Immune Correlates of Herpes Zoster in HIV-Infected Children and Youth

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To identify immunologic factors that modulate the risk of herpes zoster (HZ), we compared varicella-zoster virus (VZV)-specific and nonspecific T-cell subpopulations of 47 HIV-infected children before they developed HZ with those of 141 VZV-positive HZ-negative matched controls. Compared with controls, HZ cases had lower VZV-specific CD8⁺ CD107a⁺ cell percentages independently of CD4⁺ percentages or HIV loads, suggesting that VZV-specific cytotoxic T cells are protective against HZ. In contrast, high nonspecific regulatory and activated T cells were associated with an increased risk of HZ.

Herpes zoster (HZ) was a very common complication of HIV disease before the advent of highly active antiretroviral therapy (HAART) (7, 40) and remains relatively frequent in spite of the widespread application of HAART (39, 23). This is consistent with previous observations that HIV-infected individuals on HAART may not fully recover varicella-zoster virus (VZV)-specific cell-mediated immune (CMI) responses (14, 45).

Patients at high risk of developing HZ typically have lower VZV-specific functional responses in CD4⁺ and CD8⁺ T-cell subsets (31) than low-risk individuals. However, it is not known if one or both T-cell subsets are directly involved in preventing VZV reactivation and/or spread (2).

An HZ vaccine is efficacious in individuals who are ≥ 50 years old (30, 34). HIV-infected children on HAART are candidates for this vaccine due to their persistently high incidence of HZ. However, HIV-infected children have deficient responses to vaccines in spite of HAART (3, 24, 25, 43). Since immunogenicity will be an important endpoint for a study of the HZ vaccine in this population, establishing immune correlates of protection against HZ would represent an important outcome variable.

Mechanisms responsible for poor functional immune reconstitution in HIV-infected individuals on HAART are not well understood. HIV-infected individuals have high proportions of regulatory T cells (Treg) (1, 5), which may contribute to failure to fully reconstitute immunity to VZV and other pathogens (8). Both human and mouse CD4⁺ Treg characteristically express FoxP3 (4, 11–13, 20). Other markers that identify Treg are expression of CD25, lack of CD127, and synthesis of transforming growth factor β (TGF- β) or interleukin 10 (IL-10) (15, 26, 27, 29, 35, 38). Less is known about CD8⁺ Treg, which also contribute to deactivation of the immune response and tolerance (10, 19, 22, 41, 42).

We investigated associations between the incidence of HZ in HIV-infected children and the presence of VZV-specific effector T cells (Teff) and nonspecific Treg in their peripheral blood mononuclear cells (PBMC).

This was a case-control study using cryopreserved PBMC from subjects enrolled in the IMPAACT/PACTG 219/219c long-term follow-up cohort. HZ cases from 1994 to 2004 were matched 1:3 to controls (previous varicella but without HZ) by age, nadir CD4 percentages, utilization of HAART, and time since the last active VZV infection (≤ 1 year or > 1 year) (44).

For Treg, freshly thawed PBMC were surface stained with anti-

CD4, -CD8, -CD25 (Beckman Coulter), and -TGF- β (IQP Products) and intracellularly with anti-TGF- β , -FoxP3 (eBioscience), -PD-1, and -IL-10 (BD Biosciences). For Teff, PBMC were stimulated for 5 days with the cell-associated VZV Oka vaccine strain (9.6×10^4 PFU/ml) or medium. The virus was grown in primary human lung fibroblasts. On day four, brefeldin A (Sigma) and anti-CD107a (BD Biosciences) were added (6). On day five, PBMC were surface stained for CD4, CD8, CD25, and TGF- β and intracellularly for IL-2, tumor necrosis factor alpha (TNF- α), (eBioscience), MIP-1 β , and gamma interferon (IFN- γ) (BD Biosciences). Flow cytometric analyses were done with the Guava EasyCyte Plus system (Millipore) and the FlowJo software program (Treestar). VZV-specific Teff and Treg were expressed as percentages of the parent CD4⁺ or CD8⁺ lymphocytes.

Fisher's exact test was used for categorical variables and a generalized estimating equation (GEE) was used for continuous variables while accounting for matching. For continuous variables with small sample sizes, the Wilcoxon rank-sum test was used. Spearman's rank order correlation analysis was performed to examine the strength and statistical significance of associations. Multivariable analyses used standard logistic regression to examine the predictive value of Treg or Teff while controlling for CD4⁺ cell percentages and/or plasma HIV RNA copies/ml. Since this was an exploratory study, no adjustments were made for multiple comparisons, which may have created an overly conservative criterion resulting in our missing suggestive findings. The *P* values of the differences may be overestimates.

