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Dossier: AIDS

Antibodies to Tat and Vpr in the GRIV cohort: differential association with maintenance of long-term non-progression status in HIV-1 infection

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Abstract

The HIV-1 regulatory protein Tat and the accessory protein Vpr are thought to stimulate viral replication and contribute to viral pathogenesis as extracellular proteins. Humoral immune responses to these early viral proteins may therefore be beneficial. We examined serum anti-Tat and anti-Vpr IgG by ELISA in the GRIV cohort of HIV-1 seropositive slow/non-progressors (NP) and fast-progressors (FP), and in seronegative controls. Based on information obtained during a brief follow-up period (median = 20 months), NPs were sub-grouped as those maintaining non-progression status and therefore stable (NP-S), and those showing signs of disease progression (NP-P). As the primary comparison, initial serum anti-Tat and anti-Vpr IgG (prior to follow-up) were analyzed in the NP sub-groups and in FPs. Anti-Tat IgG was significantly higher in stable NP-S compared to unstable NP-P ($P = 0.047$) and FPs ($P < 0.0005$); the predictive value of higher anti-Tat IgG for maintenance of non-progression status was 92% ($P = 0.029$). In contrast, no-difference was observed in anti-Vpr IgG between NP-S and NP-P, although both were significantly higher than FPs ($P \leq 0.001$). Serum anti-Tat IgG mapped to linear epitopes within the amino-terminus, the basic domain and the carboxy-terminal region of Tat in stable NP-S. Similar epitopes were identified in patients immunized with the Tat-toxoid in a Phase I study in Milan. High titer serum anti-Tat IgG from both GRIV and Milan cohorts cross-reacted in ELISA with Tat from diverse viral isolates, including HIV-1 subtype-E (CMU08) and SIVmac251 Tat; a correlation was observed between anti-Tat IgG titers and cross-reactivity. These results demonstrate that higher levels of serum anti-Tat IgG, but not anti-Vpr IgG, are associated with maintenance of non-progression status in HIV-1 infection. Evidence that vaccination with the Tat toxoid induces humoral immune responses to Tat similar to those observed in stable non-progressors is encouraging for vaccine strategies targeting Tat.

Keywords: HIV-1; GRIV; Tat; Vpr; Non-progression

1. Introduction

Development of a safe and effective vaccine for AIDS, even one that does not provide complete protection, is a top priority internationally. Although two large vaccine studies in humans targeting gp120 are still in progress [25], vaccines based solely on outer coat proteins do not appear to be effective. Induction of cytotoxic T-lymphocyte (CTL) responses to other more conserved viral proteins such as Gag now appear more promising [14] at least in the SHIV 89.6P model [9,54]. However, because smaller proteins contain fewer potential CTL epitopes, viral escape may be a real problem with single target vaccine strategies [2,3,8]. Similarly, vaccines based on single small viral proteins may not always induce CTL responses in genetically diverse populations. For these reasons, multi-subunit vaccines targeting several well-conserved key viral proteins may ultimately prove more effective. As a potential vaccine subunit, the early regulatory protein Tat is increasingly being incorporated in experimental vaccine studies, with mixed results in terms of outcome but clear immunogenicity [2,11,15,21,22,34,44,55]. In addition, other non-structural proteins such as Nef and Rev are also being studied as vaccine targets [18,23]. Despite varying results using Tat, Nef and Rev as vaccine targets in SHIV and SIV models of HIV-1 infection, the

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frequent targeting of these viral proteins by CTL responses in humans with HIV-1 infection supports their further evaluation [1]. CTL targeting of Tat also appears to be important in the SIV model for control of primary viremia [3], and viral escape from immune selective pressure on Tat has been documented [2,3]. Optimization of vaccination regimens may significantly improve the efficacy of experimental vaccines in the SHIV and SIV models [8,54]. Similarly, rational identification and incorporation of other potential viral protein targets in experimental vaccines based on their conservation, importance in the viral life cycle and apparent immunogenicity in both cellular and humoral branches of the immune system—particularly in long-term non-progressors—may speed the development of an effective vaccine for AIDS.

The HIV-1 early regulatory protein Tat [5] is essential in the viral life cycle [12], and key functional domains of Tat are well conserved [19,20]. Tat is required to stimulate efficient elongation of viral transcripts, and acts as an RNA specific viral transcription factor to recruit the cellular transcriptional machinery via its interaction with the TAR RNA element present in all nascent viral transcripts [16] and host cellular proteins such as Cyclin-T/CDK9 [35]. Interestingly, Tat appears to be expressed even prior to viral integration [63], and is capable of stimulating a variety of cellular genes as an intracellular protein [17]. The HIV-1 accessory protein Vpr is also involved in viral transcription [58] and may interact with Tat directly [51]. In addition, Vpr is present in the virion [65], and acts as an immediate early protein to induce cell cycle arrest in G_2 and activate the viral LTR co-operatively with the Tat protein [51]. Vpr also appears to be involved in binding of the viral pre-integration complex to the nuclear pore complex [36], and may contribute to infectivity [53]. Like Tat, Vpr appears to be fairly well conserved [52], and although it is not essential for viral replication, mutations in Vpr impact viral replication adversely [50].

