

# HIV-INFECTED INDIVIDUALS WITH DISCORDANT IMMUNOLOGICAL RESPONSES FOLLOWING COMBINATION ANTIVIRAL THERAPY HAVE SIMILAR CONCENTRATION OF T-CELL RECEPTOR EXCISION CIRCLES AND INTERMEDIATE LEVELS OF T CELL PROLIFERATION

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## Abstract

**Background:** Many HIV-1 infected patients treated with combination antiviral therapy develop rebounds in viral load (VL) in association with improvement in CD4+ T cell counts. Recent evidence suggests that protease inhibitor (PI) resistant viruses replicate less efficiently in thymic tissue favoring sustained T cell production in the presence of PI-resistant viruses. **Methods:** We performed a cross sectional study of HIV-1 infected individuals (n=73) who had been compliant with combination antiviral therapy (including either a PI or NNRTI) for at least 12 months. Individuals were classified according to degree of viral suppression, rate of change in CD4 (using least-squares linear regression for log<sub>10</sub> transformed CD4 counts). Individuals were classified into 4 groups: those with persistent viral replication and an increasing CD4 count (discordant immunological responders, DIR, n=22), persistent viral replication and a falling CD4 count (non-responders, NR, n=10), suppressed viral replication and an increasing CD4 count (complete responders, CR, n=38) and suppressed viral replication and a falling CD4 count (discordant virological responders, n=3). Peripheral blood mononuclear cells (PBMC) were separated into purified CD4+ and CD8+ naïve (CD45RA+) and memory (CD45RA-) T cells. Thymic function was assessed by quantification of T-cell receptor excision circles (TREC) in each T cell population using real-time PCR. T cell proliferation and apoptosis were assessed by intracellular staining for Ki67 and TUNEL respectively. **Results:** CD4 counts (total and naïve) were significantly lower in NR compared with CR and DIR (p<0.05). There was a significantly lower viral load (VL) in DIR compared with NR (p=0.02). VL was undetectable (<400 copies/ml) in all CR and DIR. There was no significant difference in TREC concentration in CD4+ or CD8+ naïve T cells in DIR, CR and NR. The %Ki67 staining in CD4+ memory T cells was significantly lower in CR compared with DIR (p<0.01) and NR (p<0.05). The percentage of activated CD8+ T cells was higher in NR and DIR compared with CR. **Conclusion:** These results are consistent with reduced viral pathogenicity in peripheral T cells, leading to reduced rates of proliferation, activation and death in DIR. The mechanism of this reduced peripheral viral pathogenicity requires further investigation.

## Introduction

Treatment of HIV-1-infected individuals with highly active antiretroviral therapy (HAART) results in a dramatic decline in viral load and a significant increase in circulating CD4+ T lymphocytes in the majority of individuals. In 20-40% of individuals there is an increase in CD4+ T cells in the absence of complete virological control. This pattern is more commonly observed in children who frequently demonstrate CD4+ T lymphocyte regeneration even in the absence of significant and durable virological suppression (Essajee, et al., *AIDS* 1999. **13**: 2523-2532). The pathogenesis of this "discordant" CD4+ T cell response is unclear. Possible explanations include an alteration in viral "fitness" leading to a reduction in T lymphocyte proliferation, activation or death. Recent evidence suggests that protease inhibitor (PI) resistant viruses replicate less efficiently in thymic tissue favoring sustained T cell production in the presence of PI-resistant viruses (Stoddart, et al., *Nat Med* 2001. **7**: 712-8). Alternatively, sustained T cell increases in the presence of virus replication could be dependent on host factors such as greater thymus function at the time of infection, which may explain why discordant responses are more frequently observed in HIV-1-infected children compared with adults. To explore the pathogenesis of a discordant immunological response to HAART, we performed a cross-sectional study to examine several indices of T cell production and turnover.