Forty-seven subjects met inclusion criteria for HZ cases; 141 matched controls were identified (Table 1). HZ cases had significantly lower CD4⁺ cell percentages and higher HIV loads but no other differences.

Nine of 47 cases and 33 of 141 controls had sufficient PBMC to perform the stimulation assays (Table 2). Compared with controls, cases had higher or similar but not lower expression of Th1

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TABLE 1 Demographic and HIV disease characteristics when PBMC were obtained

Characteristic	Value for group		<i>P</i> value ^a
	Cases	Controls	
No. of subjects	47	141	
Age (yr)			
Mean (SD)	11.0 (3.8)	10.9 (3.9)	0.91‡
Range	5-21	4-22	
No. (%) male	25 (53)	68 (48)	0.61*
No. (%) with race/ethnicity			
White	5 (11)	21 (15)	0.35*
Black	24 (51)	84 (60)	
Hispanic	16 (34)	33 (23)	
Mean CD4 % (SD)	21.6 (11.4)	28.7 (11.7)	0.0003‡
Mean CD8 % (SD)	54.9 (16.0)	51.0 (16.7)	0.13‡
Mean log ₁₀ HIV RNA copies/ml (SD)	3.97 (0.96)	3.26 (1.21)	0.002‡
Mean age (yr) at varicella (SD) ^c	6.0 (3.0)	5.1 (3.2)	0.08‡
Mean no. of mos from varicella (SD) ^d	59.9 (36.9)	69.2 (40.0)	0.19‡
No. of mos prior to HZ			
Mean (SD)	4.4 (4.6)	NA ^b	
Range	0-18		
No. (%) on HAART	32 (68)	94 (67)	0.99‡
Mean no. of mos of HAART (SD)	28.3 (26.5)	32.6 (25.5)	0.33‡

^a *, chi-square statistic; ‡, Wilcoxon test. Boldfacing indicates statistical significance.^b NA, not applicable.^c Age when subject had varicella.^d Time elapsed from varicella to blood draw.

markers by CD4⁺ T cells. Specifically, CD4⁺ MIP-1 β ⁺ and CD4⁺ IL-2⁺ cell percentages were higher under VZV-stimulated and unstimulated conditions. No significant differences were observed in CD4⁺ TNF- α ⁺ or CD4⁺ IFN- γ ⁺ percentages or in any double-positive populations under VZV-stimulated or unstimulated conditions. In contrast, VZV-stimulated CD8⁺ CD107a⁺ cell percentages were significantly lower for cases than for controls (*P* = 0.02). No significant differences were observed in unstimulated CD8⁺ CD107a⁺ cell percentages (*P* = 0.42) or other CD8⁺ subpopulations.

In a multivariable analysis that included VZV-specific CD8⁺ CD107⁺ cell percentages, CD4⁺ percentages, and plasma HIV RNA copies/ml, only a low CD8⁺ CD107⁺ cell percentage remained significantly associated with HZ, indicating its strong predictive value for protection against HZ in HIV-infected children and adolescents.

Treg were measured in PBMC of 33 cases and 100 controls (Table 3). Compared with controls, HZ cases had significantly higher CD4⁺ TGF- β ⁺, CD4⁺ IL-10⁺, and CD4⁺ PD1⁺ cell percentages. The total CD4⁺ FoxP3⁺ percentages and the CD4⁺ FoxP3⁺ CD25⁺ Treg percentages tended to be higher in HZ cases. No significant differences were observed for CD8⁺ Treg.

In a multivariable analysis controlling for CD4⁺ cell percentages, higher CD4⁺ TGF- β ⁺ and CD4⁺ PD1⁺ percentages remained significantly associated with the occurrence of HZ (*P* = 0.03), but CD4⁺ IL-10⁺ percentages did not. When plasma HIV RNA levels were controlled for, however, neither the Treg sub-

TABLE 2 VZV-specific and nonspecific Teff in HIV-infected children and adolescents with HZ and their matched controls^a