In addition to its intracellular activities, Tat is secreted from infected cells (Ensoli et al., 1993; Chang et al., 1997) and is present in serum [60,64]. Extracellular Tat is thought to exert numerous deleterious effects on the host immune system, including inducing immunosuppression [60,61] (Zagury, 1997). Interestingly, the HIV-1 accessory protein Vpr also appears to be present extracellularly [28], and may similarly exert immunomodulatory effects on the host immune system, including immunosuppression [6,29]. Because Tat and Vpr are present and apparently biologically active in the extracellular milieu, humoral immune responses to these viral proteins may impact viral replication and pathogenesis, and hence slow disease progression. Although both Tat and Vpr appear to be targeted by cellular immune responses in humans [1,4,27], the humoral responses to these proteins appear to be somewhat different. A number of reports have demonstrated that antibodies to Tat are associated with non-progression to AIDS [39,40,49,66], and can block viral replication in vitro [39]. In contrast, antibodies to Vpr have not been shown to be associated with non-progression to AIDS [42,43], although antibodies to Vpr can block induction of viral replication by Vpr in vitro [28]. In order to examine and compare the association of antibodies to Tat and Vpr with slow/non-progression to AIDS, we analyzed levels of serum IgG to these viral proteins in the GRIV cohort [24,38,46,66] of HIV-1 seropositive slow/non-progressors (NP) and fast-progressors (FP). Of particular importance in the analysis, NP samples were later sub-grouped based information obtained during a brief follow-up period (median = 20 months) as those maintaining non-progression status during follow-up and therefore stable (NP-S), and those showing signs of disease progression (NP-P). Initial levels of serum IgG to Tat and Vpr (prior to follow-up) were compared between the two sub-groups, and the predictive value of antibodies to Tat and Vpr in maintenance of non-progression status was determined.

2. Materials and methods

2.1. Human serum samples

Human serum samples from HIV-1 seropositive slow/non-progressors (NP) and fast-progressors (FP) were obtained from the genetic resistance to human immunodeficiency virus (GRIV) cohort [24,38,46,66]. The GRIV cohort was established in France to identify immune responses and genetic polymorphisms associated with slow/non-progression to AIDS, and consists solely of serum and PBMC DNA samples from HIV-1 seropositive Caucasian individuals. Briefly, NPs were defined as having CD4+ T-cell counts > 500 cells/μl, despite being HIV-1 seropositive for at least 8 years without the benefit of anti-retroviral therapy (ART). Based on information obtained during a brief follow-up period (median = 20 months), NPs were sub-grouped as maintaining non-progression status and therefore stable (NP-S), and those showing signs of disease progression (NP-P); initial serum samples (prior to follow-up) were used for analysis of antibodies to Tat and Vpr in these sub-groups. FPs were defined as having CD4+ T-cell counts < 300 cells/μl within 2 years of seroconversion; some FPs received ART, including HAART regimens incorporating protease inhibitors. Control serum samples were collected from HIV-1 seronegative Caucasians with informed consent at Mt. Sinai Medical Center (New York, NY, USA), and Hahnemann Hospital of Drexel University (Philadelphia, PA, USA). Seronegative and HIV-1 seropositive serum samples from patients immunized with the Tat toxoid were obtained from a Phase I clinical trial in Milan, Italy [21,22].

2.2. Recombinant Tat and Vpr proteins

For analysis of anti-Tat IgG in the GRIV cohort, HIV-1 IIIB (pCV1) 86 amino-acid Tat was expressed in *E. coli* as a non-fusion protein and purified under native conditions by cation exchange chromatography, phase separation and reverse phase chromatography; the purified IIIB Tat was essen-

tially endotoxin-free by LAL assay (BioWhittaker, Walkersville, MD, USA). For reciprocal endpoint dilution and cross-reactivity ELISA, HIV-1 IIIB (pCV1) 86 amino-acid Tat, HIV-1 subtype E (CMU08) Tat, full-length S/HIV 89.6P Tat and a truncated 86 amino-acid 89.6P Tat, and SIVmac251 Tat were expressed in *E. coli* as N-terminal polyhistidine fusion proteins and purified essentially as previously described [44]. Briefly, polyhistidine Tat fusion proteins were purified initially by metal-chelate affinity chromatography under denaturing conditions. N-terminal polyhistidine fusion domains were then removed by Cyanogen Bromide (CnBr) cleavage and cleaved Tat proteins were re-folded at pH 5.0 by diafiltration. Following endotoxin removal by phase separation, cleaved Tat proteins were further purified by cation exchange chromatography, desalted by reverse phase chromatography and lyophilized by freeze-drying.

For analysis of serum anti-Vpr IgG, a cDNA encoding the 96 amino-acid HIV-1 89.6 Vpr protein was first amplified by RT–PCR from total RNA of 89.6 infected PBMC. RNA was purified using Tri-Reagent (Sigma, St. Louis, MO, USA). RT was performed with 1 μg of total RNA and oligo dT priming using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). PCR was performed using the primers F1 5' cagaggatagatggaacaagc 3' and B1 5' agcagttttaggctgacttc 3' and the following conditions: a 105 s hold at 94 °C, then five cycles of 94°/37°/72° each for 30 s, followed by 30 cycles of 94°/60°/72° each for 30 s, and 7 min extension at 72°. A cDNA encoding the 89.6 *vpr* gene was then gel purified using a gel extraction quick kit (Qiagen, Chatsworth, CA, USA) and ligated directly into the pCR3.1 TA cloning vector (Invitrogen, Carlsbad, CA, USA). The 89.6 *vpr* coding sequence was then amplified by PCR using primers F2 5' ggatccgatggaacaagccccagaagac 3' and B2 5' cccaagcttctaggatttactggctccatt 3', and sub-cloned into the *Bam*H1 and *Hin*dIII sites of pRSETB (Invitrogen) for expression in *E. coli* as an N-terminal polyhistidine fusion protein. The sequences of 89.6 *vpr* clones were confirmed by automated sequencing. 89.6 *vpr* was expressed in BL21CodonPlusRIL cells (Stratagene, La Jolla, CA, USA) using the CE6 bacteriophage system to deliver T7 RNA polymerase. N-terminal polyhistidine fusion 89.6 Vpr was purified by metal chelate affinity chromatography using Talon resin (Clontech, Palo Alto, CA, USA) under denaturing conditions and used directly in ELISA.

2.3. Tat peptides

Overlapping IIIB (pCV1) Tat peptides were synthesized at Hahnemann Hospital of Drexel University; serines were used in place of cysteines. BH10 Tat peptides *86*–101 and *cys* (20–39) were purchased from Tecnogen/Xeptagen, SpA, Piana di Monte Verna (CE), Italy.

