

HLA Class I Restricted CD8+ and Class II Restricted CD4+ T Cells are implicated in the Pathogenesis of Nevirapine Hypersensitivity

Short Title: Class I/Class II restricted T-cells and NVP HSR

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ABSTRACT

Objectives: This study sought to examine nevirapine hypersensitivity (NVP HSR) phenotypes and their relationship with differing MHC Class I and Class II alleles and the associated CD4⁺ and CD8⁺ T cell NVP specific responses and their durability over time. **Methods:** A retrospective cohort study compared HIV⁺ patients with NVP HSR, defined by fever and hepatitis and/or rash, with those tolerant of NVP > 3 months. Covariates included class I (HLA-A, B, C) and class II (HLA-DR) alleles. Cellular studies examined NVP specific CD4⁺ and CD8⁺ T cell responses by IFN γ ELISpot assay and intracellular cytokine staining (ICS). **Results:** NVP HSR occurred in 19/451 (4%) NVP exposed individuals between March 1993 and December 2011. HLA associations were phenotype dependent with HLA-DRB1*01:01 associated with hepatitis (P = 0.02); HLA-B*35:01 and HLA-Cw4 were associated with cutaneous NVP HSR (P = 0.001, P = 0.01), and HLA-Cw*08 was associated with NVP HSR with eosinophilia (P = 0.04) and multisystemic NVP HSR (P = 0.02). NVP specific IFN γ responses waned significantly > 3 months from the original reaction and were diminished or completely abrogated when either CD4⁺ or CD8⁺ T cells were depleted from the PBMC culture. **Conclusions:** The association of specific class I and II allele pairings with specific phenotypes of NVP HSR, and cellular studies showing both CD4⁺ and CD8⁺ T cell NVP specific responses suggest that specific combinations of NVP reactive class I restricted CD8⁺ and class II restricted CD4⁺ T cells contribute to the immunopathogenesis of NVP HSR.

KEYWORDS

Nevirapine, HLA, drug hypersensitivity, DRESS/DIHS, HIV, CD4⁺, CD8⁺, regulatory, T cells

INTRODUCTION

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) used in combination HIV therapy with an excellent long-term safety profile. A treatment-limiting hypersensitivity syndrome (NVP HSR) characterized by fever, and/or rash and/or hepatitis and often accompanied by eosinophilia occurs in 5% of those starting NVP [1, 2]. NVP HSR is suggested to be T-cell mediated by both human and rat models. In rats, the sensitivity to NVP-induced skin rash can be transferred with CD4+ T cells from NVP-rechallenged rats to naive recipients and partial depletion of CD4+ T cells delays and decreases the severity of the rash. This is consistent with the decreased incidence of rash in patients with a low CD4+ T cell count [3-9].

A CD4+ dependent-MHC Class II restricted immune response directed against NVP was first reported in Caucasians as the association of hepatic symptoms with a combination of CD4+ T cells $\geq 25\%$ and HLA-DRB1*01:01 [10]. Multiple class I and/or II MHC associations have now been described with different phenotypes of NVP HSR across several ethnicities [11-19]. These differing Class I and Class II HLA associations with different NVP HSR phenotypes across distinct populations and the increased risk of cutaneous phenotypes of NVP HSR in African Americans with CYP2B6 561 G>T [20] suggest that genetic, immunological and metabolic pathways may be important. NVP may trigger class I-restricted CD8+ or class-II restricted CD4+ T-cell mediated immune responses in the presence of the relevant class I and II major histocompatibility complex alleles, respectively.

METHODS

As part of a prospective cohort study we identified the association of class I and II HLA alleles with clinical and immunological phenotypes of NVP HSR.

Patient and Control population

Definitive ascertainment of NVP-associated reaction status was achievable in 375 of 451 individuals in the West Australian HIV Cohort identified as NVP-exposed. Ethnicity and/or HLA genotyping was unavailable for 54 of these individuals and 41 individuals lacked clinical information required as part of the inclusion criteria. Associations between HLA alleles, severity of CD4+ T lymphocyte-dependent immune deficiency and predisposition to NVP HSR were explored in analyses restricted to Asians and Caucasians without symptoms after 3 months NVP exposure (n = 262) or who had developed NVP-induced drug reactions (n=19) (Table 1). NVP-associated reactions were identified prospectively in the database, and the case definition was retrospectively validated by a clinician blinded to HLA typing, who utilized standardized diagnostic criteria, including a minimum of fever in combination with rash and/or hepatitis (grade 2 toxicity or greater: alanine aminotransferase >2.5 times the upper limit of normal) and/or eosinophilia (eosinophils > 0.5 x 10⁹/litre). HBV/HCV serology was also examined for all patients displaying hepatotoxicity. Serology results taken together with clinical symptoms such as eosinophilia or rash which indicated drug HSR and not HBV/HCV infection excluded viral infection in all cases which exhibited hepatic symptoms. Selection of patient PBMCs for cellular experiments was based on availability. Experiments were conducted with the understanding and consent of each participant as approved by the Royal Perth Hospital and Murdoch University Human Research Ethics Committees.

