11

Natural Killer Cells from HIV Infected Slow Progressors Who Carry the Protective HLA-B*27 Allele and Inhibitory KIR3DL1 Receptors Have Elevated Poly-Functional Potential Compared to Bw6 Homozygotes

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1. Introduction

NK cells are a key component of the innate immune system, which can act early in defences against virally infected and tumor cells (Robertson and Ritz 1990; Bottino, Moretta, and Moretta 2006; Bancroft 1993). They have the capacity to secrete proinflammatory cytokines and lyse their targets without prior antigen sensitization (Cooper, Fehniger, and Caligiuri 2001). They are also involved in regulation of the adaptive immune response through their interaction with dendritic cells (DCs)(Sanabria et al. 2008; Smyth et al. 2005).

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Activation of NK cells is regulated through the integration of signals from a number of activating and inhibitory receptors(Lanier 2005). Many of the inhibitory receptors use major histocompatibility complex (MHC) class I or class I-like proteins as their ligands(Lanier 2005). The interaction between inhibitory NK receptors and their ligands during NK cell development is important in educating these cells for subsequent function and for avoiding reactivity to normal cells expressing self MHC class I (Kim et al. 1969; Anfossi et al. 2006). In humans one large family of NK receptors are encoded by the Killer Immunoglobulin-like Receptors (KIR) region that maps to chromosome 19q13.4 (Lanier 2005). The most polymorphic locus among the KIR region genes is *KIR3DL1*, which encodes both inhibitory KIR3DL1 (3DL1) and activating KIR3DS1 (3DS1) alleles (Norman et al. 2007). *3DL1* alleles can be further classified according to their expression levels on the cell surface into high (**h*), low/intermediate (**l*) and null (**004*) (not cell surface expressed) alleles (Yawata et al. 2006; Norman et al. 2007; Gardiner et al. 2001; Pando et al. 2003). Genotypes homozygous for *3DL1* can be divided into 2 groups: **h/*y*, where **y* can be either another **h* allele or **004* with no **l* alleles, and **l/*x*, where **x* can be an **l*, **x* or **004* allele (Martin et al. 2007). 3DL1 receptors recognize a subset of MHC class I HLA-B molecules known as Bw4. HLA-Bw4 differ from the remaining HLA-Bw6 antigens encoded at this locus, which do not interact with 3DL1, in the amino acids present between positions 77 and 83 of the HLA heavy chain (Wan et al. 1986). Bw4 allotypes with isoleucine at position 80 (Bw4*80I) have been reported to be better ligands for many of the 3DL1 alleles (Cella et al. 1994). However, there is evidence that Bw4 antigens with threonine at position 80 (Bw4*80T), particularly HLA-B*2705, interact strongly with certain 3DL1 receptors (Luque et al. 1996).

Epidemiological studies have reported that several *KIR/HLA* combinations are associated with slower progression to AIDS and suppression of viral load (VL) (Martin et al. 2007). Compared to *Bw6* homozygotes (hmz) the *3DL1/HLA-B* combination having the most potent influence on slowing time to AIDS and VL control is *3DL1*h/*y* with *HLA-B*57* (**h/*y+B*57*)(Martin et al. 2007). Previous work from our group showed that NK cells from individuals carrying this genotype combination demonstrated higher functional potential than those from carriers of either the NK receptor genotype or *HLA-B*57* alone or from *Bw6 hmz* (Boulet et al. 2010). In these studies functional potential was defined as the percent contribution of NK cells secreting interferon-γ (IFNγ) and tumor necrosis factor-α (TNF-α) and expressing CD107a, a marker of degranulation to the total response to stimulation with the HLA devoid K562 cell line. Furthermore, NK cells from carriers of **h/*y+B*57* had higher functional potential than carriers of *3DL1*h/*y* with other *Bw4* alleles(Boulet et al. 2010). HLA-B*57 is an HLA antigen considered to be protective in the context of HIV infection(Kaslow, Dorak, and Tang 2005; Altfeld et al. 2003; Leslie et al. 2004; Carrington, Martin, and van Bergen 2008). While the protective effect conferred by HLA-B*57 is mediated at least in part through $CD8^+$ T cell recognition of HIV epitopes restricted by this antigen, epidemiological studies and our results support the possibility that HLA-B*57's protective effect may also be mediated through its ability to educate NK cells for superior functional potential (Martin et al. 2002; Martin et al. 2007; Leslie et al. 2004; Miura et al. 2009; Leslie et al. 2005).

Murine models have shown using single MHC class I transgenic mice that MHC class I molecules can differ from each other in the potency of their NK education signals, which directly translates into activation potency upon encountering cells lacking that MHC ligand (Brodin, Karre, and Hoglund 2009). Since NK cells from **h/*y+B*57* carriers had higher functional potential than carriers of *3DL1*h/*y* with other *Bw4* alleles, HLA-B*57 may be an

example in humans of an MHC class I antigen with an NK education potency that is superior to that of most other HLA-Bw4 molecules. HLA-B*27 is another allele considered to be protective in the context of HIV infection (Kaslow et al. 1996; Carrington and Bontrop 2002; Goulder, Edwards et al. 1997; Trachtenberg et al. 2003). Like HLA-B*57, its protective effect is mediated at least in part through immune pressure exerted by $CD8^+$ T cells (Schneidewind et al. 2009; Goulder, Phillips et al. 1997; den Uyl, van der Horst-Bruinsma, and van Agtmael 2004; Goulder et al. 2001). In this report we questioned whether HLA-B*27, like HLA-B*57, could also act as a ligand for 3DL1 NK receptors that was superior to other HLA-Bw4 alleles in terms of its ability to educate NK cells for functional potential. *HLA-B*27* is found at a higher frequency among the approximately 5% of HIV infected individuals that we have classified as Slow Progressors (SP) compared with HIV infected individuals exhibiting a typical rate of disease progression or uninfected subjects (Carrington, Martin, and van Bergen 2008; Kaslow, Dorak, and Tang 2005). SP are defined by either exhibiting spontaneous control of viremia or maintaining CD4 counts >400 cells/mm3 for at least 7 years post-infection (Madec et al. 2005; Deeks and Walker 2007). In order to determine whether HLA-B*27, like HLA-B*57, could educate NK cells from *3DL1* hmz individuals for superior functional potential we compared the functional potential of NK cells from *3DL1 hmz+B*27* (*3DL1+B*27*) carriers with that from *3DL1 hmz* who were *Bw6 hmz* (*3DL1+Bw6*) or who expressed at least 1 *Bw4* alleles other than *B*57* or *B*27* (*3DL1+Bw4*). NK cells from *3DL1+B*27* carriers had a significantly higher functional potential than those from *3DL1+Bw6.* When the functional potential of NK cells from carriers of the 3DL1 NK receptor /HLA-B ligand pairs **h/*y+B*57*, *3DL1+B*27* and *3DL1+Bw4* were compared we observed decreasing levels in the functional potential where NK cells responded to missing self with a *h/*y+B*57* > *3DL1+B*27* > *3DL1+Bw4* hierarchy.