2.4. ELISA

Enzyme linked immunosorbent assay (ELISA) was performed essentially as previously described (Engvall, 1971; [44]). Briefly, Lockwell C8 Maxisorb 96-well microtiter plates (Nalge Nunc, Rochester, NY, USA) were coated for 16 h at 4 °C with 0.5 μg/well Tat, Vpr or IIIB Tat peptides in 50 μl of 50 mM Na_2CO_3/$NaHCO_3$ pH 9.0. Plates were then washed 6× with 300 μl of 1 × PBS/0.05% Tween-20 using an automated 96-well microtiter plate washer (Dynex, Chantilly, VA, USA); plates were washed similarly between each subsequent step. Plates were then blocked for 4 h with 250 μl of 1 × PBS/3% Immunoglobulin-free BSA (Sigma, St. Louis, MO, USA). Plates were washed again, and serum was added at a dilution of 1:500 in 50 μl of 1 × PBS/1% BSA/0.05% Tween-20 for 16 h at 4 °C while shaking gently; titers were determined similarly using a range of dilutions. Positive and negative controls were used on each plate as standards. Plates were then washed again, and secondary antibody was added in 50 μl of 1 × PBS/1% BSA/0.05% Tween-20 for 2 h at RT while shaking gently. Protein G-HRP (BioRad, Hercules, CA, USA) was used at 1:1000 dilution for anti-Tat and anti-Vpr IgG ELISA, including anti-Tat IgG titers, cross-reactivity and linear epitope peptide mapping ELISA. Each sample was run in duplicate or triplicate; results are reflective of two or more separate experiments. Each serum sample was tested in parallel on BSA coated wells; background BSA reactivity was subtracted from each sample's mean optical density in Tat, Tat peptide, or Vpr coated wells. Background subtraction was particularly important for peptide mapping. The signal to noise ratio was low for unvaccinated GRIV HIV-1 seropositive samples; without subtracting the background and using seronegative controls in parallel it would be easy to conclude erroneously that all of Tat is immunogenic given higher backgrounds in many HIV-1 seropositive GRIV samples, presumably due to higher levels of IgG overall coincident with viral infection. After background subtraction, positivity in peptide mapping ELISA was determined based on a cutoff value calculated as the mean optical density reading of seronegative controls plus three standard deviations for each peptide. For cross-reactivity ELISA, samples were first determined to be positive or negative for each Tat protein based on a cutoff value determined as the mean optical density (O.D.) of HIV-1 seronegative controls ($n = 9$) plus three standard deviations.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 10.0 (SPSS Inc., Chicago, IL) for Windows (Microsoft, Redmond, WA, USA). ANOVA with Dunnett's T3 correction for multiple comparisons was performed to compare serum anti-Tat and anti-Vpr IgG between groups; *P*-values are reported on an adjusted scale with significance set at $\propto = 0.05$ (SPSS 10.0). As the primary comparison, differences in anti-Tat and anti-Vpr IgG were compared between the two NP sub-groups (NP-S and NP-P) and FPs by ANOVA with Dunnett's T3 correction for multiple comparisons (three groups). The two NP sub-groups together comprise all the NP samples, with the exception of four NP samples for which follow-up information was unavailable; all samples were collected prior to

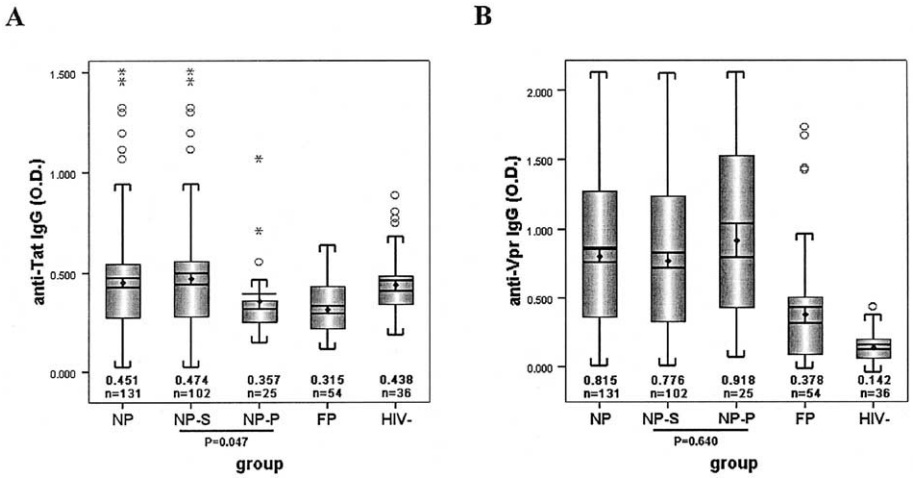

Fig. 1. Serum anti-Tat and anti-Vpr IgG levels in the HIV-1 seropositive GRIV cohort and in seronegative controls. (A) Anti-Tat IgG was significantly higher in the slow/non-progressor (NP) sub-group stable during subsequent follow-up (NP-S) compared to both NP unstable during follow-up (NP-P; $P = 0.047$) and fast-progressors (FP; $P < 0.0005$). There was no difference in anti-Tat IgG between NP-S and seronegative controls, although levels were higher in seronegatives compared to FPs ($P = 0.002$). (B) There was no difference in anti-Vpr IgG between NP-S and NP-P, although levels in both were significantly higher compared to FPs ($P < 0.0005$). Anti-Vpr IgG levels were significantly lower in seronegative controls compared to NP-S, NP-P and also FPs ($P < 0.0005$).

subsequent follow-up. Differences in distribution of NP-S and NP-P samples with high and low anti-Tat and anti-Vpr IgG were evaluated by Chi-square analysis. Correlations were assessed using Spearman's Rank test.