HLA Typing

HLA A B C and DR, DQ typing was performed using sequence base typing as previously described [21].

Isolation of Nevirapine and 12-OH-NVP

NVP solution was prepared by dissolving 200 mg NVP tablets (Viramune, Boehringer-Ingelheim) in DMSO and the absorbance at 260nm was checked with the nanodrop spectrophotometer. The NVP stock solution was diluted to 1mg/ml in PBS, filtered and resuspended 1:10 in culture medium (10%FCS-RPMI-1604) and added to cell cultures in the ELISpot assay at a final concentration of 40 μ m. The 12-OH-NVP metabolite was prepared similarly and then added to cell cultures at 50 μ m final concentration.

T-Cell depletions and IFN γ ELISpot assay

PBMC were depleted of T cells using CD4 or CD8 or CD25 Dynal beads (Invitrogen) according to the manufacturer's instructions. Cryopreserved PBMC were thawed and left to settle overnight or freshly isolated PBMC were used in the T-cell depletion assays. The T-cell depleted fractions were counted prior to use in the ELISpot assay. Specific CD4, CD8 or CD25^{high} T-cell depletions were confirmed by flow cytometry (Gallios Flow Cytometer, Beckman Coulter).

The IFN γ ELISpot assay was performed in triplicate as described previously using NVP and 12-OH NVP at final concentrations of 40 μ m and 50 μ m respectively [22]. A positive response was defined as greater than 50 spots/million cells after background removal [23].

Intracellular cytokine staining

Intracellular cytokine staining (ICS) was set up at 2 million cells/ml with either 40µm NVP or 50µm 12-OH-NVP, and positive (PHA) and negative controls (cells alone). Brefeldin A (20µg/ml) was added after 2 hours. The reaction was stopped after 6 hours. Cells were then washed and 10µls each of CD4 and CD8 fluorochrome-conjugated antibodies (CD4-PE and CD8-APC-H7) were added (20minutes room temperature). Cells were fixed and permeabilised using Intraprep reagents 1 and 2 (Beckman coulter), then anti-human IFN γ antibody was added (ALEXA Fluor 488) 15 minutes room temperature. Cells were then washed and analysed on the Gallios flow cytometer. A 9 colour flow antibody panel (CD3-V450, CD4-PE, CD8-PE CF594, CD45RA-APC, CCR7-PECY7, IFN γ -ALEXA Fluor 488 IL-2- PerCP Cy5.5, TNF α AF 700 and MIP-1 β -APC-H7) was used to assess the immune response after overnight stimulation with 40µm NVP and/or unstimulated cells. Staining procedure was similar to that described with the exception of Brefeldin A (10µg/ml) which was added at set up of the overnight cultures. All antibodies were supplied by BD Pharmingen. In addition, for membrane staining for T regulatory cell markers (CD4⁺ CD25^{high} CD127^{low}), antibodies (CD3 PerCP Cy5.5, CD4 PE-Cy7, CD8 FITC, CD25 APC and CD127 PE) were added to isolated PBMC and incubated for 20 minutes at room temp. Cells were then washed, resuspended in flow buffer (PBS-1% FCS) and analysed on the Gallios flow cytometer (Beckman Coulter, Miami, FL, US).

Statistical Analysis

Statistical analyses were restricted to individuals of known Caucasian or East/South-east Asian race, with corresponding stratification, because of the known impact of race

on genetic associations and the potential ascertainment bias arising from incomplete cohort information. Demographic and clinical/immunological differences between nevirapine sensitive and tolerant groups within each racial group were assessed by either a Fisher exact test (sex, HCV co-infection, undetectable viral load at baseline, treatment experience), or Wilcoxon test (age, CD4+ and CD8+ T cells). Initial genetic analyses which investigated specific HLA alleles by means of a race-stratified Mantel-Haenszel test were confirmed by logistic regression analyses incorporating adjustments for race, NRTI backbone and CD4+ T cell count at initiation of NVP.

RESULTS

19 patients (13 Caucasian, 6 Asian) were identified with NVP HSR (Figure 1). Reactions to NVP were more prevalent amongst the fully ascertained Asians ($P = 0.009$, Fisher exact test), but comparisons of the NVP sensitive and tolerant patient groups did not show any significant within-race differences in terms of age, sex, or clinical and immunological factors (Table 1).