2. Materials and methods

2.1 Study population

A total of 51 HIV-infected SP were studied. Forty four were from the Canadian Cohort of HIV Infected Slow Progressors and 7 were from a cohort followed at the National Institutes of Allergy and Infectious Diseases (NIAID) (Migueles et al. 2008). The term SP was used here to define treatment naïve HIV infected subjects who maintained absolute CD4 counts above 400 cells/mm^3 for more than 7 years or who were followed for at least 1 year with VL <3000 copies/ml of plasma. Information on CD4, CD8 T cell counts, VL and duration of infection at time of testing, *3DL1* genotype and *HLA-B* allotype of the study population is provided in Table 1. All individuals in the study populations are *3DL1 hmz* to eliminate the possible confounding effect on NK function of expressing the activating 3DS1 receptor, which is an allele at this locus (Martin et al. 2007; Boulet et al. 2008; Yawata et al. 2006). The study population is classified into 4 groups: group 1 (n=12) carry the *3DL1+B*27* genotype, group 2 (n=13) are *3DL1+Bw4*, group 3 (n=14) are *3DL1+Bw6* and group 4 (n=12) are **h/*y+B*57*. Informed consent was obtained from all study subjects and research conformed to the ethical guidelines of the authors' institutions.

2.2 MHC and KIR typing

All subjects were typed for MHC class I alleles by sequence-based typing using kits from Atria Genetics (South San Francisco, CA) and Assign software to interpret sequence information for allele typing (Conexio Genetics, Perth, Australia) as previously described

(Boulet et al. 2010). *HLA-Bw6 hmz* subjects lacked any *HLA-Bw4* alleles at the *HLA-A* or *B* locus. *3DL1/S1* genotyping was performed using two sets of primers specific for the *3DL1* and *3DS1* alleles at the 3DL1 locus as previously described (Boulet et al. 2008). Subjects were subsequently *3DL1* allotyped by identifying single nucleotide polymorphisms (SNP) corresponding to high frequency *3DL1* alleles as previously described (Boulet et al. 2010). In our study we categorized *3DL1*005, *006, *007, *053, *054* as **l* alleles, *3DL1*001, *002, *008, *009, *015, *020* as **h* alleles and **004* as a null allele.

Subjects ID	gender[1]	Age[2]	Time infected[2]	Group[3]	*HLA-B1*	*HLA-B2*	*KIR* genotype[4]	CD4[5]	CD8[5]	VL[6]
1001	M	59	2.9	1	B15:01	B27:05	*h/*y	760	900	1.7
1002	M	59	14.6	1	B15:01	B27:05	*h/*y	788	2498	4.12
1003	M	61	23.5	1	B27:02	B67:01	*h/*y	670	1050	4.7
1004	M	49	20.2	1a	B27:05	B57:01	*h/*y	830	1700	3.91
1005	M	37	2.5	1	B27:05	B40:02	*h/*y	1040	1090	1.7
1006	M	40	3.7	1	B14:02	B27:05	*h/*y	820	570	1.7
1007	M	71	5.2	1	B27:03	B51:01	*l/*x	590	114	1.7
1008	F	31	1.2	1a	B27:05	B57:01	*l/*x	489	672	1.7
1009	F	59	13.2	1	B27:03	B49:01	*l/*x	692	627	1.7
1010	M	38	2.3	1	B15:01	B27:05	*l/*x	396	936	1.7
1011	M	63	25.0	1	B27:05	B51:01	*h/*y	340	748	1.7
1012	M	41	6.4	1	B07:02	B27:05	*l/*x	710	970	3.25
2001	M	35	6.5	2	B15:02	B51:02	*l/*x	500	NA	3.24
2002	F	39	15.9	2	B49:01	B58:02	*h/*y	400	510	3.54
2003	M	15	8.0	2	B38:01	B51:01	*h/*y	596	1614	2.08
2004	M	45	16.2	2	B07:02	B51:01	*h/*y	440	350	3.54
2005	F	46	14.7	2	B07:02	B38:01	*l/*x	720	720	1.7
2006	M	47	17.0	2	B39:01	B35:01	*h/*y	546	572	5.21
2007	M	46	1	2	B53:01	B58:01	*l/*x	870	550	1.7
2008	M	49	7.3	2	B44:03	B53:01	*l/*x	360	1610	2.8
2009	F	40	2.8	2	B07:02	B13:01	*h/*y	1487	712	3.76
2010	F	50	6.9	2	B15:03	B44:02	*h/*y	590	1650	3.73
2011	F	44	22.2	2	B44:02	B52:02	*h/*y	420	1180	3.15
2012	F	32	5.1	2	B14:02	B44:03	*l/*x	715	384	1.7
2013	F	36	6.3	2	B49:01	B53:01	*h/*y	535	793	2.43
3001	M	34	3.0	3	B07:02	B14:02	*l/*x	680	890	2.85
3002	M	40	14.4	3	B14:02	B15:01	*h/*y	343	804	1.7
3003	M	40	4.7	3	B14:02	B14:02	*h/*y	350	730	3.41
3004	M	40	3.4	3	B07:02	B07:02	*h/*y	985	605	3.3
3005	M	46	5.6	3	B15:10	B41:01	*l/*x	530	390	1.7
3006	F	42	4.0	3	B07:05	B35:01	*l/*x	919	1557	1.7
3007	F	57	3.1	3	B07:02	B42:01	*l/*x	636	663	1.7