3. Results

3.1. Serum anti-Tat and anti-Vpr IgG

In order to determine if humoral immune responses to the viral proteins Tat and Vpr are associated with slow/non-progression to AIDS, and/or maintenance of non-progression status, ELISA was performed to evaluate serum anti-Tat and anti-Vpr IgG in GRIV slow/non-progressors (NP), non-progressor follow-up sub-groups stable (NP-S) and unstable (NP-P), fast-progressors (FP) and seronegative controls (HIV–). Comparing all five groups, serum anti-Tat IgG was significantly higher in NPs overall ($P < 0.0005$) and the stable sub-group NP-S ($P < 0.0005$) compared to FPs (Fig. 1A). Although serum anti-Tat IgG was higher in the stable NP-S sub-group compared to unstable NP-P, the difference was not significant after correcting for comparisons between five groups ($P = 0.145$). Surprisingly, serum anti-Tat IgG was also significantly higher in seronegative controls (HIV–) compared to FPs ($P = 0.003$). Similarly, anti-Tat IgG was not significantly different in seronegatives compared to NP overall or the stable NP-S and NP-P sub-groups, suggesting natural IgG recognizing domains in Tat may be present prior to infection, as has been demonstrated with IgM [49].

Examining HIV-1 seropositive samples for serum anti-Tat IgG more closely, comparison of the NP sub-groups NP-S and NP-P, and FPs (three groups) revealed that anti-Tat IgG was significantly higher in stable NP-S relative to both unstable NP-P ($P = 0.047$) and FP ($P < 0.0005$). As an additional comparison, Chi-square analysis was performed with samples from NP-S and NP-P sub-groups classified as either anti-Tat positive or negative based on a cutoff value calculated as the mean of the HIV-1 seronegative group plus two standard deviations (O.D. = 0.758). Chi-square analysis was also performed with samples from NP-S and NP-P sub-groups classified as either anti-Tat "high" or "low" based on a cutoff value calculated as the upper bound of the 95% confidence interval of the mean of the NP group overall (O.D. = 0.498). There was no difference in the distribution of anti-Tat positive samples between NP-S (13/102; 12.7%) and NP-P (1/25; 4%) by Chi-square analysis ($P = 0.211$). However, the difference in distribution of anti-Tat "high" or "low" between NP-S (35/102; 34.3%) and NP-P (3/25; 12%) was significant (Table 1; $P = 0.029$). Furthermore, the predictive value of higher initial anti-Tat IgG (prior to follow-up) in ELISA for maintenance of non-progression status during subsequent follow-up was 92% (Table 1), comparable to

Table 1
Distribution of the NP sub-groups NP-S and NP-P with high and low anti-Tat and anti-Vpr IgG

(A)			
NP sub-groups	High anti-Tat IgG	Low anti-Tat IgG	Total
NP-S	35*	67	102
NP-P	3	22	25
Total	38	89	127
(B)			
NP sub-groups	High anti-Vpr IgG	Low anti-Vpr IgG	Total
NP-S	38**	64	102
NP-P	13	12	25
Total	51	76	127

* Higher initial levels of anti-Tat IgG were 92% predictive of maintenance of non-progression status during subsequent follow-up; the number of NP-S with high anti-Tat IgG was significantly different than NP-P by Chi-square analysis ($P = 0.029$).

** In contrast, higher initial levels of anti-Vpr IgG were not predictive of continued non-progression during follow-up; the number of NP-S with high anti-Vpr IgG was not significantly different than NP-P.

TREC, CD4+ T-cell count, viral load and p24 antigenemia [45]; the sensitivity was 34.3%. In contrast, the predictive value of lower initial anti-Tat IgG for progression was 24.7%, also comparable to TREC, CD4+ T-cell count, viral load and p24 antigenemia. Hence, high anti-Tat IgG levels were associated with slow/non-progression to AIDS, and higher initial levels of anti-Tat IgG were predictive of maintenance of non-progression status during a subsequent follow-up period. An inverse correlation was observed in NP-S between anti-Tat IgG and p24 antigenemia ($r_s = –0.467$; $P_s < 0.0005$), as previously reported [66].

A different pattern was observed with humoral immune responses to Vpr. Comparing all five groups, serum anti-Vpr IgG was significantly higher in NPs overall ($P < 0.0005$), the stable NP-S sub-group ($P < 0.0005$) and the unstable NP-P sub-group ($P \leq 0.001$) compared to both FPs and seronegative controls (Fig. 1B). Anti-Vpr IgG was also significantly higher in FPs compared to seronegative controls ($P < 0.0005$). Examining HIV-1 seropositive samples for serum anti-Vpr IgG more closely, comparison of the NP sub-groups NP-S and NP-P, and FPs (three groups) revealed no difference between NP-S and NP-P sub-groups, unlike anti-Tat IgG, although both were higher compared to FPs ($P < 0.0005$). As an additional comparison, Chi-square analysis was performed with samples from NP-S and NP-P sub-groups classified as either anti-Vpr positive or negative based on a cutoff value calculated as the mean of the HIV-1 seronegative group plus three standard deviations (O.D. = 0.586). Chi-square analysis was also performed with samples from NP-S and NP-P sub-groups classified as either anti-Vpr "high" or "low" based on a cutoff value calculated as the upper bound of the 95% confidence interval of the mean of the NP group overall (O.D. = 1.071). There was no significant difference in the distribution of anti-Vpr positive samples between NP-S (79/102; 77.5%) and NP-P (21/25; 84%) by Chi-square analysis ($P = 0.364$). Similarly, there was also no significant difference in distribution of anti-Vpr IgG "high" or "low" between NP-S (38/102; 37.3%) and NP-P (13/25; 52%) by Chi-square (Table 1; $P = 0.178$). Although the difference was not significant, clearly a different pattern was observed with antibodies to Vpr compared to Tat. Initial anti-Vpr IgG levels appeared to be somewhat lower in NP-S compared to NP-P; fewer NP-S had high levels of anti-Vpr IgG compared to NP-P on a percentage basis. There was no correlation between serum anti-Vpr and anti-Tat IgG levels. Hence, although higher levels of anti-Vpr IgG were associated with slow/non-progression to AIDS, higher initial levels of anti-Vpr IgG were not predictive of maintenance of non-progression status during a subsequent follow-up period.