Genetic analysis

The association of the alleles HLA-Cw*04, -Cw*08 and -B*3501 with NVP HSRs move in the same direction in both Asians and Caucasians (Table 2, Table S2). The allele DRB1*0101 did show differences in its association with NVP induced hepatotoxicity between Caucasians and SEAs. The carriage of HLA DRB1*01:01 was associated with hepatic symptoms of NVP HSR ($P = 0.02$), particularly for Caucasians commencing therapy with CD4+ T cell counts $\geq 25\%$ (OR = 10.2, $P = 0.005$, Fisher exact test), but notably lacking amongst Caucasians with lower baseline CD4+ T cell

counts ($P > 0.9$), and amongst Asians (Table 2). The presence of HLA-B*35:01 significantly associated with NVP HSR ($P = 0.005$) and specifically with NVP HSR with cutaneous symptoms ($P = 0.001$). The HLA-Cw*04 allele was also associated with NVP HSR with rash ($P = 0.01$). The association of HLA-B*35 with cutaneous phenotype NVP HSR is similar to observations in Thai populations with HLA-B*35:05 although our observation specifically associating HLA-B*35:01 with cutaneous phenotype HSR across Caucasian and Southeast Asian populations is new. HLA-Cw*08 was associated with multisystemic reactions including eosinophilia ($P = 0.02$).

Cellular responses in genetically susceptible individuals

Cellular experiments with available PBMC collected from 12/19 NVP HSR patients (median of 14.4 months ranging from 2 days to 144 months after NVP stop) were conducted to evaluate the phenotype/HLA class I and II specific host immune responses.

IFN γ response wanes with time since NVP HSR

NVP-specific responses were detected by IFN γ ELISpot assay in PBMC from 4/12 patients evaluated. Mean responses for the four patients were 115, 400, 300 and 1600 SFU / million cells in the first PBMC samples evaluated post NVP HSR for patients 1, 2, 3 and 4 respectively (mean days from reaction 26, range 2-92 days). *Patient 1* and *Patient 2* expressed the B*35 and C*04 allele pairings and experienced the rash associated phenotype. *Patient 1* (HLA-A*24:07, -A24:10, -B*35:30, -B*38:02, -C*04:01, -C*07:02, -DR*12:01, -DR*15:02) experienced fever and cutaneous symptoms of NVP HSR within 7 days and was re-challenged with NVP 1 month later

with recurrence of symptoms. NVP-induced IFN γ responses were detected in five longitudinal PBMC samples collected from this patient, after re-challenge, up to 144 days post NVP HSR (Figure 2A, Table S1). A stored sample available from *Patient 1* collected one year prior to first NVP administration showed a negative IFN γ response to NVP in an ELISpot assay. *Patient 2* (HLA-A*11:01, -A*24:02, -B*13:01, -B*35:01, -C*03:04, -C*04:01, -DR*11:01, -DR*16:01) presented with fever, rash, altered liver function and eosinophilia within 14 days of NVP start and NVP induced IFN γ responses were observed on day 97 (400 SFU) and had diminished by day 476 post NVP HSR (20 SFU, less than the positive cut off of 50 SFU/million cells) (Table S1) [23]. *Patient 2* had experienced trimethoprim-sulfmethoxazole HSR 2-3 months prior to the NVP HSR and showed evidence of elevated and activated CD4⁺ and CD8⁺ T cells (32% CD4 T cells/ μ L, 31% CD4⁺HLA-DR⁺, 37% CD8⁺HLA-DR⁺, 71% CD8⁺CD38⁺ T cells) in a sample tested 1 month post NVP HSR compared to a sample tested 4 months later (24% CD4 T cells/ μ L, 5% CD4⁺HLA-DR⁺, 1% CD8⁺HLA-DR⁺, 24% CD8⁺CD38⁺ T cells).

Patient 3 was a 5 month old infant with Asian ancestry diagnosed with NVP HSR (HLA-A*02:06, -A*34:01, -B*15:21, -B*56:01, -C*04:03, -C*07:01, -DR*04:06, -DR*15:02), with fever, rash, eosinophilia and hepatitis on day 7 of NVP/abacavir/3TC treatment. NVP specific IFN γ responses were detected in *Patient 3* on day 2 (300 SFU) and had diminished by day 62 post NVP HSR (45 SFU, < positive cut off of 50 SFU/million cells).

Patient 4 (HLA- A*29:02, -A*31, -B*14:01, -B*44:03, -C*08:02, -C*16:01, -DR*07:01) presented with rash, fever and eosinophilia two weeks after commencing NVP/Abacavir/3TC. IFN γ responses to NVP were examined for *Patient 4* on days 5, 8, 12, 18 and 49 post NVP HSR (Table S1, Figure 2A) with the highest frequency of responses detected on day 5 and no response by day 18 post HSR. Plasma cytokine profiles showed detectable IFN γ release at days 1 and 5 post NVP HSR which then sharply decreased (Figure S1). ALT levels rose from 68U/L eight days before NVP start to a peak of 2670 U/L by day 3 post NVP stop coinciding near the highest NVP specific IFN γ responses and then in parallel slowly declined to 29 U/L by day 49 post NVP HSR. Eosinophils peaked when the NVP specific IFN γ responses had declined to undetectable levels (Figure 2B).