## Methods:

**Patients:** Patients were recruited from the Royal Melbourne and Alfred Hospitals in Melbourne, Australia. Inclusion in the study required HIV-infected individuals to be compliant with combination antiviral therapy (at least 3 antiretroviral agents, including either a PI or non-nucleoside reverse transcriptase inhibitor (NNRTI)) for at least 12 months prior to enrolment. Informed consent was obtained from all study subjects in accordance with the policies of the institutional review boards of both institutions. Individuals were classified according to degree of viral suppression and rate of change in CD4 (using least-squares linear regression for log<sub>10</sub> transformed CD4 counts). Individuals were classified into 4 groups: those with persistent viral replication (viral load > 400 copies/ml) and an increasing CD4 count (discordant immunological responders, DIR, n=22), persistent viral replication and a falling CD4 count (non-responders, NR, n=10), suppressed viral replication and an increasing CD4 count (complete responders, CR, n=38) and suppressed viral replication and a falling CD4 count (discordant virological responders, n=3).

**Immunological and virological analyses:** Peripheral blood mononuclear cells (PBMC) were isolated and frozen at -70°C for future analysis. Using three and four-colour flow cytometry, PBMC were stained with monoclonal antibodies to CD4, CD8, CD45RA, CD45RO, CD38 and HLA-DR. T cell proliferation and apoptosis were assessed by intracellular staining for Ki67 and TUNEL respectively. PBMC were separated into purified CD4+ and CD8+ naïve (CD45RA+) and memory (CD45RA-) T cells and thymic function was assessed by quantification of T-cell receptor excision circles (TREC) in each T cell population using real-time PCR. Plasma HIV-1 RNA was measured using either RT-PCR (Amplicor, Roche, NJ) or bDNA (Bayer, Foster City, CA) and adjusted to the bDNA 3.0 scale. HIV-1 RNA reverse transcriptase (RT) and protease genotype were analysed using a kit assay (Applied Biosystems).

**Statistical analyses:** Quantitative data is summarised as the geometric mean  $\pm$  95% confidence intervals. Categorical data are presented as absolute frequencies and compared using the chi-square test. Each variable was compared amongst groups by analysis of variance (ANOVA) using SAS version 8.0.