3.2. Linear epitope peptide mapping ELISA

In order to identify linear epitopes within Tat recognized by anti-Tat IgG, peptide mapping ELISA was performed using linear overlapping peptides derived from HIV-1 IIIB (pCV1) Tat. Linear epitopes of serum anti-Tat IgG were mapped in HIV-1 seronegative controls ($n = 7$), GRIV non-progressors stable during subsequent follow-up (NP-S; $n = 19$), unstable non-progressors (NP-P; $n = 1$), fast-progressors (FP; $n = 3$), and HIV-1 seronegative ($n = 3$) and HIV-1 seropositive ($n = 8$) Milan patients vaccinated with the Tat toxoid. GRIV samples used in peptide mapping ELISA had anti-Tat IgG against Tat protein designated as "high" in ELISA—defined as having a mean optical density above the upper 95% confidence interval of the NP group overall (O.D. = 0.498). For each sample, background subtraction of reactivity with BSA was performed prior to determining positivity. Positivity in peptide mapping ELISA was determined for each peptide based on a cutoff value calculated as the mean optical density (O.D.) of seronegative controls plus three standard deviations. Anti-Tat IgG from GRIV stable NP-S recognized linear epitopes primarily within the amino-terminus, the basic domain, and the carboxy-terminal region of Tat; several samples reacted with peptide 6.1 spanning the activation domain, but not a similar peptide "*cys*" containing cysteines (Fig. 2A). In contrast, anti-Tat IgG in fast-progressors (FP) and in a single unstable non-progressor (NP-P) mapped only to the basic domain (data not shown). Unvaccinated HIV-1 seronegative controls also recognized the basic domain of Tat to some degree, and perhaps the activation domain in a two samples (data not shown). In both seronegative and HIV-1 seropositive Milan patients vaccinated with the Tat toxoid, anti-Tat IgG recognized linear epitopes primarily within the amino-terminus and the carboxy-terminal region, although some reactivity was observed with the basic domain; one sample also reacted with peptide 6.1 spanning the activation domain, but not a similar peptide "*cys*" containing cysteines (Fig. 2B). Interestingly, in addition to being present in fewer samples, reactivity with peptides overlapping the basic domain region was much less intense compared to the amino-terminus and carboxy-terminal regions; only a small boost in reactivity to the basic domain was detected after immunization, in three samples. Unlike GRIV HIV-1 seropositive NP-S, no reactivity was detected in Milan HIV-1 seropositives with peptide "86-" spanning the extended carboxy-terminal region present in some Tat proteins; this epitope was not present in the 86 amino-acid Tat toxoid derived from IIIB (pCV1) Tat.

3.3. Titers of serum anti-Tat IgG

Titers of serum anti-Tat IgG were analyzed by serial endpoint dilution in ELISA. Titers were determined for GRIV NP-S ($n = 11$), NP-P ($n = 1$), and FP ($n = 2$) samples with "high" levels of anti-Tat IgG; one NP sample for whom follow-up information was unavailable was also tested. In addition, titers were determined for seronegative ($n = 3$) and HIV-1 seropositve ($n = 7$) patients vaccinated with the inactive, carboxymethylated Tat toxoid [21]. Unvaccinated seronegative controls ($n = 6$) were tested in parallel; the mean O.D. reading of seronegative controls plus three standard deviations was used as the cutoff for positivity at each dilu-