When IFN γ responses were examined from all 4 patients it was clear that NVP-induced T-cell responses decrease over time. The highest frequency of NVP-induced IFN γ responses were observed in PBMC collected from *Patients 1* and *2* within approximately 90 days of stopping NVP. In contrast the peak response for *Patient 3* and *4* was within 1 week of stopping NVP and complete abrogation of NVP-stimulated responses was observed in PBMC collected from *Patient 4*, 18 days post NVP stop (Figure 2A). The abrogation of the IFN γ responses over time was not observed in samples from *Patient 1*, possibly because he was re-challenged with NVP one month after first being administered the drug and this may have provided a potent stimulus to the memory T-cell pool and ensured their prolonged survival. Indeed, an increased NVP-stimulated IFN γ response of 870 SFU/million cells was detected in freshly isolated PBMC obtained from *Patient 1*, 144 days after his initial reaction (4 months

after re-challenge) compared with 115 SFU/million cells detected in a sample collected from this patient 6 days after *first* commencing NVP. Similarly, a prolonged response was seen in *Patient 2*, 97 days post NVP HSR, and this patient had experienced sulfamethoxazole HSR only 2 months before being exposed to NVP. T cell activation markers were elevated in *Patient 2* one month post NVP HSR. Blocking of inhibitory signals during immune activation may have contributed to the persistence of NVP specific T cells at day 97 post HSR. T cell activation markers decreased by 5 months NVP HSR and NVP specific T cells were not detected in this patient when tested at a later time point 476 day post NVP HSR. For the 8 patients with no detectable NVP stimulated IFN γ responses PBMCs had been collected at ≥ 11 months from the NVP HSR reaction (median 2 yrs. 11 months, range 11months-12years), consistent with the abrogation of NVP-induced IFN γ responses over time. The 12-OH-NVP metabolite did not illicit significant IFN γ responses in 3 of the 4 patients tested at time points when NVP responses were detected (*Patient 1*, 50 SFU, day 144 after re-challenge; *Patient 2*, 0 SFU day 97; *Patient 3*, 0 SFU days 2 post NVP HSR).

T reg numbers increase in the recovery phase of NVP HSR

High frequencies of regulatory T cells have been reported following drug reaction with eosinophilia with systemic symptoms (DRESS) [24], thus we hypothesized the abrogation of NVP-induced T-cell responses was due to expansion of CD4⁺ regulatory T cell (T regs) populations. The percentage of T regs present in the day 5 sample (3.9%) collected from *Patient 4* was similar to levels of T regs detected in 3 samples from 2 healthy controls (3.6, 4.0. and 5.1% T regs respectively) and was also similar to T reg levels detected in an HIV-infected patient controlling their viral load in the

absence of medication (4.9%). However T reg percentages in *Patient 4* increased to 7.3, 8.2 and 8.0% in samples collected on days 8, 18 and 49 post NVP HSR respectively (Figure 2C) and this corresponded with the decline in the IFN γ response, however, depletion of CD25^{high} cells did not restore the NVP-induced IFN γ responses (data not shown).

Elevated Tregs and relapse of DRESS symptoms may accompany viral reactivation [25, 26], however viral serology for all NVP HSR patients failed to show any evidence of reactivation (i.e. IgM +) of HHV-1,-2,- VZV, CMV, EBV or HHV-6 and whole blood qualitative PCR for HHV-6 was negative in patients 2, 3 and 4 (data not shown). The infant, *Patient 3* only remained positive for IgG to HHV-6 at age 14 months consistent with early infection with HHV-6 and waning of passive immunity and lack of infection with EBV, CMV, HSV 1 / 2 and VZV.

CD4+ and CD8+ T cells play dual roles in NVP HSR

T-cell depletion studies showed that both CD4+ and CD8+ T cells contributed to the NVP-specific immune response (Figure 3A). In *Patient 4*, CD4+ T-cell depletion marginally reduced the responses detected (21% decrease) when compared with whole PBMC, whereas CD8+ T-cell depletion resulted in a greater reduction (83% decrease compared with whole PBMC) in the frequency of detectable NVP-specific T-cell responses (1600, 1265 and 270 SFU/million cells for whole PBMC, CD4+ depleted and CD8+ T-cell depleted PBMC respectively), data suggesting CD8 T cells may play a larger role in the NVP-induced immune response in *Patient 4*. CD4+ and CD8+ T-cell depletion in cells from *Patient 1* showed a complete abrogation of the NVP specific

responses when either CD4⁺ or CD8⁺ cells were depleted (Figure 3A). The presence of cytokine producing CD8⁺ and CD4⁺ T cells was confirmed and investigated further by ICS (*Patient 3* and *4*). The frequency of IFN γ producing cells was low but detectable by CD4⁺ and CD8⁺ T cells in NVP-stimulated PBMC (Figure 3B).