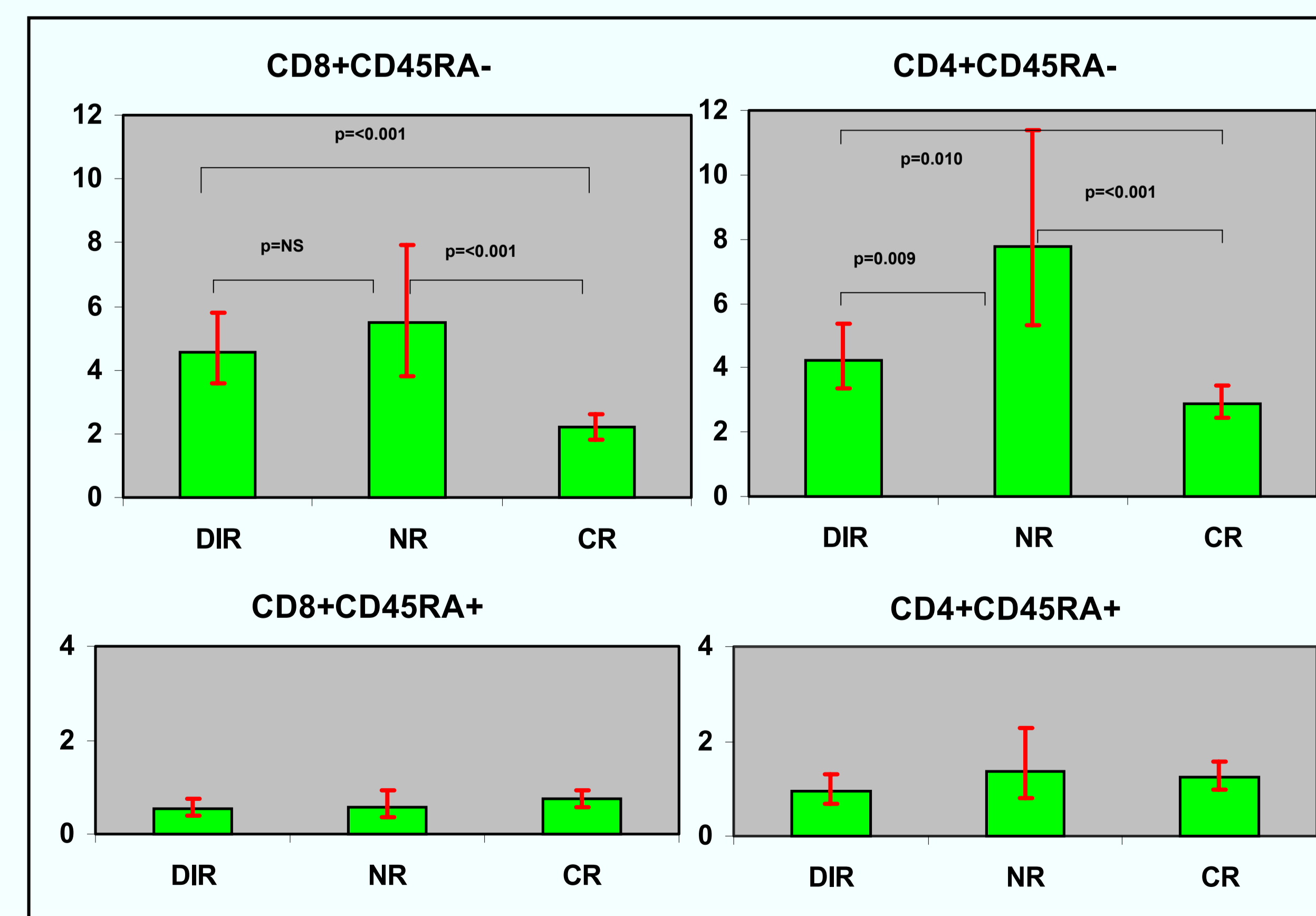
## Results:

### 1. Characteristics of study subjects, antiviral use and lymphocyte phenotypes.

	DIR	NR	CR	DVR	p value
<b>Number</b>	22	10	38	3	
<b>Age (years)</b>	45 (41-49)	47 (41-52)	44 (41-47)	50 (42-58)	NS
<b>Duration HAART (months)</b>	40	48	45	60	NS
<b>CD4 T cells (x10<sup>6</sup> cells/L)</b>					
At time of analysis	511 (387-635)	274 (90-458)	553 (459-648)	188	0.008
Nadir	167	112	176	164	NS
Slope (per year)	55	-22	76	-32	<0.0001
<b>T cell subsets at time of analysis (x10<sup>6</sup> cells/L)</b>					
CD4+/CD45RA+	123 (58-263)	23 (7-75)	91 (159-278)		0.002
CD4+/CD45RO+	222 (116-423)	30 (11-82)	293 (182-471)		0.001
CD8+/CD45RA+	385 (227-652)	229 (99-523)	507 (342-751)		NS
CD8+/CD45RO+	372 (219-630)	214 (93-414)	317 (214-470)		NS
<b>Plasma HIV-1 viral load (bDNA copies/ml)</b>					
At time of analysis	8405	78333	400	400	0.0005
Before HAART	174127	231209	157930	169749	NS
<b>Antiretroviral therapy</b>					
At time of analysis					
NRTI	22 (100%)	10 (100%)	38 (100%)	3 (100%)	NS
NNRTI	7 (32%)	4 (40%)	17 (45%)	1 (33%)	NS
PI	15 (68%)	22 (70%)	22 (58%)	2 (66%)	NS
Previous PI use	21 (95%)	10 (100%)	27 (71%)	3 (100%)	NS
<b>Genotype mutations</b>					
Total number genotyped	9	7	ND	ND	
NRTI res only	3	1			
NRTI and NNRTI	1	1			
NRTI and PI	3	1			
Multi-class res.	2	4			