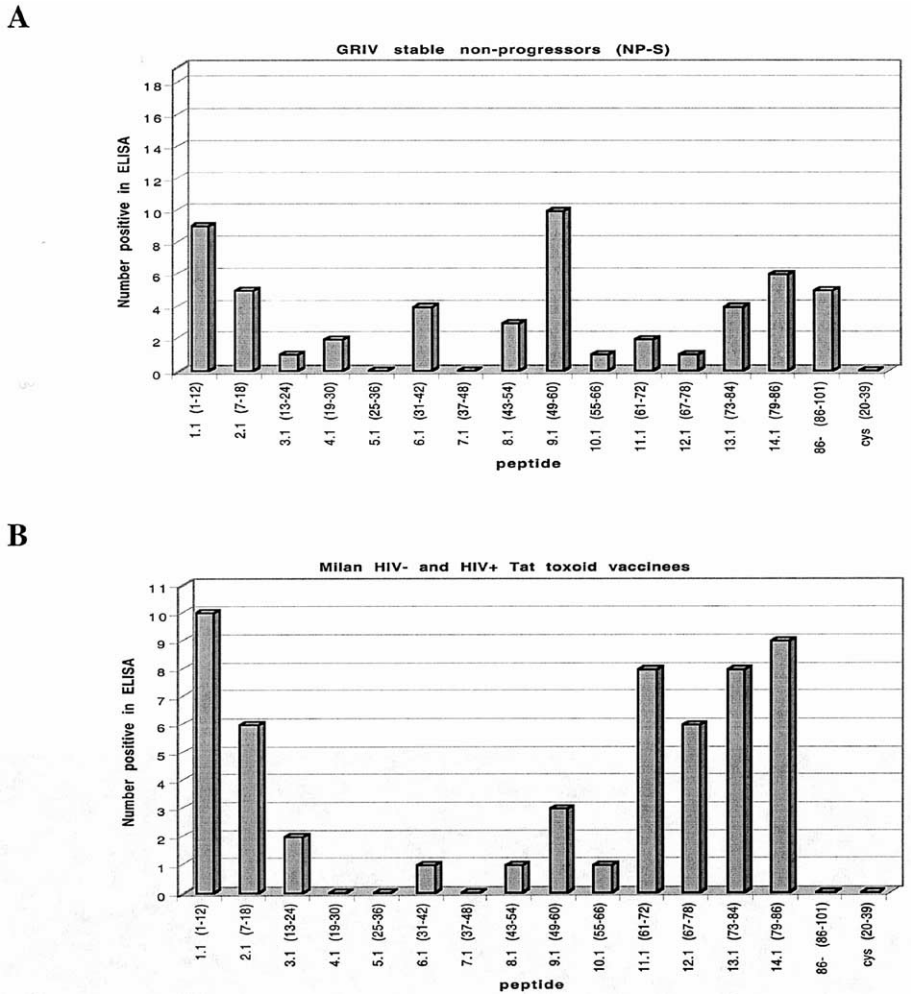

Fig. 2. Linear epitope peptide mapping ELISA of serum anti-Tat IgG. (A) Anti-Tat IgG in the GRIV HIV-1 seropositive non-progressor sub-group stable during subsequent follow-up (NP-S) maps to linear epitopes primarily within the amino-terminus, the basic domain, and the carboxy-terminal region, and possibly the activation domain. (B) Anti-Tat IgG in seronegative and HIV-1 seropositive Milan patients vaccinated with the Tat toxoid maps primarily to linear epitopes within the amino-terminus and the carboxy-terminal region, and to a lesser extent the basic domain.

tion tested. Titers ranged from 1:500 to above 1:10,000 – the highest dilution tested (data not shown).

3.4. Cross-reactivity of anti-Tat IgG

Because Tat is a well-conserved protein and broadly reactive immune responses to viral proteins may be beneficial, particularly for vaccine induced immunity in different geographic regions, the cross-reactivity of serum anti-Tat IgG with Tat proteins from diverse viral isolates was analyzed in ELISA. GRIV HIV-1 seropositive stable non-progressors (NP-S) and two fast-progressors (FP) with "high" levels of anti-Tat IgG were analyzed for cross-reactivity with a truncated 86 amino-acid S/HIV 89.6P Tat, full-length 102 amino-acid 89.6P Tat, HIV-1 subtype E (CMU08) Tat, and SIVmac251 Tat in ELISA (Fig. 3A). Anti-Tat IgG in a subset of GRIV NP-S and FP samples with "high" anti-Tat IgG levels cross-reacted in ELISA with the truncated 86 amino-acid 89.6P Tat, and to a lesser extent the full-length 102 amino-acid 89.6P Tat. Cross-reactivity was also apparent in several samples with HIV-1 subtype E (CMU08) Tat. One NP-S sample (136) reacted strongly with SIVmac251 Tat; this sample appears to recognize the extended carboxy-terminal region of Tat preferentially (data not shown). Cross-reactivity with the truncated 89.6P Tat was also observed in two FP samples with "high" levels of anti-Tat IgG; one sample also cross-reacted with full-length 89.6P Tat, CMU08 Tat and SIVmac251 Tat. To determine if vaccination induces broadly cross-reactive antibodies to Tat, serum from seronegative and HIV-1 seropositive Milan patients immunized with the Tat toxoid were also examined for cross-reactivity with Tat from diverse viral isolates in ELISA (Fig. 3B). Broad cross-reactivity was observed in a subset of Milan vaccines with the truncated 86 amino-acid 89.6P Tat, full-length 102 amino-acid 89.6P Tat, CMU08 Tat, and with SIVmac251 Tat. A strong correlation was observed between serum anti-Tat IgG titers and cross-reactivity in ELISA ($r_s \geq 0.508$; $P_s \leq 0.011$) in GRIV and Milan samples combined ($n = 24$). No correlation was observed between serum anti-Tat IgG titers or cross-reactivity with serum anti-Vpr IgG.