Variable	No. of cases	No. of controls	Median % of cells (case/control)	Wilcoxon test value ^b
CD4 ⁺				
CD4 ⁺ TNF- α ⁺ (medium)	9	33	2.8/2.6	0.81
CD4 ⁺ TNF- α (VZV)	9	33	4.6/2.8	0.45
CD4 ⁺ MIP-1 β ⁺ (medium)	9	33	14.5/3.6	0.04*
CD4 ⁺ MIP-1 β ⁺ (VZV)	9	33	19.9/3.6	0.02*
CD4 ⁺ IL-2 ⁺ (medium)	9	33	12.5/2.5	0.05*
CD4 ⁺ IL-2 ⁺ (VZV)	9	33	13.4/3.4	0.05*
CD4 ⁺ CD107a ⁺ (medium)	9	32	4.0/4.1	0.94
CD4 ⁺ CD107a ⁺ (VZV)	9	32	3.4/7.4	0.41
CD4 ⁺ IFN- γ ⁺ (medium)	9	32	1.06/0.87	0.28
CD4 ⁺ IFN- γ ⁺ (VZV)	9	32	2.24/1.22	0.61
CD8 ⁺				
CD8 ⁺ CD107a ⁺ (medium)	9	31	3.3/6.5	0.42
CD8⁺ CD107a⁺ (VZV)	9	32	3.1/9.6	0.02
CD8 ⁺ TNF- α (medium)	12	27	1.34/1.12	0.36
CD8 ⁺ TNF- α (VZV)	12	28	1.25/1.19	0.46
CD8 ⁺ MIP-1 β ⁺ (medium)	12	27	1.40/1.42	0.84
CD8 ⁺ MIP-1 β ⁺ (VZV)	12	28	1.66/1.73	0.26
CD8 ⁺ IL-2 ⁺ (medium)	12	39	2.06/1.22	0.26
CD8 ⁺ IL-2 ⁺ (VZV)	12	39	1.51/1.24	0.50
CD8 ⁺ IFN- γ ⁺ (medium)	9	32	0.70/0.94	0.67
CD8 ⁺ IFN- γ ⁺ (VZV)	9	32	1.08/1.23	0.71

^a Boldfacing indicates significant differences only in VZV-stimulated Teff.^b *, Significant or marginally significant differences in both VZV-stimulated and unstimulated Teff.

populations nor the CD4⁺ cell percentages maintained significance.

To understand the factors that affect the frequency of Treg in HIV-infected children and adolescents, we performed correlation analyses of the CD4⁺ Treg subpopulations with CD4⁺ percentages and plasma HIV RNA concentrations (Table 4). Higher CD4⁺ FoxP3⁺ and CD4⁺ TGF- β ⁺ percentages were weakly associated with lower CD4⁺ percentages and higher plasma HIV RNA levels. In contrast, higher CD4⁺ IL-10⁺ and CD4⁺ PD1⁺ percentages were associated with higher CD4⁺ percentages and lower plasma HIV RNA levels. CD4⁺ CD25⁺ FoxP3⁺ percentages were

TABLE 3 Nonspecific Treg in HIV-infected children and adolescents with HZ and their matched controls

Variable	No. of cases	No. of controls	Median % of cells (case/control)	GEE analysis ^a
CD4 ⁺ CD25 ⁺ FoxP3 ⁺	39	100	1.4/1.1	0.08
CD8 ⁺ CD25 ⁺ FoxP3 ⁺	37	88	0.18/0.12	0.16
CD4 ⁺ FoxP3 ⁺	39	100	4.6/2.9	0.08
CD8 ⁺ FoxP3 ⁺	37	88	1.39/0.83	0.35
CD4 ⁺ TGF- β ⁺	39	100	2.8/1.6	0.003
CD8 ⁺ TGF- β ⁺	37	88	1.29/0.74	0.50
CD4 ⁺ IL-10 ⁺	38	96	2.2/1.7	0.04
CD8 ⁺ IL-10 ⁺	38	96	2.2/1.8	0.86
CD4 ⁺ PD1 ⁺	38	96	5.4/3.4	0.007
CD8 ⁺ PD1 ⁺	38	96	1.6/1.8	0.77

^a Boldfacing indicates significant Treg differences; italics indicate marginally significant Treg differences.

TABLE 4 Correlations between Treg and HIV disease characteristics^a

Variable 1	Variable 2	No. of children tested	Spearman coefficient	P value
% CD4 ⁺ cells	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	158	-0.08	0.34
	CD4 ⁺ FoxP3 ⁺	158	-0.16	0.048
	CD4 ⁺ TGF- β ⁺	158	-0.25	0.001
	CD4 ⁺ IL-10 ⁺	155	0.40	0.0001
	CD4 ⁺ PD1 ⁺	155	0.37	0.0001
No. of HIV RNA copies/ml	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	127	-0.03	0.72
	CD4 ⁺ FoxP3 ⁺	127	0.18	0.038
	CD4 ⁺ TGF- β ⁺	127	0.32	0.0003
	CD4 ⁺ IL-10 ⁺	124	-0.24	0.006
	CD4 ⁺ PD1 ⁺	124	-0.28	0.001

^a Boldfacing indicates statistical significance.

not significantly associated with CD4⁺ percentages or plasma HIV RNA levels.