Subjects ID	gender[1]	Age[2]	Time infected[2]	Group[3]	*HLA-B1*	*HLA-B2*	*KIR* genotype[4]	CD4[5]	CD8[5]	VL[6]
3008	M	53	4.1	3	B07:02	B14:02	*h/*y	447	455	3.3
3009	M	29	4.2	3	B07:02	B14:02	3DL1 hmz	516	788	2.89
3010	F	61	14.3	3	B07:02	B18:01	*l/*x	810	1050	1.7
3011	F	39	6.6	3	B42:01	B45:01	*h/*y	620	408	2.69
3012	M	39	9.9	3	B14:02	B40:06	*h/*y	720	1820	2.59
3013	M	46	7.0	3	B07:02	B07:05	*h/*y	970	1210	1.94
3014	M	62	17.5	3	B14:01	B81:01	*h/*y	650	2110	4.46
4001	F	39	3.0	4	B57	B7	*h/*y	1443	895	1.7
4002	F	55	23.8	4	B57	B57	*h/*y	277	385	1.7
4003	M	58	20.5	4	B57	B15	*h/*y	883	590	1.7
4004	M	41	11.2	4	B40:01	B57:01	*h/*y	1200	860	1.7
4005	M	61	11.0	4	B40:02	B57:01	*h/*y	770	990	3.9
4006	M	45	14.9	4	B07:02	B57:01	*h/*y	650	1460	4.21
4007	F	39	2.6	4	B35:01	B57:01	*h/*y	530	NA	1.7
4008	M	24	2.0	4	B07:02	B57:01	*h/*y	680	880	3.1
4009	M	49	10.0	4	B57	B13	*h/*y	955	881	1.7
4010	M	58	18.0	4	B57	B44	*h/*y	1329	1243	1.7
4011	M	46	21.0	4	B57	B81	*h/*y	1362	1055	1.7
4012	M	36	7.0	4	B57	B52	*h/*y	780	739	1.7

Table 1. Study population characteristics. [1] M=male/ F=female, [2] in years, [3] 1= 3DL1+B*27; 2=3DL1+Bw4; 3=3DL1+Bw4; 4=*h/*y+B*57. The 2 subjects with the 1a designation carry both a B*27 and B*57 allele, [4] the individual classified as 3DL1 hmz has not been allotyped for KIR3DL1 alleles, [5] cells/mm^3, [6] VL= $\log_{10}$ viral load copies/ml plasma.

2.3 Cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Paque Pharmacia Upsala, Sweden) from whole blood obtained by venipuncture into tubes containing EDTA anti-coagulant or by leukaphersis as previously described (Boulassel et al. 2003). Cells were cryopreserved in 10% DMSO (Sigma-Aldrich, St. Louis, MO) with 90% fetal bovine serum (FBS, Wisent, St. Bruno, Quebec, Canada).

2.4 NK cell functional potential

Cryopreserved PBMC were thawed and resuspended at 10^6 cells/ml in RPMI 1640 (Wisent) that contained 10% FBS (Wisent), 2mM L-glutamine and 50 IU penicillin and 50µg/ml streptomycin (Wisent). Brefeldin A (at 5µg/ml, Sigma-Aldrich), Monensin (at 6µg/ml, Golgi-Stop; BD Biosciences, Mississauga, Ontario, Canada) and anti-CD107a–FITC mAb (BD Biosciences) were added to the cells. One million PBMCs were stimulated with media alone or HLA devoid K562 cells (American Type Culture Collection Manassas, VA) at a PBMC to K562 cell ratio of 5:1 or with 1.25µg/ml phorbol 12-myristate 13-acetate (PMA); 0.25µg/ml ionomycin, (Sigma-Aldrich) as a positive control for 6 hours at 37°C in a humidified 5% CO_2

incubator. All stimulation data shown is from cells that generated a positive result in the PMA and ionomycin stimulation condition.

Cells were stained for viability using the Aqua LIVE/DEAD® fixable dead cell stain kit (Invitrogen, Burlington, Ontario, Canada) following manufacturer's instructions. Cells were then stained for cell surface markers with anti-CD56-APC, anti-CD16-Pacific Blue (BD Biosciences), anti-CD3-ECD and CD158e-PE (ie: Z27-PE, Beckman Coulter, Mississauga, Ontario, Canada) for 30 min. After washing with phosphate buffered saline (PBS) containing 1% FBS (Wisent), cells were fixed and permeabilized using the Fix and Perm Kit (Invitrogen) and stained for intracellular cytokines using anti-IFN-γ-Alexa 700 and anti-TNF-α-PE-Cy7 (BD Biosciences). Cells were washed and fixed with 1% paraformaldehyde solution (Fisher Scientific, Ottawa Ontario, Canada) and kept in the dark at 4°C until acquisition.

2.5 Flow cytometry analysis

Between 400,000 and 500,000 events were acquired per sample using an LSRII flow cytometer (BD Biosciences). Analysis for NK cell activation was performed using FlowJo software version 9.1 (Tree Star, San Carlos, CA). The functional profiles of stimulated NK cells were determined using a gating strategy where NK cells were defined as $CD3^{-}CD56^{+/-}CD16^{+/-}$. Boolean gating was used to identify seven NK cell functional profiles, i.e. tri-functional NK cells ($CD107a^{+}$ IFN-γ^{+} TNF-α^{+}), bi-functional NK cells (any combination of two of these functions) and mono-functional NK cells (any single one of these functions). All results for the frequency of individual functional subsets were background corrected by subtracting the frequency of positive NK cells in the unstimulated subset. Corrected results were used to generate the percent contribution of each functional subset to the total NK cells response to K562. Results reported as subset frequency or percent contribution of a subset to the total K562 response showed a high level of correlation with each other (Boulet et al. 2010).