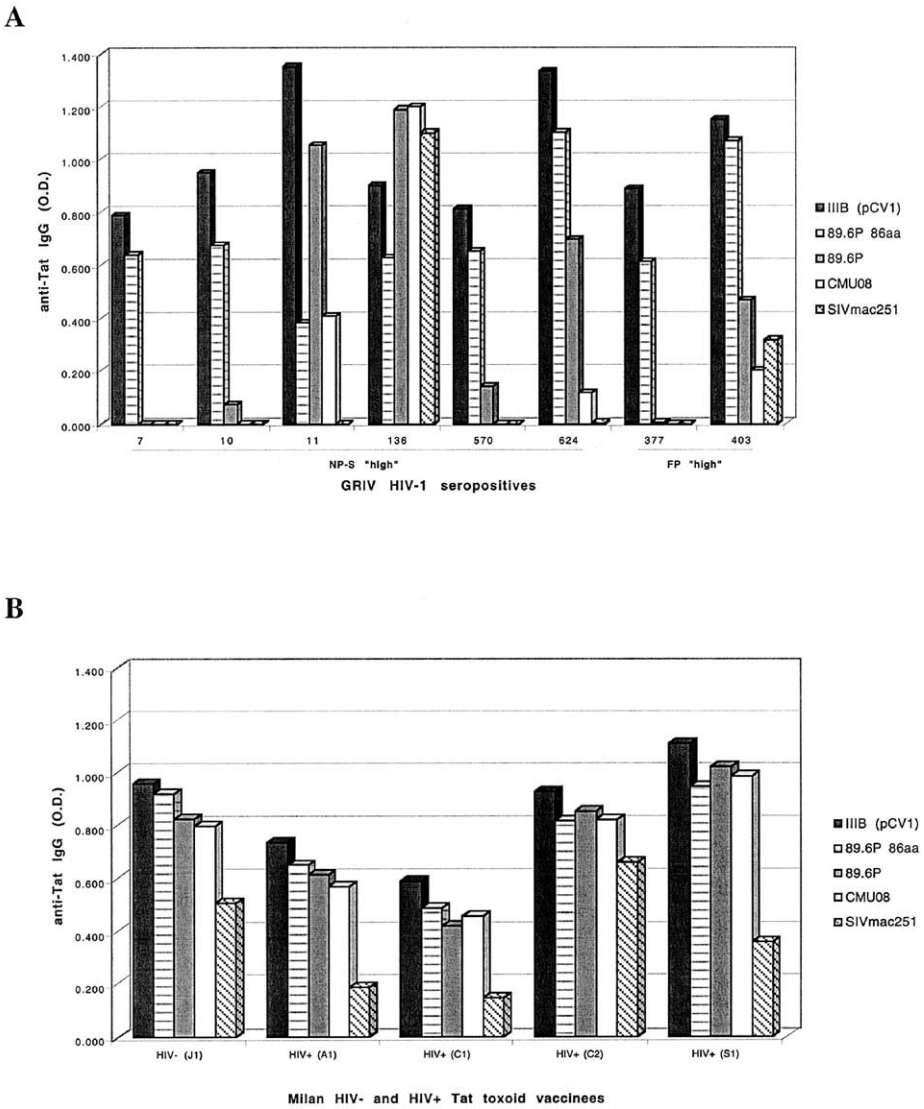

Fig. 3. High titer serum anti-Tat IgG cross-reacts with Tat from diverse viral isolates in ELISA. (A) Anti-Tat IgG in GRIV NP-S cross-reacts to varying degrees with a truncated 86 amino-acid S/HIV 89.6P Tat, full-length 89.6P Tat, HIV-1 subtype E (CMU08) Tat, and SIVmac251 Tat; cross-reactivity was also observed in two GRIV FP samples with "high" levels of anti-Tat IgG. (B) Cross-reactivity of high titer anti-Tat IgG in HIV-1 seronegative and seropositive Milan patients vaccinated with the Tat toxoid.

4. Discussion

Our results confirm the association of higher initial levels of anti-Tat IgG with maintenance of non-progression status during a subsequent follow-up period (median = 20 months). This association was previously demonstrated in a slightly smaller number of stable GRIV non-progressors (NP-S; $n = 78$) by stratifying the NP group overall based on "high" and "low" antibody responses to Tat, and analyzing the distribution of NP-S and NP-P samples by Chi-square analysis [66]. Furthermore, using the same dichotomized variable approach in a multivariate regression model, antibody to Tat was the most prominent predictor of disease progression, followed by viral load, antibodies to p24, Tetanus-toxoid and Nef, and p24 antigenemia. However, there was no difference in mean or median levels of anti-Tat IgG overall between unstratified NP-S and NP-P sub-groups. Here we demonstrate that there is a significant difference in anti-Tat IgG overall between unstratified NP-S and NP-P sub-groups. The difference in results between the two studies most likely reflects the use of a non-fusion Tat protein in ELISA in the current study, compared to the use of an N-terminal polyhistidine fusion protein in the previous study. The N-terminus of Tat is a key immunogenic domain in primates [44,55], and N-terminal polyhistidine fusion Tat proteins are considerably less reactive in ELISA compared to their non-fusion or cleaved Tat counterparts (data not shown). In addition, the predictive value of higher initial anti-Tat IgG levels for maintenance of non-progression status was calculated, and found to be similar to that of TREC, CD4+ T-cell count, p24 antigenemia and viral load [45].

In contrast to Tat, higher initial levels of antibodies to Vpr were not associated with maintenance of non-progression

status during subsequent follow-up. In fact, although the difference was not significant, mean levels of anti-Vpr IgG overall and both the number of samples positive for anti-Vpr IgG and the number with "high" levels of anti-Vpr IgG were somewhat higher in the unstable NP-P sub-group compared to NP-S. These results are somewhat in agreement with previous studies that found no association of antibodies to Vpr with non-progression to AIDS, although no association was found with disease progression either [42,43]. Interestingly, a separate study demonstrated a rise in serum Vpr in HIV-1 infected patients in parallel with p24 antigenemia [28], as might be expected given the presence of Vpr in the virion [65]. Hence, higher levels of anti-Vpr IgG in unstable NP-P may reflect higher viral loads and p24 antigenemia in this group compared to NP-S. These results do not preclude the potential benefit of humoral immune responses to Vpr earlier in infection; GRIV NPs overall had years of persistent infection (median = 11 years) at the time of sample collection, prior to follow-up. Indeed, although antibodies to Vpr were not predictive of maintenance of non-progression status during follow-up, higher levels were associated with slow/non-progression overall given significantly higher levels in both NPs compared to FPs. In view of evidence Vpr is targeted as frequently as Tat by CTL responses in humans [1,4], Vpr may warrant further evaluation as a vaccine component provided its potent immunosuppressive properties [6] can be removed by chemical or genetic modification.

Unlike previous studies in the GRIV [66] and other cohorts were measured in parallel with HIV-1 seronegative controls, levels of antibodies to Tat and Vpr in the GRIV cohort [42,43]. The lack of a significant difference in levels of anti-Tat IgG even in stable NP-P compared to seronegative controls supports now considerable evidence of natural antibodies to Tat [47–49], and suggests they may not be limited to IgM. It is conceivable that innate immunity to Tat may contribute to maintenance of stable non-progression status, or may prime the humoral immune response for subsequent exposure to Tat antigen. Alternatively, the highly basic nature of Tat may cause a higher background in seronegative samples due to non-specific interaction with various serum components, for example rheumatoid factor. In contrast, levels of antibodies to Vpr were significantly lower in seronegative controls even compared to fast-progressors (FP). Innate immunity to Vpr therefore does not seem to be a consideration. Of note, despite the lack of association with stable non-progression status, antibody levels to Vpr were quite high overall compared to Tat. Vpr is clearly immunogenic, perhaps in part due to its presence in the virion, although at somewhat variable copy number [56].

