of CD31 expression on CD4⁺ cells in the absence of further subtyping would provide

a good measure of CD4⁺ RTE cells. If this was the case, a simple procedure for measuring RTE suitable for use in clinics lacking sophisticated hardware could be available.

RTEs have well established properties including decline with increasing age,^{5,11} equivalency in numbers for HIV-free (HIV[−]) and disease-well-controlled HIV⁺ individuals,^{2,7,10,12} and that within age-normalized groupings, values are higher for females.¹¹ In this report, we reviewed medical records for HIV[−] and HIV⁺ individuals that included measurements of CD4⁺CD31⁺ T-cells to determine whether the findings were consistent with those expected if CD4⁺CD31⁺ measurements were surrogates for RTE.

The retrospective record review found 69 perinatally HIV⁺ (median age [interquartile range (IQR)] = 13.0 (8.6) years, 54% female, 71% black) and 51 HIV[−] children (median [IQR] = 1.6 (5.6) years, 51% female, 63% black) who attended UTHealth, Houston clinics between January 2010 and September 2012 for whom CD4⁺CD31⁺ outcomes were available and medical records accessible. Informed consent was obtained from all adult participants and from parents or legal guardians of minors. The study was approved by the UTHealth Committee for the Protection of Human Subjects. The studied perinatally HIV⁺ population was in good clinical status with respect to HIV disease (2.2 [1.5] log₁₀ HIV RNA copies/mL, 33% [12%] CD4⁺, median [IQR]; and, 81% on cART [defined as dual-nucleoside/nucleotide reverse transcriptase inhibitors with either a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor without interruption for at least 3 months before CD4⁺CD31⁺ measurement]).

We first tested the hypothesis that the measured numbers of CD4⁺CD31⁺ cells and their change with age in the HIV⁺ children were similar to published values and patterns of CD31⁺ naive CD4⁺ cells.^{5,13} The fraction of CD4⁺CD31⁺ cells measured in our HIV⁺ patients was plotted against patient age (Figure 1A). Then, for each measured patient, an expected RTE for age and the range of this estimate were calculated from published values.^{5,13} The dispersion of observed and expected values was indistinguishable, and the rates of decline with increasing age were essentially identical.

Next, we plotted CD4⁺CD31⁺% versus age for the pediatric HIV⁺ and HIV[−] patients to test the hypothesis that for patients with well controlled HIV disease, the measured values were similar for both groups as would be expected if the measurements were of RTE (Figure 1B). The values for HIV⁺ and HIV[−] had similar dispersion, and regression analysis found slopes that were indistinguishable.

Lastly, to determine whether measured CD4⁺CD31⁺ cells were more frequent in females, a finding expected for RTE,¹¹

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FIGURE 1. (A) Measured CD4⁺CD31⁺ cells (open circles) were indistinguishable from predicted recent thymus emigrant (RTE) % cells (solid squares) for age. Predicted CD4⁺CD31⁺ values were calculated, and the measured outcomes were tested against estimated outcomes. Estimation: Predicted CD31⁺ among naive CD4⁺ T-cells for each observed subject's age was calculated using predicted CD4⁺CD45RA⁺ T-cells for age as reported by Kimmig and others.⁵ Predicted CD4⁺CD45RA⁺ was estimated by calculating predicted CD4⁺CD45RA⁺ and CD4⁺ absolute cell counts for each patient using the model published by Huenecke and others to estimate normal numbers of lymphocyte subsets in children according to age.¹³ 95% upper and lower limits were calculated based on the same model. Measured outcomes from perinatally Human immunodeficiency virus (HIV)⁺ subjects were compared with predicted outcomes. The upper and lower dashed lines represent the 95% upper and 95% lower limits of predicted values (the down or up pointing arrowheads). (B) Demonstration that CD4⁺CD31⁺ cells decreased with increase in age as expected for RTE (Spearman's $\rho = -0.525$, $P < 0.001$ and $\rho = -0.475$ ($P < 0.001$) for HIV⁻ and HIV⁺, respectively) and were maintained at similar numbers for clinically well HIV⁺. Solid circles and solid line represent HIV⁺ data and open squares and dashed line represent HIV⁻ data. (C) Demonstration that within age-similar groupings, females (F) had more CD4⁺CD31⁺ cells. These findings were expected for RTE.¹¹ Comparison between different groups and P values obtained were through using χ^2 test. For both figures 1A and 1B, linear regression models were generated to fit the lines on the graphs using GraphPad Prism v6 (San Diego, CA). Distributions of measured and predicted CD4⁺CD31⁺ by age were statistically identical: no difference in slopes or intercepts of fitted lines ($P = 0.56$ and $P = 0.76$, respectively). Fitted lines to CD4⁺CD31⁺ distribution by age for HIV⁻ and HIV⁺ showed no statistically significant difference in slopes or intercepts either ($P = 70$ and $P = 0.56$, respectively).

findings for the HIV⁺ and HIV⁻ patients, established as indistinguishable in the preceding analysis, were combined to give sufficient sample size for subsequent grouping on age and then subgrouping on gender. The findings showed the expected and previously observed decline with age, and that within each age grouping, the females had higher values than males, which were expected findings for RTE (Figure 1C).