2.6 Statistical analysis

GraphPad Instat 3.05 and GraphPad Prism 5.04 were used for statistical analyses and graphical presentations. A Kruskal-Wallis test was used to assess the significance of between group differences in age, duration of infection at the time point tested, CD4 counts, CD8 counts and VL. Mann-Whitney *U* tests were used to test the significance of between group differences in the percent contribution of an NK cell functional subset to the total NK cell response. A Spearman correlation test was used to test the significance of the trend towards declining tri-functional potential with 3DL1/HLA-B genotype combinations. A p value of <0.05 was considered significant.

3. Results

3.1 Study population

Table 2 provides information on the average and standard deviation for age, duration of infection, CD4 count, CD8 count and $\log_{10}$VL at the time point tested for NK functional potential for SP classified as *3DL1+B*27*, *3DL1+Bw4*, *3DL1+Bw6* and **h/*y+B*57* described in Table 1. No between-group differences were seen for any of these parameters (Krukal-Wallis test).

	*3DL1+B*27* (n=12)	*3DL1+Bw4* (n=13)	*3DL1+Bw6* (n=14)	**h/*y+B*57* (n=12)
Age[1]	50.7 ± 12.8[3]	40.1 ± 9.5	44.9 ± 10.0	45.9 ± 11.0
Duration of infection[1]	10.1 ± 8.9	10.0 ± 6.5	7.2 ± 4.8	12.1 ± 7.6
CD4[2]	677 ± 198	619 ± 306	655 ± 211	904 ± 363
CD8[2]	989 ± 606	887 ± 494	963 ± 533	907 ± 292
Log_{10} VL	2.53 ± 1.2	2.95 ± 1.0	2.57 ± 0.86	2.21 ± 0.95

Table 2. Summary of study population characteristics. [1] In years, [2] Cells/mm[3], [3] Means ± standard deviation.

3.2 NK cells from *3DL1+B*27* carriers have a higher functional potential than those from *3DL1+Bw6*

To investigate whether NK cells from HIV-infected SPs carrying the *3DL1+B*27* genotype for an NK receptor/HLA-B ligand pair differ in their NK cell functional potential compared to *Bw6 hmz* with no ligand for 3DL1 receptors, we measured the frequency of NK cells expressing CD107a and secreting IFN-γ and TNF-α from K562-stimulated NK cells using eight colour multi-parametric flow cytometry. In this analysis CD107a expressing NK cells were the sum of the mono-, bi- and tri-functional functional subsets expressing CD107a. This was also the case for IFN-γ and TNF-α secreting NK cells. As shown in Figure 1A the median (range) frequency of NK cells from *3DL1+B*27* versus *3DL1+Bw6* carriers expressing CD107a was 6.92% (3.01%, 17.15%) versus 4.27% (0.74%, 13.32%), secreting IFN-γ was 8.91% (4.25%, 21.52%) versus 5.17% (0.55%, 25.92%) and secreting TNF-α was 0.92% (0.11%, 3.67%) versus 0.23% (0.01%, 3.06) ($p<0.05$ for all comparisons; Mann-Whitney test). Therefore, the frequency of NK cells with any of these functions was significantly greater when NK cells were from *3DL1+B*27* individuals than from *3DL1+Bw6*.

Since we simultaneously measured these three functions following K562 stimulation we were able to assess the frequency of seven possible NK cell functional profiles and their percent contribution to the total K562 response. Figure 1B shows the percent contribution of each NK cell functional profile in PBMC from 12 *3DL1+B*27* and 14 *3DL1+Bw6* subjects. Of the seven possible NK cell functional profiles, only the percent contribution of tri-functional NK cells to the total K562 response was significantly higher in the *3DL1+B*27* group versus *3DL1+Bw6* SPs (2.99% [0.00%, 6.43%]) and (0.52% [0.00%, 7.36%]) for 3DL1+*B*27* and *3DL1+Bw6*, respectively, p=0.019; Mann-Whitney *U* test). Based on these results we concentrated on the tri-functional NK subset in subsequent analyses.

We next compared the K562 stimulated NK cells from *3DL1+B*27* individuals to that from carriers of *3DL1+Bw4*. NK cells from **h/*y+B*57* carriers have previously been shown to have superior tri-functional potential compared to *3DL1+Bw6* and *h/*y+*Bw4* subjects (Boulet et al. 2010; Kamya et al. 2011). Since *HLA-B*57* is a *Bw4* allele we excluded carriers of either *HLA-B*57* or *HLA-B*27* alleles from the *3DL1+Bw4* group. Although the tri-functional potential of NK cells from 13 *3DL1+Bw4* subjects was lower (1.78% [0.00%, 5.71%]) than that from *3DL1+B*27* individuals the difference did not achieve statistical significance (p=0.2644; Mann-Whitney *U* test) (**Figure 2A**).