Because higher levels of antibodies to Tat, but not Vpr were associated with maintenance of non-progression status, linear epitope peptide mapping ELISA was performed to identify key immunogenic domains of Tat recognized by stable NP-S and associated with maintenance of non-progression status. Anti-Tat IgG mapped to linear epitopes within the amino-terminus, the basic domain and the carboxy-terminal region of Tat. Some reactivity was also seen with peptides overlapping the activation domain and containing serines in place of cysteines, but not with a peptide spanning this region and containing cysteines. Similar results were obtained with both seronegative and HIV-1 seropositive samples from individuals immunized with the Tat toxoid in a Phase I study in Milan, Italy [21,22]. We have previously observed a similar pattern of immunogenicity in rhesus macaques vaccinated with either HIV-1 IIIB or SHIV 89.6P Tat or Tat toxoid [44,55]. As seen with 89.6P versus IIIB vaccinated macaques, GRIV NP-S sample reactivity with the carboxy-terminal region of Tat seemed to extend somewhat further compared to vaccinated Milan patient samples—even in HIV-1 seropositive samples; the Milan patients were vaccinated with a Tat toxoid derived from the 86 amino-acid IIIB(pCV1) Tat, which has a truncated carboxy-terminus. Interestingly, unlike vaccinated rhesus macaques, [44,55] the Milan samples showed lower reactivity with the basic domain of Tat compared to the amino-terminus and the carboxy-terminal region, and only a small boost in reactivity to the basic domain following vaccination. This may reflect differences in vaccine preparations; clearly, the basic domain is immunogenic in humans. For example, three GRIV fast-progressor (FP) samples and one unstable NP-P with "high" antibodies to Tat mapped in parallel with NP-S and Milan samples recognized the basic domain exclusively (data not shown). It is worth noting that Tat may also contain non-linear B-cell epitopes [32], for example possibly spanning the amino and carboxyl termini [7].

Domains of Tat corresponding to linear epitopes identified in peptide mapping ELISA are associated with distinct biological activities of Tat. For example, the amino-terminus may be involved in inducing immuno-suppression through an interaction with CD26 [62]. Similarly, the basic domain of Tat facilitates its cellular uptake (Chang et al., 1997), is a strong nuclear localization signal [57], and mediates the interaction between Tat and the TAR RNA element in Tat mediated LTR transactivation [16]. The carboxy-terminal domain of Tat has been implicated in Tat mediated induction of IL-2 production [33], apoptosis [10] and is required for maximal LTR activation [26,37,59]. Antibodies recognizing these functional domains of Tat may impair Tat's biological activities as an extracellular protein, decrease viral replication and pathogenesis, and therefore contribute to maintenance of non-progression status.

Other studies have reported different results with linear epitope peptide mapping of anti-Tat IgG in HIV-1 infected humans. The entire Tat protein has been reported to be immunogenic [13,41]. However, these studies did not adjust for differences in background IgG reactivity, or use protein based blocking agents, although presumably they incorporated standard ELISA rather than high binding plates. Clearly, individuals with viral infections frequently have higher levels of IgG in serum, and hypergammaglobulinemia is a feature of HIV-1 infection in some individuals [30,31]. We observed higher levels of background reactivity in HIV-1

seropositive samples compared to seronegative controls, and therefore subtracted background reactivity of each sample (including controls) prior to scoring samples for positivity based on the mean observed in seronegatives plus three standard deviations. It is quite possible this approach is too conservative and may miss relevant epitopes. However, our results in humans are in good agreement with epitopes identified in Tat vaccinated rhesus macaques, and predicted using standard antigenicity algorithms. Furthermore, similar results were observed in GRIV NP-S samples compared to Milan samples vaccinated with the Tat toxoid—with the exception of reactivity to the extended carboxy-terminus of Tat present in the majority of primary isolates but not present in the IIIB based Milan Tat toxoid.

In order to determine how broad-based antibody responses to Tat were, we examined the ability of anti-Tat IgG in GRIV and Milan samples to cross-react with Tat from diverse viral isolates in ELISA. Several GRIV NP-S samples with "high" levels of anti-Tat IgG cross-reacted with a truncated 86 amino-acid SHIV 89.6P Tat, and to a lesser extent the full-length 102 amino-acid Tat. In addition, cross-reactivity was observed in several samples with HIV-1 subtype-E (CMU08) Tat, and in one sample with SIVmac251 Tat, demonstrating the ability of anti-Tat IgG to recognize Tat from diverse viral isolates. Cross-reactivity was also observed in two FP samples with "high" levels of anti-Tat IgG, demonstrating that recognition of a single conserved domain alone may provide broad-based responses to diverse Tat proteins. It is also possible, however, that protection in vivo may require recognition of more than one conserved domain given the differences in linear epitopes identified in stable NP-S versus unstable NP-P and FPs. Milan Tat toxoid vaccine samples also cross-reacted with diverse Tat proteins in ELISA. The larger, less homologous SIVmac251 Tat was less reactive in both GRIV and Milan samples compared to the SHIV 89.6P Tat and HIV-1 subtype E (CMU08) Tat. A strong correlation was observed with anti-Tat IgG titers and cross-reactivity with diverse Tat proteins. Similar results were observed previously with Tat vaccinated macaques [44,55].

5. Conclusions

Humoral immune responses to the early HIV-1 regulatory protein Tat appear to be associated with maintenance of non-progression status in HIV-1 infection. Furthermore, vaccination with the Tat toxoid induces humoral immune responses to Tat similar to those present in stable GRIV slow/non-progressors (NP-S). In contrast to Tat, humoral responses to the viral accessory protein Vpr do not appear to be associated with maintenance of non-progression status, although we cannot exclude the possibility they may be of benefit early in infection. Tat appears to be a more promising vaccine candidate at this point based on the immunogenicity of Tat in vaccine studies in rhesus macaques and humans, the association of humoral immune responses to Tat with maintenance of non-progression status in humans, and the frequent targeting of Tat by CTL responses in humans and macaques. Evidence that Tat targeted immunization in the SIV model induced immune selective pressure leading to viral escape [2] is particularly encouraging. Similarly, although the rapid decline of CD4+ T-cells appears to preclude the potential role of Tat vaccine induced immune responses in controlling viral replication in the SHIV model [44,55], the immunogenicity of Tat in both humoral and cellular branches of the immune system was impressive. Further studies in SIV models of HIV-1 infection using Tat as a component of a multi-targeted approach are underway, as are larger Phase I clinical trials of Tat toxoid alone in humans.

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