The avidity and lytic efficiency of the CTL response to HTLV-1[1]

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Abstract

In human T-lymphotropic virus type 1 (HTLV-1) infection, a high frequency of HTLV-1-specific CTLs can co-exist stably with a high proviral load and the proviral load is strongly correlated with the risk of HTLV-1-associated inflammatory diseases. These observations led to the hypothesis that HTLV-1 specific CTLs are ineffective in controlling HTLV-1 replication but contribute to the pathogenesis of the inflammatory diseases. But evidence from host and viral immunogenetics and gene expression microarrays suggests that a strong CTL response is associated with a low proviral load and a low risk of HAM/TSP. Here, we quantified the frequency, lytic activity and functional avidity of HTLV-1-specific $CD8^+$ cells in fresh, unstimulated PBMCs from individuals with natural HTLV-1 infection. The lytic efficiency of the $CD8^+$ T-cell response – the fraction of autologous HTLV-1-expressing cells eliminated per $CD8^+$ cell per day – was inversely correlated with both the proviral load and the rate of spontaneous proviral expression. The functional avidity of HTLV-1-specific $CD8^+$ cells was strongly correlated with their lytic efficiency. We conclude that efficient control of HTLV-1 in vivo depends on the CTL lytic efficiency, which depends in turn on CTL avidity of antigen recognition. CTL 'quality' determines the position of virus-host equilibrium in persistent HTLV-1 infection.

Introduction

The retrovirus human T-lymphotropic virus type 1 (HTLV-1) causes two distinct types of disease: a malignancy of $CD4^+$ T cells known as adult T-cell leukaemia/lymphoma (ATLL) and a range of inflammatory diseases, of which HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is the best recognized and most widely studied. Over 90% of HTLV-1-infected individuals develop no associated disease. The reasons why the minority develop these conditions are not yet identified. A high proviral load of HTLV-1, i.e. a high frequency of provirus-carrying cells in the circulation, is associated with a high risk of developing HTLV-1-associated diseases (1); however, the factors that determine an individual's proviral load of HTLV-1 are poorly defined.

HTLV-1 infection elicits a strong cytotoxic T lymphocyte (CTL) response (2). The frequency of HTLV-1-specific CTLs is often very high: 1 to 10% of circulating $CD8^+$ T cells can recognize a single epitope in the immunodominant CTL target antigen, Tax protein (3). Furthermore, the frequency of specific CTLs may be positively correlated with the proviral load of HTLV-1 (4), and can be particularly high in patients with HAM/TSP (5). These observations gave rise to the suggestion that HTLV-1-specific CTLs not only fail to eradicate the virus but may in fact cause the inflammatory tissue damage seen in HAM/TSP (6). However, there is also evidence from a number of experimental approaches that the CTL response to HTLV-1 is a major determinant of the control of the proviral load and is thus important in the protection against HTLV-1-associated diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). An immunogenetic study showed a strong association between a lower risk of developing HAM/TSP and a particular MHC class I

genotype in a group of HTLV-1 patients in Japan (3). Using DNA expression microarrays it was found that genes that encode cytotoxicity effector proteins are expressed at a higher level in $CD8^+$ cells of patients with low proviral load than in those with high proviral load (7). Finally, Tax protein, the immunodominant antigen recognized by HTLV-1-specific CTLs (8) (9) (10) is subject to positive selection in vivo (11) (12) (13); the only plausible selection force that has been suggested is immune selection exerted by the strong HTLV-1-specific CTL response.

We have hypothesized that the steady-state magnitude of the HTLV-1 proviral load, which is strongly correlated with the risk of HAM/TSP (1), is determined by the balance between the survival and proliferation of infected cells and clearance of productively infected cells by the abundant virus-specific CTLs (14) (15). Further, we propose that a critical factor that determines the outcome of HTLV-1 infection is the 'efficiency' of the HTLV-1-specific CTL response (14, 15).

We have therefore investigated methods of quantifying antiviral CTL 'efficiency'. In HTLV-1 infection, both effector CTLs and infected target cells are often present in fresh blood at frequencies sufficiently high to obviate the need for enrichment of specific subpopulations. We have exploited this feature to develop an assay of $CD8^+$ cell-mediated suppression of HTLV-1 expression in fresh PBMCs (16). As a marker of proviral expression we use the viral protein Tax, a regulatory protein expressed early in the life cycle of HTLV-1 (16). We previously showed that this suppression of HTLV-1 depended on $CD8^+$ T cell frequency and required both perforin and a match in class 1 MHC genotype between effector and target cells (17), consistent with classical class 1 MHC-restricted CTL lysis. Mathematical modelling can

be used to quantify the rate of killing of Tax-expressing $CD4^+$ cells per $CD8^+$ cell per day. We use the term 'lytic efficiency' to denote this per-$CD8^+$-cell rate of lysis. This assay of lytic efficiency showed that the rate of CTL-mediated lysis of HTLV-1-infected cells in fresh PBMCs was inversely correlated with the proviral load, both in patients with HAM/TSP and in asymptomatic HTLV-1 carriers (ACs) (16).