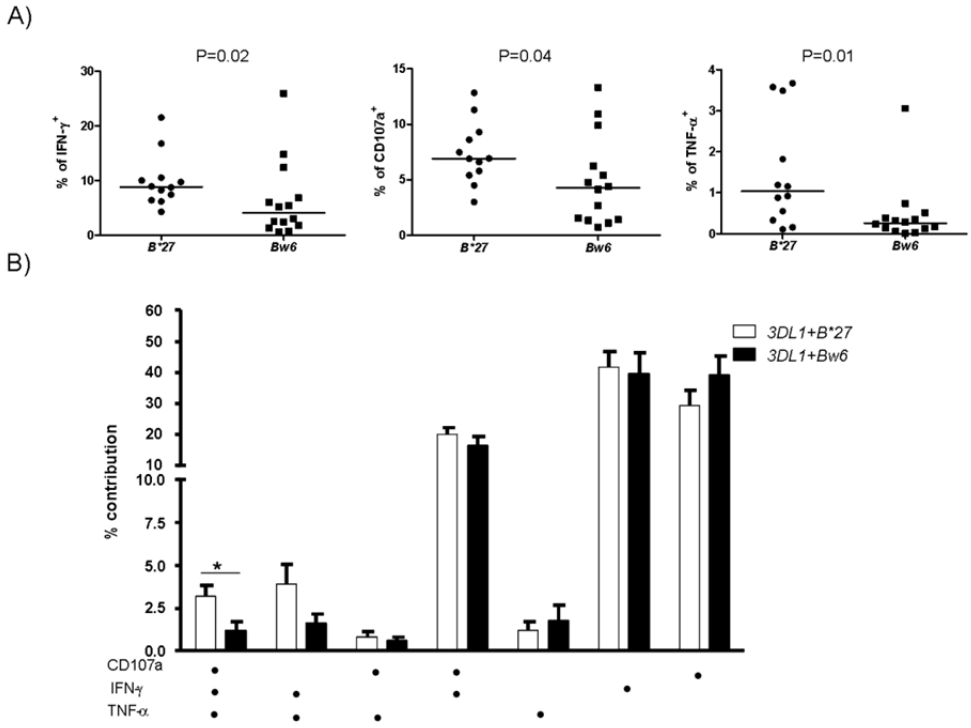

Fig. 1. NK cells from individuals carrying the 3DL1+HLA B*27 NK receptor-ligand pair have an increased functional potential. The frequency of NK cells that secrete IFN-γ (left), express CD107a (middle) or secrete TNF-α (right) in 3DL1 *hmz* individuals who carry *HLA-B*27* or *HLA-Bw6*. Each data point represents a separate individual. The bar through each scatter plot indicates the median frequency for the group. A Mann-Whitney U test was used to assess the significance of between-group comparisons (A). The percent contribution of seven different functional profiles to the total NK cell response to K562 *HLA*-devoid cells from individuals carrying *3DL1+B*27* (n=12) and *3DL1+Bw6* (n=14). Below the *x*-axis, dots refer to the presence of each functional marker (CD107a, IFN-γ and TNF-α) in that profile. The height of each bar represents the median for the group and the height of the error bar the interquartile range for that group. An asterisk (*) over the line linking two bars indicates that the contribution of that functional subset of the NK cell response was significantly different in the two study populations. A Mann-Whitney U test was used to assess the significance of between-group comparisons and $p<0.05$ was considered significant (B).

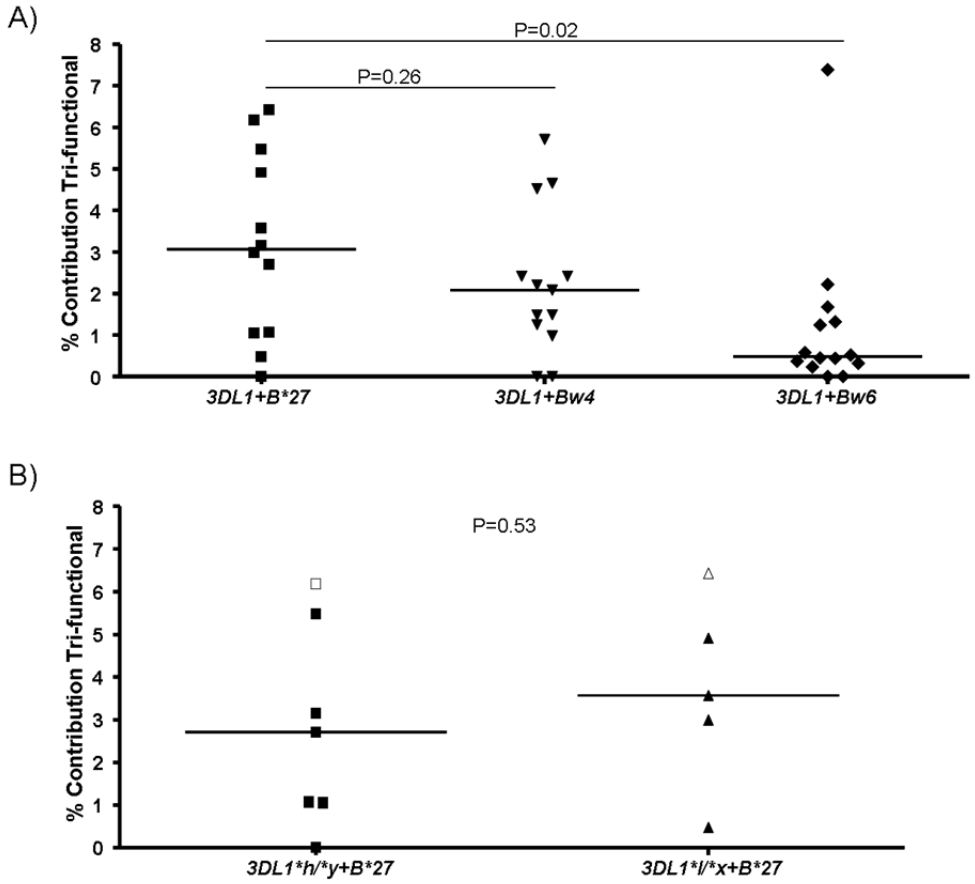

Fig. 2. Comparisons of the tri-functional potential of NK cells from study subjects with defined *KIR/HLA* combinations. Scatter plots show percent contribution of tri-functional NK cells to the total K562 stimulated response in individuals who are *3DL1+B*27, 3DL1+Bw4* and *3DL1+Bw6* carriers (A), and **h/*y+B*27* and **l/*x+B*27* carriers (B). Each data point represents results from a separate individual. The data point represented by an empty square and triangle refer to individuals that carry both *B*27* and *B*57*. The bar through each scatter plot indicates the median frequency for the group. A Mann-Whitney U test was used to assess the significance of between-group differences.

3DL1 hmz genotypes can be classified as either **h/*y* or **l/*x* depending on the 3DL1 alleles expressed. Previous work showed that NK cells from **h/*y+B*57* subjects had a significantly higher tri-functional potential than those from **l/*x+B*57* subjects. We therefore questioned whether NK cells from the 7 **h/*y+B*27* and 5 **l/*x+B27* individuals differed from each other in their tri-functional potential. The median (range) NK tri-functional potential was 2.56% (0.00%, 6.18%) and 3.57% (0.47%, 6.43%) for NK cells from carriers of the **h/*y+B*27* and **l/*x+B*27* genotypes, respectively (p=0.53; Mann-Whitney test), a difference that was not statistically significant (Figure 2B). Therefore, HLA-B*27 appears to be able to interact with NK receptor alleles in either the 3DL1 *h/*y and *l/*x genotype categories to educate NK cells for equivalent NK functional potential.