This retrospective record review shows that the direct measurement of CD4⁺CD31⁺ cells in peripheral blood gives results with numerical and biological characteristics consistent with those expected for CD4⁺ RTE. The practical importance of our finding is that it shows the addition of a single reagent to the long-established routine patient sample collection, sample processing, and sample testing procedures used for CD4 cell measurements may enable quantitative determinations of RTE; the test is usable in clinical settings lacking research grade infrastructure.

A practicable RTE assay could enable rapid identification of patients with cART effected control of HIV viremia where thymus output is low and prognosis is poor. Interventions to stimulate thymus activity may be needed. Most of the patients who could benefit reside in resource-disadvantaged settings.

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REFERENCES

- Li T, Wu N, Dai Y, Qiu Z, Han Y, Xie J, Zhu T, Li Y, 2011. Reduced thymic output is a major mechanism of immune reconstitution failure in HIV-infected patients after long-term antiretroviral therapy. *Clin Infect Dis* 53: 944–951.
- Resino S, Galan I, Perez A, Leon JA, Seoane E, Gurbindo D, Muñoz-Fernandez MA, 2004. HIV-infected children with moderate/severe immune-suppression: changes in the immune system after highly active antiretroviral therapy. *Clin Exp Immunol* 137: 570–577.
- De Rossi A, Walker AS, Klein N, De Forni D, King D, Gibb DM, 2002. Increased thymic output after initiation of antiretroviral therapy in human immunodeficiency virus type 1-infected children in the Paediatric European Network for Treatment of AIDS (PENTA) 5 Trial. *J Infect Dis* 186: 312–320.
- Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, Polis MA, Haase AT, Feinberg MB, Sullivan JL, Jamieson BD, Zack JA, Picker LJ, Koups RA, 1998. Changes in thymic function with age and during the treatment of HIV infection. *Nature* 396: 690–695.
- Kimmig S, Przybylski GK, Schmidt CA, Laurisch K, Mowes B, Radbruch A, Thiel A, 2002. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J Exp Med* 195: 789–794.
- Junge S, Kloeckener-Gruissem B, Zufferey R, Keisker A, Salgo B, Fauchere JC, Scherer F, Shalaby T, Grotzer M, Siler U,

- Seger R, Güngör T, 2007. Correlation between recent thymic emigrants and CD31⁺ (PECAM-1) CD4⁺ T cells in normal individuals during aging and in lymphopenic children. *Eur J Immunol* 37: 3270–3280.
7. Tanaskovic S, Fernandez S, Price P, Lee S, French MA, 2010. CD31 (PECAM-1) is a marker of recent thymic emigrants among CD4⁺ T-cells, but not CD8⁺ T-cells or $\gamma\delta$ T-cells, in HIV patients responding to ART. *Immunol Cell Biol* 88: 321–327.
8. Klein N, Sefe D, Mosconi I, Zanchetta M, Castro H, Jacobsen M, Jones H, Bernardi S, Pillay D, Giaquinto C, Walker AS, Gibb DM, De Rossi A, Paediatric European Network for Treatment of AIDS 11 Trial Team, 2013. The immunological and virological consequences of planned treatment interruptions in children with HIV infection. *PLoS One* 8: e76582.
9. Blanche S, Scott-Algara D, Le Chenadec J, Didier C, Montange T, Avettand-Fenoel V, Rouzioux C, Méléard A, Viard JP, Dollfus C, Bouallag N, Warszawski J, Buseyne F, 2014. Naive T lymphocytes and recent thymic emigrants are associated with HIV-1 disease history in French adolescents and young adults infected in the perinatal period: the ANRS-EP38-IMMIP study. *Clin Infect Dis* 58: 573–587.
10. Rallon N, Sempere-Ortells JM, Soriano V, Benito JM, 2013. Central memory CD4 T cells are associated with incomplete restoration of the CD4 T cell pool after treatment-induced long-term undetectable HIV viraemia. *J Antimicrob Chemother* 68: 2616–2625.
11. Pido-Lopez J, Imami N, Aspinall R, 2001. Both age and gender affect thymic output: more recent thymic migrants in females than males as they age. *Clin Exp Immunol* 125: 409–413.
12. Resino S, Resino R, Micheloud D, Gurbindo Gutierrez D, Leon JA, Ramos JT, Ciria L, de José I, Mellado J, Muñoz-Fernández A, Spanish Group of Paediatric HIV Infection, 2006. Long-term effects of highly active antiretroviral therapy in pretreated, vertically HIV type 1-infected children: 6 years of follow-up. *Clin Infect Dis* 42: 862–869.
13. Huenecke S, Behl M, Fadler C, Zimmermann SY, Bochennek K, Tramsen L, Esser R, Klarmann D, Kamper M, Sattler A, von Laer D, Klingebiel T, Lehrnbecher T, Koehl U, 2008. Age-matched lymphocyte subpopulation reference values in childhood and adolescence: application of exponential regression analysis. *Eur J Haematol* 80: 532–539.