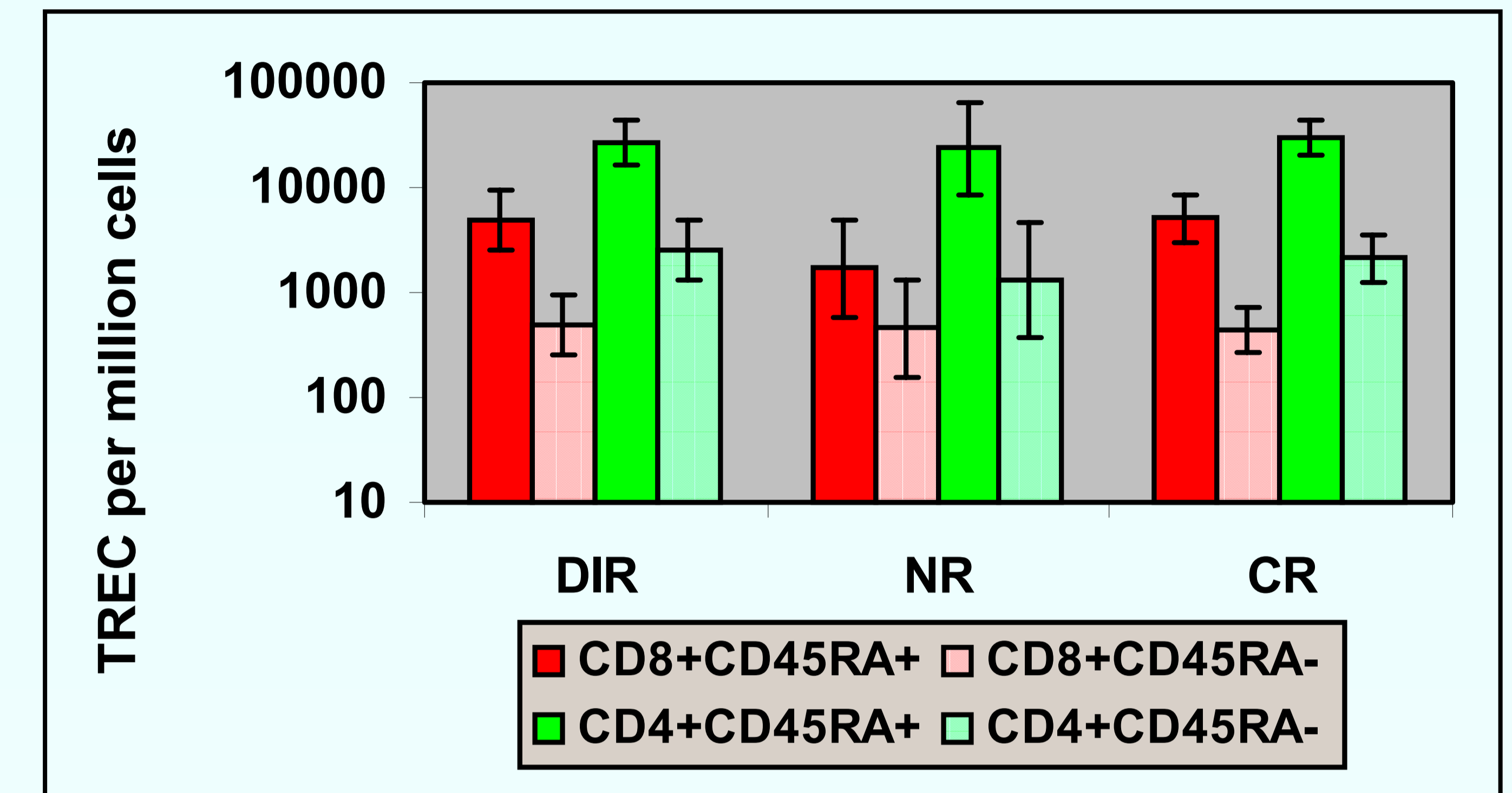
**Table 1: Comparison of patient characteristics of DIR, CR, NR and DVR. The geometric mean ( $\pm$ 95% C.I.) are shown.**