3.3 NK cells from *3DL1+B*27 and *h/*y+B*57* carriers have a similar tri-functional potential

Next we questioned whether the level of tri-functional potential of NK cells from *3DL1+B*27* carriers was of a similar magnitude to that seen in NK cells from **h/*y+B*57* carriers. For this analysis we excluded the 2 *3DL1+B*27* individuals who also expressed a *B*57* allele and compared NK tri-functional potential in this group to that in 12 **h/*y+B*57* carriers. As seen in Figure 3A, although the tri-functional potential of NK cells from **h/*y+B*57* carriers was higher than that from *3DL1+B*27* carriers this difference did not achieve statistical significance (p=0.09; Mann-Whitney test).

Pair-wise comparisons of the tri-functional potential of NK cells from carriers of **h/*y+B*57*, *3DL1+B*27* and *3DL1+Bw4* revealed that only the comparison of **h/*y+B*57* and *3DL1+Bw4* was statistically significant. NK cells from each of these groups had higher tri-functional potential than those from the *3DL1+Bw6* group (not shown). Small group sizes and large variability within groups likely contributed to the lack of statistical significance in the tri-functional potential between the **h/*y+B*57* and *3DL1+B*27* and the *3DL1+B*27* and *3DL1+Bw4* groups. We performed another analysis testing for the significance of a trend towards declining NK functional potential among subjects with these *3DL1/HLA-B* genotypes. The rationale for this came from epidemiological studies showing that the effect of *B*57* on time to AIDS and VL control was greater in the presence of **h/*y* than **l/*x 3DL1* genotypes and greater than that of *B*27* on these outcomes in the presence of either *3DL1* genotype (Martin et al. 2007). When the tri-functional potential of NK cells from these 3 *3DL1/HLA-B* genotypes were assessed. Using a test of trend we observed that this measure decreased as follows: **h/*y+B*57* > *3DL1+B*27* > *3DL1+Bw4* (r=-0.40, p=0.01; Spearman's correlation test). Together these results suggest that there is a hierarchy in the impact on NK cell education of HLA-B variants where B*57 is the most potent in the context of **h/*y* genotypes followed by B*27 in the context of *3DL1* genotypes. Furthermore the impact of these alleles is superior to that of other Bw4 alleles co-expressed with *3DL1* genotypes.

4. Discussion

We have presented results showing that NK cells from carriers of the *3DL1+B*27* KIR/HLA genotype combination have tri-functional responses to missing self that are 1) significantly higher than those from *3DL1+Bw6* and 2) have a tri-functional potential that falls between that of carriers of **h/*y+B*57* and *3DL1+Bw4*.

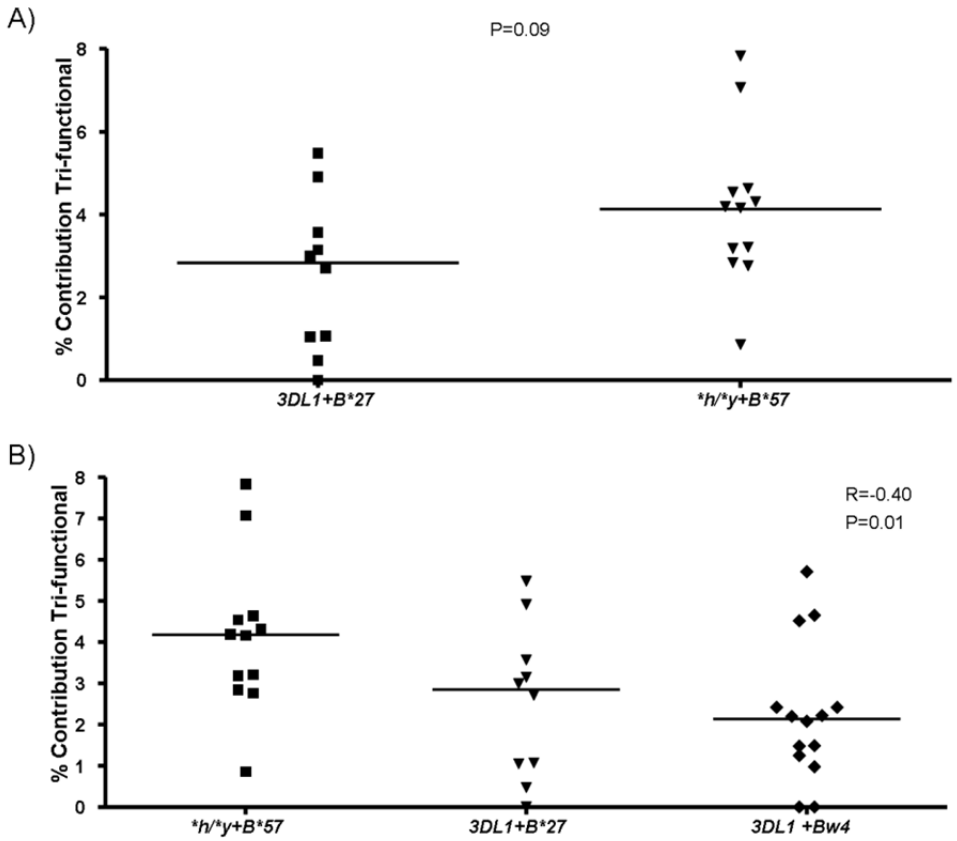

Fig. 3. Comparisons of the tri-functional potential of NK cells from individuals carrying *3DL1+B*27, *h/*y+HLA-B*57* and *3DL1+Bw4*. Scatter plots show percent contribution of tri-functional NK cells to the total K562 stimulated response in individuals who are *3DL1+B*27* and **h/*y+B*57* carriers (A) or **h/*y+B*57, 3DL1+B*27* and *3DL1+Bw4* carriers (B). Each data point represents results from a separate individual. The bar through each scatter plot indicates the median frequency for the group. A Mann-Whitney U test was used to assess the significance of between-group differences (A). A Spearman's correlation test was used to assess the significance of a trend towards decreasing functional potential in carriers of these genotype combinations (B).