This measure of lytic efficiency has two chief limitations. First, the antiviral activity is expressed per $CD8^+$ cell, not per virus-specific $CD8^+$ cell, since there is no currently available method to measure in the same assay the lytic activity and the total frequency of $CD8^+$ T cells specific to all viral epitopes in each individual. Second, rate of lysis is likely to be a composite parameter that is a function of both the frequency of the antigen-specific $CD8^+$ cells and the "quality" of their effector functions at the single-cell level (18). In an acute viral infection, efficient elimination of the virus is associated with a high frequency of antigen-specific T cells (19). But in persistent infections, the complexity of the equilibrium dynamics makes it impossible to infer the efficiency of virus-specific CTLs directly from their steady-state frequency (20).

In the present study, we used the sensitivity of viral peptide recognition in an IFN-γ ELISpot assay as a measure of the 'quality' of the HTLV-1-specific $CD8^+$ response. This measure is often called the 'functional avidity' of the T cell; for brevity, we refer to it here as 'avidity'(21) (22). The aim of the present study was to test the hypothesis that specific $CD8^+$ T cell avidity determines the lytic efficiency of HTLV-1-specific CTLs and thereby determines the individual's proviral load.

Materials and Methods

PBMC separation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation using Histopaque-1077 (Sigma, Poole, United Kingdom) from EDTA-anticoagulated blood samples taken from HTLV-1 infected individuals. All individuals attended the HTLV-1 clinic at St Mary's Hospital, London and gave written informed consent, and the study was approved by the St Mary's NHS Trust Local Research Ethics Committee. Isolated PBMCs were washed twice in PBS then cryopreserved in fetal calf serum (FCS, Sigma) with 10% dimethyl sulfoxide (DMSO, Sigma).

Cell culture

Cells were thawed, washed twice in PBS and then cultured in complete medium consisting of RPMI-1640 medium (Sigma) supplemented with 10% FCS, 2 mM L-Glutamine, 100 U/ml Penicillin and 100µg/ml Streptomycin (Gibco). Cells were incubated for different times at 37^{o}C in 5% CO_2. When required, $CD8^+$ or $CD4^+$ cells were depleted by positive selection using antibody coated magnetic microbeads following the manufacturer's instructions (Miltenyi Biotec, Surrey, United Kingdom).

ELISpot assay

To stimulate IFN-γ^+ HTLV-1 Tax-specific $CD8^+$ T cells we used two pools of overlapping 20 mer peptides (offset by 6 amino acids) spanning the full length of the Tax protein, as previously described (8). The use of 20mer peptides, rather than peptides closer in length to the typical physiological optimum of ~9 amino acids may reduce the sensitivity of detection of

antigen-specific $CD8^+$ T cells. However, the difference between the response to13mers and 20mers was not statistically significant (8). Eighty to one hundred thousand CD4-depleted PBMCs were incubated at 37°C for 6 hours in the presence a range of concentrations (0, 0.01, 0.1, 1, 5 and 10 μM) of the Tax peptide pools in duplicate wells. IFN-γ production by Tax-specific CTLs was quantified by ELISpot (Mabtech) according to the manufacturer's instructions. Spot-forming cells (SFCs) were counted using an automated ELISpot reader (AID Autoimmun Diagnostika GmbH). For each peptide pool concentration, the frequency of IFN-γ^+Tax-specific CTLs was calculated as follows:

$$(SFC^{poolA} + SFC^{poolB} - 2 * SFC^{no\ peptide}) / (\text{Total number of } CD8^+ \text{ T cells})$$

Log_{10}[peptide] was then plotted against the number of IFN-γ^+ Tax-specific CTLs. Using Graphpad Prism software, the following equation was fitted to the data:

$$y = (Max) / (1 + 10^{((LogEC50 - x) * Hillslope)})$$

where x = $\log_{10}$ (peptide concentration); y = % $CD8^+$ cells producing IFN-γ at a given peptide concentration; Hillslope = gradient of fitted curve; and Max = predicted maximum SFC (i.e. where peptide concentration is not a limiting factor in IFN-γ production by antigen specific cells). The effective concentration of peptide that induced IFN-γ production by half the maximum number of Tax-specific cells (EC50) was estimated using this equation, and $CD8^+$ T cell avidity was defined as the reciprocal of this value (1/EC50). Avidity is expressed in units of $10^6 M^{-1}$.

Flow cytometric detection of Tax expression

After incubation, cells were surface-stained with monoclonal antibodies specific to CD4 and CD8 at 15 μg/mL in each case (Beckman Coulter, Marseille, France). Cells were fixed with 2% paraformaldehyde (PFA, Sigma) and then permeabilized using PBS/0.1% Triton X-100 (Sigma). Finally, cells were stained intracellularly with the FITC conjugated antibody anti-Tax protein Lt-4 (41), diluted 1/100. Cells were analyzed on a Coulter Epics XL flow cytometer. Thirty thousand events were routinely collected during acquisition of the data. The data were analysed using Coulter Expo32 software (Beckman Coulter).