The phenotype of the NVP-stimulated CD4⁺ and CD8⁺ T-cell responses was further investigated by examining expression of CCR7 and CD45Ra in PBMC from *Patient 4* confirming that the active T-cell populations were central memory cells (Figure 3C). In addition NVP-induced TNF α and IL-2 production was detected. TNF α was detected in CD4⁺ and CD8⁺ T cells, however IL-2 was detected predominantly in central memory CD4⁺ T cells. IFN γ producing cells were primarily single cytokine producing cells whilst TNF α producing cells were also IL-2 producing (Figure 3D). We postulate that NVP-stimulated CD4⁺ T-cell production of IL2 and TNF α induced the expansion of effector CD4⁺ and CD8⁺ T cells to produce IFN γ and TNF α .

DISCUSSION

Contrary to early animal and human studies supporting an HLA class II CD4⁺ T-cell mediated model for NVP HSR our work supports the role of both CD8⁺ and CD4⁺ T-cell mediated responses in the immunopathogenesis of NVP HSR. A role for MHC Class I specific CD8⁺ T cells is supported by the association of HLA-B*35:01 and HLA-Cw*04 in particular with cutaneous phenotype NVP HSR, the association of HLA-Cw*08 with multisystemic NVP HSR and eosinophilia, also the detection of IFN γ production by CD8⁺ T cells in an NVP HSR infant (*Patient 3*) carrying HLA-C*04 and in an adult male patient with the B*14:01/Cw8 haplotype (*Patient 4*). The significant

reduction of NVP specific responses observed with CD8⁺ T-cell depletion in *Patient 1* and *Patient 4* (B*35/C*04 and B*14/Cw*08) supports the contribution of the class I CD8⁺ T-cell restriction. A dual role for MHC Class II specific CD4⁺ T cells in NVP HSR has also been demonstrated through confirmation of the association of HLA-DRB1*01:01 with NVP HSR featuring hepatitis and the abrogation of, or decreased INF γ production by the PBMC pool in response to NVP in CD4⁺ T-cell depletion studies.

It is still unclear whether the HLA-B*35 and HLA-Cw*04 associations with NVP HSR featuring rash are independent allele or haplotype effects and whether associations between HLA alleles and NVP HSR may be confounded due to linkage disequilibrium with other genes on chromosome 6. In our cohort, the numbers are too small to perform linkage analysis between the two markers, however the 6/7 individuals who carried the HLA-Cw*04 allele also carried HLA-B*35:01. Some studies have identified HLA-Cw*04 as significant but not examined HLA-B*35 [15, 19], while others have implicated both alleles in Asian populations [20]. Individual allele analysis and a GWAS in relation to NVP induced rash in a Thai cohort showed that HLA-B*35:05 and HLA-Cw*04 were higher in the HSR group, however only HLA-B*35:05 remained significant with correction [18, 19]. Another group more recently reported an association between NVP SJS/TEN and HLA-C*04:01 in a Malawian population however SJS/TEN is a rare reaction with a differing immunopathogenesis that was not seen in our NVP HSR study [14].

Alleles outside of the MHC may contribute to phenotype specific adverse reactions observed for NVP. A GWAS and independent replication studies revealed 5 SNPs on chromosome 6 that were significantly associated with NVP-induced rash within a 30kb region containing the CCHCR1 gene, and 2 SNPs within the gene significantly associated with rash [27]. Complete screening of the CCHCR1 locus found one nonsynonymous SNP, rs1576, significantly associated with NVP induced rash. The CCHCR1 locus has previously been associated with psoriasis susceptibility and may be involved in keratinocyte proliferation [28]. Thus, it is likely that this association is specific to the rash phenotype of NVP HSR. Although the CCHCR1 gene is located 110 kb telomeric from the HLA-C locus and 210 kb from the HLA-B locus, logistic regression analysis indicated that the association of SNPs in the CCHCR1 gene with nevirapine-induced rash was independent from that of the HLA-B*3505 allele [27]. This study raises the issue that other genetic associations may also contribute to phenotype specific NVP HSR.