We previously showed that B*57 is superior to most other Bw4 alleles in educating NK cells through its interaction with 3DL1 NK receptors for functional potential as measured by responses to missing self on K562 cells (Boulet et al 2010; Kamya et al. 2011). These results suggested that B*57, an allele associated with slow time to AIDS and VL control, may contribute to viral control not only through its interaction with HIV epitopes recognized by $CD8^+$ T cell but also through its interaction with 3DL1 inhibitory receptors on NK cells. B*27 is another allele associated with slow time to AIDS and VL control. We therefore sought to determine whether B*27 was also able to educate NK cells for potent

responses to missing self. The rationale for investigating the role of B*27 in educating NK cells for NK functional potential in HIV infected SP is that this allele is over represented among SP compared to the uninfected or HIV susceptible subjects. Among 101 SPs enrolled in the Canadian Cohort of HIV Infected SP typed to date 18 (17.8%) were B*27 positive whereas in HIV susceptible subject enrolled in a Primary HIV Infection cohort 26 of 434 (6%) expressed this allele ($p<0.001$; Fisher's Exact test), an allele frequency similar to that seen in uninfected Caucasian (www.allelefrequencies.net). The frequency of individuals expressing both a *B*27* allele and a homozygous *3DL1* genotype would be expected to be even lower. This provided the rationale for investigating this phenomenon in SPs in whom the frequency of the *KIR/HLA* genotype under investigation was higher than in uninfected subjects. We also reasoned that SPs with either controlled viremia or long term non progression would constitute a population with limited NK dysfunction due to HIV infection.

Others have reported that HIV infection dysregulates NK cell subset distribution such that there is a reduction in the frequency of the $CD56^{+}CD16^{+}$ NK subset with cytolytic activity with an associated increase the frequency of an anergic $CD56^{-}/CD16^{+}$ subset (Mavilio et al. 2005; Tarazona et al. 2002). VL seems to play a key role in this redistribution of NK cell subsets. Even though a decrease of cytotoxic NK cells is observed in SPs compared to healthy controls it is not as pronounced as that observed in viremic HIV infected progressor subjects (O'Connor et al. 2007; Barker et al. 2007). NK function may be altered by direct contact with HIV. The gp120 Envelope glycoprotein has been shown to suppress the activity, proliferation and survival of NK cells (Kottilil et al. 2006). Perturbations in the NK cell receptor repertoire have been reported in HIV infection, affecting both inhibitory and activating receptors (Kottilil et al. 2004; Mavilio et al. 2003; De Maria et al. 2003; O'Connor et al. 2007). Furthermore, HIV can escape NK cell recognition by restricting upregulation of activating NK cell receptor ligands such as NKp44L (Fausther-Bovendo et al. 2009), MICA and ULBP1 and 2 (Cerboni et al. 2007) and by preventing downregulation of HLA-C/E (Bonaparte and Barker 2004). Results reported by Kamya et al. demonstrated a negative correlation between NK cell tri-functional potential and VL suggesting VL and HIV infection negatively impact NK function (Kamya et al. 2011). In summary, although NK function in SPs may be affected by HIV infection the effect would be expected to be limited in this population. Our results demonstrate that NK cells from *3DL1+B*27* SPs can produce IFN-γ and TNF-α and express CD107a following K562 stimulation to a greater extent than those from *3DL1+Bw6*. This finding is unlikely to be due to difference of VL as these 2 groups had a similar VL (Table 2).

We have previously shown that NK cells from SP who carry **h/*y+B*57* have a level of tri-functional potential that is significantly higher than those from individuals carrying either *3DL1*h/*y* or *3DL1*l/*x* genotypes with *Bw4* alleles other than *B*27* or *B*57*. The elevated tri-functional potential of NK cells from **h/*y+B*57* carriers depends on the presence of both the *3DL1*h/*y* receptor genotype and *HLA-B*57* since carriers of *3DL1*l/*x* with *HLA-B*57* also have significantly lower NK tri-functional potential (Kamya et al. 2011). In contrast, we observed no differences in tri-functional potential in NK cells from *HLA-B*27* positive SPs carrying either the *3DL1*h/*y* or **l/*x* receptor genotypes. It should be noted that a limitation in making this assertion is the small number of subject who were **h/*y+B*27* versus **l/*x+B*27* available to make this comparison. Epidemiological studies found that compared to *Bw6 hmz* the *B*57* effect on time to AIDS and VL control was

enhanced in the presence of *3DL1*h/*y* compared to *3DL1*l/*x*. Overall, the effect of *B*27* on these outcomes was more moderate than that of *B*57* in the presence of *3DL1 *h/*y*. Compared to *Bw6 hmz* the *B*27-80T* allele *B*2705* was more protective in the presence of *3DL1*l/*x* than in the presence of *3DL1*h/*y* (Martin et al. 2007). All but one of the *3DL1+B*27* group of subjects in our study population expressed *B*27-80T* alleles (Carrington, Martin, and van Bergen 2008). Furthermore, there is also evidence that B*2705 has a greater affinity for one or more of the KIR3DL1*l allotypes (Luque et al. 1996). Our findings that NK cells from **h/*y+B*27* and **l/*x+B*27* carriers have similar levels of tri-functional potential that may be more modest than of **h/*y+B*57 carriers* is in line with B*27 being able to interact with receptors encoded by *3DL1*h/*y* and **l/*x* genotypes in *3DL1+B*27* carriers and this more effectively that B*57 interacting with receptors encoded by *3DL1*l/*x* genotypes (Kamya et al. 2011). There is also a trend towards NK cells from *3DL1+B*27* carriers having higher function that those from *3DL1+Bw4* individuals. These results argue in favour of HLA-B*27 and B*57 being unique among HLA-Bw4 antigens in their impact on educating NK cells for subsequent activity, although the effect of B*27 is more modest than that of B*57.