**2. T cell proliferation is reduced in CD4+ T cells in DIR when compared with NR:** The percentage of cells positive for Ki67 was significantly reduced in CR compared with NR, as expected. The percentage of Ki67+ CD4+ T cells in DIR was reduced in comparison with NR, but not as significantly reduced as in CR ie %Ki67+ CD4+ T cells were intermediate in comparison with NR and CR. This would suggest a partial but not complete reduction in CD4+ T cell proliferation in DIR. In CD8+ T cells, the proportion of Ki67+ cells was similar in both DIR and NR. Both DIR and NR had significantly higher levels of Ki67+CD8+ T cells when compared to CR (Figure 2).



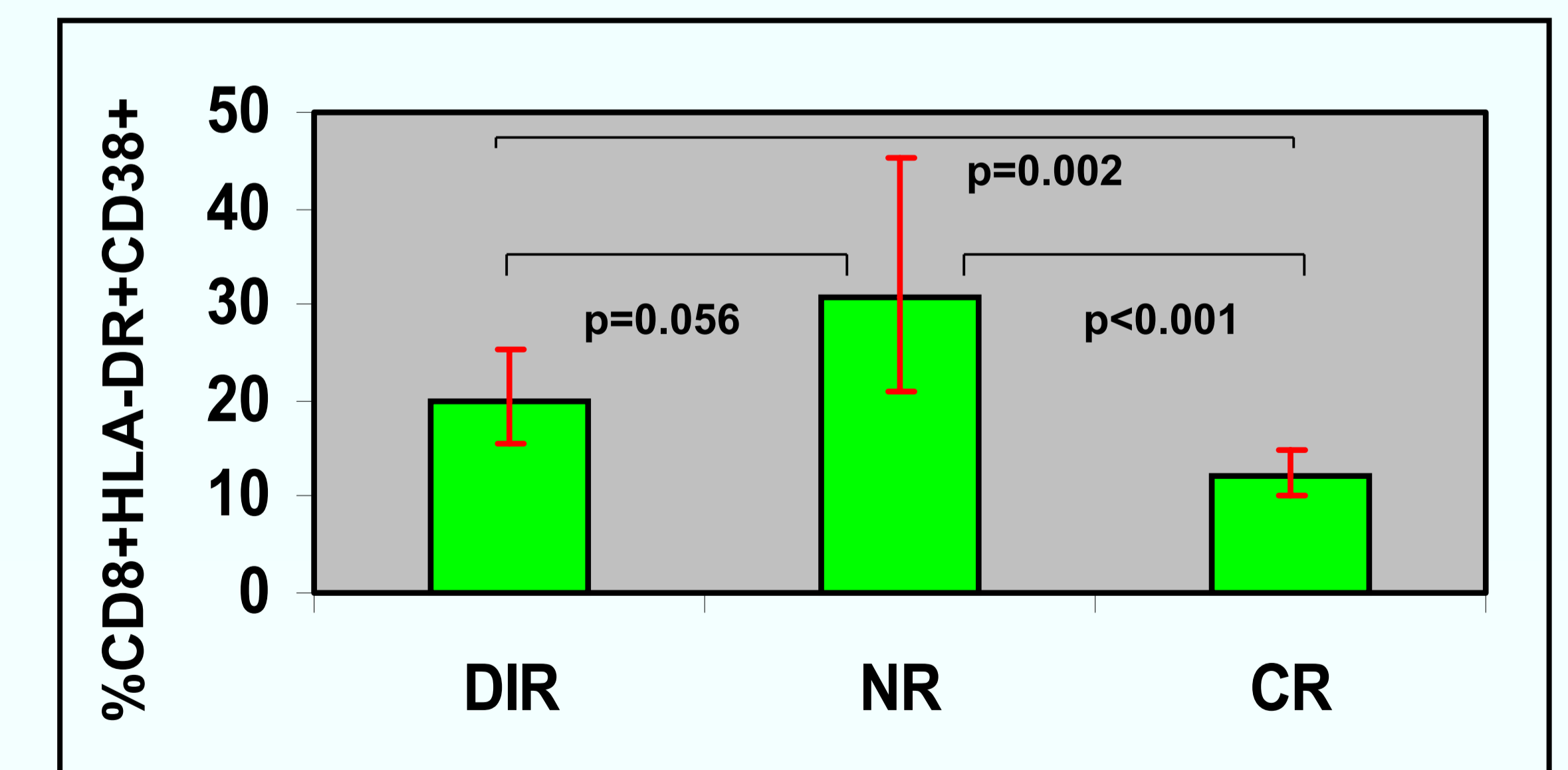
**Figure 2: %Ki67 in CD8+ (left) and CD4+ (right); memory (upper) and naïve (lower) T lymphocytes. Geometric mean  $\pm$  95% C.I. (red) is shown for DIR (discordant immunological responders), NR (non-responders), CR (complete responders) and DVR (discordant virological responders). Comparison between each group was made by analysis of variance (ANOVA).**