Polymorphism in drug metabolising enzymes and in particular CYP2B6 516G→T and 983T→C have been shown to be associated with NVP exposure [29-31]. Some who have not considered HLA have not shown a direct relationship between oral clearance and development of NVP HSR [29] while others have shown, cutaneous adverse events have been associated with CYP2B6 516G→T HSR in African Americans carrying HLA-Cw4 [32]. This suggests that the development of class I mediated NVP HSR is dependent on HLA carriage as well as accumulation of the parent drug. In contrast, hepatic symptoms have not been linked to such metabolism and suggest a MHC Class II restricted CD4+ T-cell dependent mechanism [20]. Although, nevirapine has also

shown to be a substrate for efflux transporter ABCC10, and genetic variants influence plasma concentrations, the clinical implication is not yet known [33]. Prolonged exposure to any antiretroviral therapy, coinfection with hepatitis C virus (HCV) and abnormal baseline levels of alanine aminotransferase place patients at a higher risk of developing hepatotoxicity due to NVP exposure [34, 35], however HCV and HBV coinfection were excluded in our cohort for individuals with hepatotoxicity.

Animal studies also highlight the duality of the immune response to NVP in hypersensitivity. In the NVP rash model, rats show no evidence of hepatotoxicity and animal T cells produce $\text{INF}\gamma$ in response to NVP rather than to 12-OH-NVP [36]. Taken together with the responses seen here in systemic NVP-HSR patient PBMCs, the evidence suggests another pathway for induction of the systemic immune response. Keratinocytes can upregulate the expression of MHC Class I/II molecules on APCs and stimulate antigen-experienced T cells, however the consensus is that they are unlikely to be able to prime new T-cell responses [37]. Thus, it would appear that the mechanism in the NVP induced skin rash may propagate systemic stimulatory signals for T cells activated by NVP via another pathway.

The rapid waning of *ex vivo* responses to NVP supports a delayed type hypersensitivity model in which drug induced T-cell activation is mediated by the associated HLA allotypes. Naive T cells are primed on initial exposure to NVP and a memory pool is restimulated on repeat exposure. We suggest that a required threshold of NVP specific memory cells are necessary in order to stimulate a response *ex vivo*, or perhaps that a second antigen specific T-cell activating signal/pro-inflammatory stimulus is required to

sustain the memory T cell pool to NVP. Non-drug specific T-regulatory responses may play a role in the rapid waning of immune responses. Some studies have suggested reactivation of EBV, HHV-6 and CMV, and corresponding virus specific T-regulatory cells in patients with DRESS [25, 26]. We found no evidence of viral reactivation in our patients with NVP HSR. In contrast to the HLA-B*57:01 restricted CD8+ dependent abacavir hypersensitivity reaction where CD4+ help is not needed, and abacavir specific IFN γ responses are durable over time, both CD4+ and CD8+ T cells are required for the initiation and maintenance of the immune response to NVP which appears to wane quite rapidly in most patients.

Work examining T cell populations in anti-convulsant drug-induced hypersensitivity have observed CD4(+) T-cell proliferation and drug-specific CD8(+) CTLs in the acute stages of disease and switching between the dominant cell populations [38]. In one case, drug-specific CD4(+) CD25(+) Foxp3(+) regulatory T cells (Tregs) proliferated during the recovery stage after withdrawal of the drug and a similar effect was observed here for *patient 4*. However, suppressive activity of Tregs did not explain the wane in response observed after the withdrawal of the drug in cellular assays in this case. Further evaluation in a larger patient group using cell sorting to remove the Tregs may provide further insight.

For the first time we demonstrate a role for both CD4+ and CD8+ T cells in NVP HSR through both HLA class I and II associations and characterization of cellular responses. Although future studies incorporating larger samples sizes are needed to validate these findings, the examples presented show that precisely phenotyped populations with

differing HSR clinical characteristics are important tools to further define the immunopathogenesis of HLA-restricted drug HSR. The complexity of these findings and the low positive predictive value of HLA typing for NVP HSR illustrate the restrictions of implementing such testing “at the bedside” prior to NVP prescription [39]. However, this and other studies examining NVP HSR highlight that drug metabolism genotyping, viral serology, precise phenotyping of the HSR reaction and HLA typing can potentially aid in clear diagnosis of the various drug HSR phenotypes for NVP and identify at risk patients.