According to the rheostat model of NK education, the strength of the inhibitory input received by NK cells determines the threshold of activation that is set in each NK cell. The higher the inhibitory input, the more likely the NK cell will pass the threshold required to respond to stimuli with an increased frequency of effector cells and an increased number of effector functions (Brodin, Karre, and Hoglund 2009). During development, NK cells must acquire sufficient inhibitory signals to prevent autoreactivity through recognition of ligands for inhibitory NK receptors (Valiante et al. 1997; Hoglund et al. 1997). The larger the contribution of given receptor-ligand pairing to NK cell inhibition under homeostatic conditions the more potent a missing self response will be when the ligand is lost. This situation would be encountered in a setting of HIV infection where HIV encoded Nef downmodulates HLA-A and B molecules from the cells surface abrogating inhibitory signals mediated by 3DL1 receptors (Collins et al. 1998; Cohen et al. 1999).

In this report we have focused on comparisons of the tri-functional subset of NK cells. This was done because the percent contribution of this subset to the entire K562 stimulated response was the only functional subset that differed between carriers *3DL1+B*27* and a KIR/HLA receptor-ligand combination unable to signal through 3DL1 NK receptors. NK cells able elicit 3 functions are more potent in terms of the intensity of each of their functions than corresponding mono-functional NK cells (Kamya et al. 2011). This is similar to what has been reported for poly- versus mono-functional $CD8^+$ T cells (Darrah et al. 2007; Betts et al. 2006) Poly-functional HIV-specific $CD8^+$ T cells in SPs may play a role in superior anti-HIV activity (Betts et al. 2006; Makedonas and Betts 2011). This is still a controversial area as the low VL seen in SPs may preserve multi-functional HIV-specific immune response. Although the biological relevance of poly-functional antigen specific $CD8^+$ T cells in HIV infection is not yet clear, they do serve as an indicator of an effective response to HIV. Our experiments did not directly test the anti-viral activity of NK cells since we did not use HIV infected cells as stimuli. The role of tri-functional NK cells in inhibition of viral replication warrants further investigation.

If stimulation with HIV infected cells produces higher functionality in NK cells from *3DL1+B*27* versus *3DL1+Bw6* carriers and tri-functional NK cells are endowed with a superior capacity to suppress viral replication it would be interesting to study the ability

of NK cells from *3DL1+Bw6* for other NK cells functions. Our results demonstrate that these individuals have a limited NK cell tri-functional potential upon missing self stimulation. Since these SPs are able to control viral replication and/or maintain CD4 counts above 400 for 7 or more years it is not unreasonable to assume that NK cells from these subjects possess other NK function. NK cells are known to mediated antibody-dependent cell-mediated cytotoxicity which has been shown to play a role in controlling viral replication (Forthal, Landucci, and Daar 2001) and may play a role in preventing infection (Rerks-Ngarm et al. 2009). In addition, NK cells are also able to regulate antiviral immunity by modulating DC function (Gerosa et al. 2002; O'Leary et al. 2006). In the presence of HIV replication the cross talk between NK cells and DC is impaired (Mavilio et al. 2006; Melki et al. 2010; Alter et al. 2010). It would be interesting to assess whether the interaction between NK cells and DC is maintained in SPs, particularly in *3DL1+Bw6* carriers.

5. Conclusion

We have demonstrated that NK cells from *3DL1+B*27* SPs have a higher tri-functional potential following K562 stimulation than those from *3DL1+Bw6*. A test of trend found that NK tri-functional potential declines significantly in NK isolated from carriers of the following genotypes: **h/*y+B*57* > *3DL1+B*27* > *3DL1+Bw4*. Our results suggest that although the protective effect on HIV infection conferred by HLA-B*27 is mediated in part by $CD8^+$ T cells recognizing HIV epitopes restricted by this allele, the protective effect of this alleles may also be mediated by its interaction with inhibitory 3DL1 receptors. HLA-B*27, like HLA-B*57, appears to have an impact on NK education that is superior to that of other Bw4 alleles. Although this remains to be demonstrated experimentally we hypothesize that in carriers of certain *3DL1* and *HLA-B*27* or *HLA-B*57* genotypes, virus-infected cells that have down modulated the HLA ligand for their inhibitory 3DL1 NK receptors may be able to recruit a larger number NK cells with multiple functions, which can play a role in viral control. Such information is relevant to vaccine design by providing a rationale for modulating NK activity at the time of vaccination to favor developing protective immunity.

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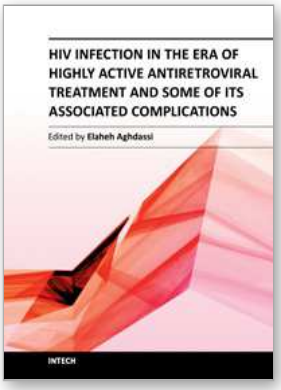

HIV Infection in the Era of Highly Active Antiretroviral Treatment and Some of Its Associated Complications

Edited by Dr. Elaheh Aghdassi

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Human immunodeficiency virus (HIV) infection is a complex illness affecting the immune system. Acquired immunodeficiency syndrome (AIDS) is an advanced form of HIV infection in which the patient has developed opportunistic infections or certain types of cancer and/or the CD4+ T cell count has dropped below 200/µL. More than 40 million persons around the world are infected with HIV, with approximately 14,000 new infections every day. The disease causes 3 million deaths worldwide each year, 95% of them in developing countries. Optimal management of human immunodeficiency virus requires strict adherence to highly active antiretroviral treatment (HAART) regimens, but the complexity of these regimens (e.g., pill burden, food requirements, drug interactions, and severe adverse effects) limits effective treatment. However, more patients with HIV are surviving longer today because of these drugs. This allows further study of commonly associated adverse effects. These may affect all body systems and range from serious toxicities to uncomfortable but manageable events. This book reviews some of HAART-related metabolic and neurological complications.

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