Among the effector T cell populations, only the CD8⁺ CD107a⁺ percentages were significantly lower prior to HZ cases than for non-HZ controls, suggesting that these cytotoxic T lymphocytes (CTL) are important in protecting against HZ. It is likely that when VZV reactivates, CTL eliminate VZV-infected cells and thereby limit viral replication and prevent disease. Studies in healthy individuals demonstrated a robust VZV-specific CD8⁺ CTL response to VZV infections (17, 36), although the protective effect of CD8⁺ CD107a⁺ effectors against VZV disease or asymptomatic reactivation had not previously been established. The strength of our results is tempered by the limited number of samples tested. However, our finding has strong biologic plausibility, and the association between high levels of CTL and a low risk of HZ was independent of CD4⁺ percentages and HIV loads, which frequently dominate this type of correlation analysis. The HZ vaccine stimulates VZV-specific CD8⁺ cells in immunocompetent vaccinees (31), and it will be important to determine if it also boosts VZV-specific CTL in HIV-infected individuals.

High frequencies of VZV-specific CD4⁺ memory T cells have been associated with protection against HZ in elderly individuals and transplant recipients, groups that are at as high a risk of developing HZ as the HIV-infected individuals (16, 28, 46, 47). In our study, HZ cases had lower CD4⁺ percentages than non-HZ controls, which is in agreement with previous reports (40). However, using traditional Treg markers, we did not find significant associations between protection against HZ and the proportions of CD4⁺ Treg. This may have been an artifact of the small number of samples tested but may also indicate that VZV-specific CD4⁺ Treg are not fully functional in HIV-infected individuals or that they may be too few for an effective anti-VZV response.

It is noteworthy that IL-2 and MIP-1 β expression was more common in CD4⁺ T cells of HZ cases, but this was not VZV specific, probably reflecting higher level of T cell activation in HZ cases. HZ cases had more HIV replication than matched controls, providing a potential explanation of the difference in T-cell activation. It is unclear if this higher level of nonspecific T-cell activation contributes to VZV reactivation and/or to the viral expansion necessary for the genesis of symptomatic disease.

We found that higher CD4⁺ IL-10⁺ percentages, CD4⁺ TGF- β ⁺ percentages, and CD4⁺ PD1⁺ percentages were associ-

ated with an increased risk of HZ. These data are the first demonstration of an association between Treg frequencies and development of HZ. Higher frequencies of Treg may increase the risk of HZ by several mechanisms: (i) cytokines and other mediators secreted by Treg may stimulate VZV reactivation, and (ii) Treg inhibition of Teff could allow reactivated VZV to spread in the dorsal root ganglion, travel to the skin and/or invade the bloodstream (i.e., become clinically apparent), and/or promote VZV infection of skin.

The multivariable and correlation analyses of Treg, CD4⁺ percentages, and plasma HIV load showed that the relationship of all Treg subpopulations with development of HZ was associated with HIV replication, while the relationship of CD4⁺ TGF- β ⁺ percentages and CD4⁺ PD1⁺ percentages with HZ was independent of CD4⁺ percentages. Moreover, higher CD4⁺ TGF- β ⁺ percentages were found in subjects with lower CD4⁺ percentages and higher plasma HIV RNA. Taken together, this suggests that CD4⁺ TGF- β ⁺ Treg increase in response to HIV replication and/or promote HIV replication and consequent CD4⁺ T-cell depletion while simultaneously promoting VZV reactivation and/or replication. This suggests that the CD4⁺ TGF- β ⁺ Treg have nonspecific regulatory activity.

The dynamics of the CD4⁺ PD1⁺ cells is more complex, since higher CD4⁺ PD1⁺ percentages were observed in subjects with higher CD4⁺ percentages and lower HIV loads. PD1 expression is found in regulatory, activated, or exhausted T cells. The regulatory activity of CD4⁺ PD1⁺ cells was originally described for mice (33, 37), where it was proposed that unlike the surface expression by conventional CD4⁺ PD1⁺ cells, CD4⁺ PD1⁺ Treg maintained PD1 in the cytoplasm (32). We measured intracellular PD1. A recent publication proposed that human CD4⁺ PD1⁺ cells represent memory cells (9). Although our findings that CD4⁺ PD1⁺ percentages increase with higher CD4⁺ percentages and decrease with higher HIV replication differ from findings for other Treg studied here, this pattern is still consistent with the behavior described in some studies for Treg subpopulations of HIV-infected individuals (18, 21). Furthermore, it is unclear how higher frequencies of memory T cells would predispose to a higher incidence of HZ. Hence, it is most likely that the CD4⁺ PD1⁺ cells detected in excess in HZ cases represent Treg.

In conclusion, in addition to the protective role of VZV-specific CD8⁺ CTL against HZ, we demonstrated in HIV-infected children and adolescents that higher frequencies of Treg and higher T cell activation are associated with an increased risk of HZ. Further studies are needed to identify the mechanisms by which these T cell subpopulations contribute to the development of HZ and if they are also associated with a higher incidence of HZ in other high-risk individuals.

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