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REFERENCES

1. Gangar M, Arias G, O'Brien JG, Kemper CA. Frequency of cutaneous reactions on rechallenge with nevirapine and delavirdine. *Ann Pharmacother* 2000; 34: 839-42.
2. Pollard RB, Robinson P, Dransfield K. Safety profile of nevirapine, a nonnucleoside reverse transcriptase inhibitor for the treatment of human immunodeficiency virus infection. *Clin Ther* 1998; 20: 1071-92.
3. Bersoff-Matcha SJ, Miller WC, Aberg JA, van Der Horst C, Hamrick Jr HJ, Powderly WG, *et al.* Sex differences in nevirapine rash. *Clin Infect Dis* 2001; 32: 124-9.
4. Bonjoch A, Paredes R, Domingo P, Cervantes M, Pedrol E, Ribera E, *et al.* Long-term safety and efficacy of nevirapine-based approaches in HIV type 1-infected patients. *AIDS Res Hum Retroviruses* 2006; 22: 321-9.
5. Gonzalez de Requena D, Nunez M, Jimenez-Nacher I, Soriano V. Liver toxicity caused by nevirapine. *AIDS* 2002; 16: 290-1.
6. Manfredi R, Calza L. Safety issues about nevirapine administration in HIV-infected pregnant women. *J Acquir Immune Defic Syndr* 2007; 45: 365-8.
7. Peters PJ, Stringer J, McConnell MS, Kiarie J, Ratanasuwan W, Intalapaporn P, *et al.* Nevirapine-associated hepatotoxicity was not predicted by CD4 count \geq 250 cells/ μ L among women in Zambia, Thailand and Kenya. *HIV Med* 2010; 11: 650-60.
8. Shenton JM, Popovic M, Chen J, Masson MJ, Uetrecht JP. Evidence of an immune-mediated mechanism for an idiosyncratic nevirapine-induced reaction in the female Brown Norway rat. *Chem Res Toxicol* 2005; 18: 1799-813.
9. Taiwo BO. Nevirapine toxicity. *Int J STD AIDS* 2006; 17: 364-9; quiz 70.
10. Martin AM, Nolan D, James I, Cameron P, Keller J, Moore C, *et al.* Predisposition to nevirapine hypersensitivity associated with HLA-DRB1*0101 and abrogated by low CD4 T-cell counts. *AIDS* 2005; 19: 97-9.
11. Phillips E, Bartlett JA, Sanne I, Lederman MM, Hinkle J, Rousseau F, *et al.* Associations between HLA-DRB1*0102, HLA-B*5801, and hepatotoxicity during initiation of nevirapine-containing regimens in South Africa. *J Acquir Immune Defic Syndr* 2013; 62: e55-7.
12. Vitezica ZG, Milpied B, Lonjou C, Borot N, Ledger TN, Lefebvre A, *et al.* HLA-DRB1*01 associated with cutaneous hypersensitivity induced by nevirapine and efavirenz. *AIDS* 2008; 22: 540-1.
13. Yang KL, Chen IH, Hsiao CK, Cherng JM, Yang KZ, Chang CC, *et al.* Polymorphism of HLA-B27 in Taiwanese Chinese. *Tissue Antigens* 2004; 63: 476-9.
14. Carr DF, Chaponda M, Jorgensen AL, Castro EC, van Oosterhout JJ, Khoo SH, *et al.* Association of human leukocyte antigen alleles and nevirapine hypersensitivity in a Malawian HIV-infected population. *Clin Infect Dis* 2013; 56: 1330-9.
15. Gao S, Gui XE, Liang K, Liu Z, Hu J, Dong B. HLA-Dependent Hypersensitivity Reaction to Nevirapine in Chinese Han HIV-Infected Patients. *AIDS Res Hum Retroviruses* 2011;
16. Gatanaga H, Yazaki H, Tanuma J, Honda M, Genka I, Teruya K, *et al.* HLA-Cw8 primarily associated with hypersensitivity to nevirapine. *AIDS* 2007; 21: 264-5.
17. Littera R, Carcassi C, Masala A, Piano P, Serra P, Ortu F, *et al.* HLA-dependent hypersensitivity to nevirapine in Sardinian HIV patients. *AIDS* 2006; 20: 1621-6.
18. Chantarangsu S, Mushiroda T, Mahasirimongkol S, Kiertiburanakul S, Sungkanuparph S, Manosuthi W, *et al.* HLA-B*3505 allele is a strong predictor for nevirapine-induced skin adverse drug reactions in HIV-infected Thai patients. *Pharmacogenet Genomics* 2009; 19: 139-46.