**3. TREC concentration is independent of immunological response to antiviral therapy.** TREC concentration in sorted CD4+RA+, CD4+RA-, CD8+RA+ and CD8+RA- T cell subsets was not significantly different between the different patient groups. When TREC concentration was adjusted for concentration of CD4+ and CD8+ naïve T cells and expressed as TREC+CD4+ and CD8+ cells per ml of blood, there was still no significant difference between the three study groups, suggesting that reduced thymic pathogenicity was an unlikely mechanism explaining a discordant immunological response.



**Figure 3. TREC concentration in T cell naïve and memory subsets in DIR (discordant immunological responders), NR (non-responders), CR (complete responders) and DVR (discordant virological responders). There was no significant difference in TREC concentration between each group.**

**4. Activated and apoptotic CD8+ T cells are increased in DIR when compared with CR:** There was a significantly higher percentage of activated CD8+ T cells in DIR in comparison to CR (p=0.002) and a trend toward lower levels of activated CD8+ T cell in DIR when compared with NR (p=0.056). In a subgroup of patients (n=16) apoptosis was assessed by measurement of TUNEL in CD4+ and CD8+ T cells. Although this was only performed on a small number of individuals, there was a lower mean level of TUNEL staining in CR (mean=5.5%, 9.7%), intermediate level in DIR (mean = 7.6%, 26.4%) and high level in NR (11.8%, 42.7%) for both CD4+ and CD8+ T cells respectively. However, these difference only reached significance for the CD8+ T cells (p=0.01).



**Figure 4. Percentage of activated CD8+ T cells, defined by HLA-DR and CD38 staining, in DIR (discordant immunological responders), NR (non-responders), CR (complete responders) and DVR (discordant virological responders).**

## Conclusion:

A discordant immunological response (DIR) to antiviral therapy is not specifically associated with either PI or NNRTI use. In DIR, levels of CD4+ T cell proliferation and CD8+ T cell activation and apoptosis were intermediate between that of NR and CR while there was no significant difference in TREC concentration. These results are consistent with reduced viral pathogenicity in peripheral T cells, leading to reduced rates of proliferation, activation and death in DIR. The mechanism of this reduced peripheral viral pathogenicity requires further investigation.