19. Likanonsakul S, Rattanatham T, Feangvad S, Uttayamakul S, Prasithsirikul W, Tunthanathip P, *et al.* HLA-Cw*04 allele associated with nevirapine-induced rash in HIV-infected Thai patients. *AIDS Res Ther* 2009; 6: 22.
20. Yuan J, Guo S, Hall D, Cammett AM, Jayadev S, Distel M, *et al.* Toxicogenomics of nevirapine-associated cutaneous and hepatic adverse events among populations of African, Asian, and European descent. *AIDS* 2011; 25: 1271-80.
21. Mallal S, Nolan D, Witt C, Masel G, Martin AM, Moore C, *et al.* Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *Lancet* 2002; 359: 727-32.
22. Keane N, Lucas A, Netto J, Almeida C-A, Martin A, Marriott D, *et al.* 2007. Use of the ELISpot assay to detect abacavir- and nevirapine-induced IFN-g responses in PBMC from HIV-infected patients with abacavir or nevirapine hypersensitivity reactions. In *AIS 2007*. Sydney, Australia
23. Keane NM, Roberts SG, Almeida CA, Krishnan T, Chopra A, Demaine E, *et al.* High-avidity, high-IFN γ -producing CD8 T-cell responses following immune selection during HIV-1 infection. *Immunol Cell Biol* 2012; 90: 224-34.
24. Hanafusa T, Azukizawa H, Matsumura S, Katayama I. The predominant drug-specific T-cell population may switch from cytotoxic T cells to regulatory T cells during the course of anticonvulsant-induced hypersensitivity. *J Dermatol Sci* 2012; 65: 213-9.
25. Picard D, Janela B, Descamps V, D'Incan M, Courville P, Jacquot S, *et al.* Drug reaction with eosinophilia and systemic symptoms (DRESS): a multiorgan antiviral T cell response. *Sci Transl Med* 2010; 2: 46ra62.
26. Criado PR, Avancini J, Santi CG, Medrado AT, Rodrigues CE, de Carvalho JF. Drug reaction with eosinophilia and systemic symptoms (DRESS): a complex interaction of drugs, viruses and the immune system. *Isr Med Assoc J* 2012; 14: 577-82.
27. Chantarangsu S, Mushiroda T, Mahasirimongkol S, Kiertiburanakul S, Sungkanuparph S, Manosuthi W, *et al.* Genome-wide association study identifies variations in 6p21.3 associated with nevirapine-induced rash. *Clin Infect Dis* 2011; 53: 341-8.
28. Asumalahti K, Veal C, Laitinen T, Suomela S, Allen M, Elomaa O, *et al.* Coding haplotype analysis supports HCR as the putative susceptibility gene for psoriasis at the MHC PSORS1 locus. *Hum Mol Genet* 2002; 11: 589-97.
29. Dickinson L, Chaponda M, Carr DF, van Oosterhout JJ, Kumwenda J, Lalloo DG, *et al.* Population pharmacokinetic and pharmacogenetic analysis of nevirapine in hypersensitive and tolerant HIV-infected patients from Malawi. *Antimicrob Agents Chemother* 2014; 58: 706-12.
30. Penzak SR, Kabuye G, Mugenyi P, Mbamanya F, Natarajan V, Alfaro RM, *et al.* Cytochrome P450 2B6 (CYP2B6) G516T influences nevirapine plasma concentrations in HIV-infected patients in Uganda. *HIV Med* 2007; 8: 86-91.
31. Wyen C, Hendra H, Vogel M, Hoffmann C, Knechten H, Brockmeyer NH, *et al.* Impact of CYP2B6 983T>C polymorphism on non-nucleoside reverse transcriptase inhibitor plasma concentrations in HIV-infected patients. *J Antimicrob Chemother* 2008; 61: 914-8.
32. Mahungu T, Smith C, Turner F, Egan D, Youle M, Johnson M, *et al.* Cytochrome P450 2B6 516G-->T is associated with plasma concentrations of nevirapine at both 200 mg twice daily and 400 mg once daily in an ethnically diverse population. *HIV Med* 2009; 10: 310-7.
33. Liptrott NJ, Pushpakom S, Wyen C, Fatkenheuer G, Hoffmann C, Mauss S, *et al.* Association of ABCC10 polymorphisms with nevirapine plasma concentrations in the German Competence Network for HIV/AIDS. *Pharmacogenet Genomics* 2012; 22: 10-9.

34. Martinez E, Blanco JL, Arnaiz JA, Perez-Cuevas JB, Mocroft A, Cruceta A, *et al.* Hepatotoxicity in HIV-1-infected patients receiving nevirapine-containing antiretroviral therapy. *Aids* 2001; 15: 1261-8.
35. Torti C, Costarelli S, De Silvestri A, Quiros-Roldan E, Lapadula G, Cologni G, *et al.* Analysis of severe hepatic events associated with nevirapine-containing regimens: CD4+ T-cell count and gender in hepatitis C seropositive and seronegative patients. *Drug Saf* 2007; 30: 1161-9.
36. Chen X, Harmanathan T, Mannargudi B, Gou H, Uetrecht J. A Study of the specificity of lymphocytes in Nevirapine-Induced skin rash. *Journal of Pharmacology and Experimental Therapeutics* 2009; 331: 836-41.
37. Gutowska-Owsiak D, Ogg GS. The epidermis as an adjuvant. *J Invest Dermatol* 2012; 132: 940-8.
38. Hung SI, Chung WH, Jee SH, Chen WC, Chang YT, Lee WR, *et al.* Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions. *Pharmacogenet Genomics* 2006; 16: 297-306.
39. Daly AK. Human Leukocyte Antigen (HLA) Pharmacogenomic Tests: Potential and Pitfalls. *Curr Drug Metab* 2014; 15: 196-201.