Proviral load

DNA was extracted from 2 x 10^6 PBMCs as described in the manufacturer's protocol (Qiagen, DNeasy Tissue Kit) and eluted in 100 μl polymerase chain reaction- (PCR-) grade H_2O. Eluted DNA was amplified for HTLV-1 DNA using the Tax sequence-specific primers previously reported (42) and for β-actin (to quantify genomic DNA). DNA was amplified by real time quantitative PCR (qPCR) in a Roche light cycler using SYBR® Green 1 Dye incorporation (Roche) and 1 μM of each primer. Standard curves were generated using the rat cell line Tarl2, which contains 1 copy per cell of the HTLV-1 provirus (1). The sample copy number was estimated by interpolation from the standard curve and expressed as percentage of PBMCs infected, assuming one proviral copy per cell.

Statistical analysis

Spearman's rank-order test was used to test for correlation between 2 parameters across all HTLV-1–infected individuals. The Mann-Whitney test was used to compare the percentage of Tax^{high} and Tax^{low} expressing cells between samples or within the same sample. All tests were 2-tailed and rejected or accepted at the 95 % level. Multiple linear regression analysis was

carried out using SPSS (v16.0), with the rate of lysis as the dependent variable and frequency and avidity as the predictors. Raw, log-transformed and ranked data for each predictor were analysed.

Rate of $CD8^+$ cell-mediated lysis

The rate ("efficiency") of $CD8^+$ cell-mediated lysis of HTLV-1-infected cells was estimated as previously described (16). $CD8^+$ cell lytic efficiency (expressed as the proportion of Tax-expressing $CD4^+$ cells killed per $CD8^+$ cell per day) was calculated using the following equation:

$$\frac{dy}{dt} = c - \epsilon yz \qquad (1)$$

where y is the proportion of $CD4^+$ cells expressing Tax, c is the rate of increase of Tax expression which is assumed to be constant during the short-term culture, ε (epsilon) is the $CD8^+$ cell-mediated lytic efficiency and z is the proportion of PBMCs that are $CD8^+$. This model was solved analytically and fitted to the data using non-linear least-squares regression (SPSS v16), providing an estimate of the lytic efficiency in each individual.

Rate of $CD8^+$ cell mediated lysis of high Tax and low Tax expressing $CD4^+$ T cells

We wished to extend the above model to estimate the rate of lysis of high Tax-expressing (Tax^{high}) and low Tax-expressing (Tax^{low}) cells. Tax^+ cells were divided in flow-cytometric analysis into two gates, corresponding respectively to Tax^{low} and Tax^{high} cells according to fluorescence intensity. The line dividing these gates was arbitrarily defined; the same definition was used in the analysis of all samples. Since we observed a continuous increase in

the number of cells expressing a high level of Tax during the 18hr incubation (TK and CRMB, unpublished observations), we modified the existing model as follows:

In this model the Tax^{low} population (as defined from the gated FACS) is produced at a constant rate c_1 and the Tax^{high} population at a rate c_2.

The following pair of linked ordinary differential equations describes the model:

$$\frac{dy}{dt} = c_1 - c_2 y \qquad (2)$$

$$\frac{dw}{dt} = c_2 y \qquad (3)$$

Here, y is the proportion of Tax^{low} $CD4^+$ cells and w is the proportion of Tax^{high} $CD4^+$ cells. Solving these equations, we have:

$$y = \left(\frac{c_1}{c_2}\right)(1 - e^{-c_2 t}) \qquad (4)$$

$$w = c_1\left(t + \frac{e^{-c_2 t}}{c_2} - \frac{1}{c_2}\right) \qquad (5)$$

Equations 4 and 5 were fitted to the data in the absence of $CD8^+$ cells using non-linear least-squares regression, providing an estimate of c_1 and c_2 for each individual. Equations 2 and 3 were then modified to describe the rate of $CD8^+$ cell-mediated lysis of Tax^{low} cells and Tax^{high} cells separately:

$$\frac{dy}{dt} = c_1 - c_2 y - \epsilon^{low} yz \qquad (6)$$

$$\frac{dw}{dt} = c_1 \left(1 - \frac{1}{e^{c_2 t}}\right) - \epsilon^{high} wz \qquad (7)$$

These equations (Model 2) were solved analytically and fitted to the data using non-linear least-squares regression, to produce estimates of the rate of lysis of Tax^{low} and Tax^{high} cells for each individual.

Results

Control of HTLV-1 expression by CTLs

Progressive addition of $CD8^+$ cells to freshly isolated, unstimulated PBMCs resulted in a progressive decrease in the survival of Tax^+CD4^+ cells at 18h. Representative data from a single patient are shown in Figure 1A. Since this suppression of HTLV-1-expressing cells is proportional to $CD8^+$ cell frequency and requires both perforin and a Class 1 MHC match between $CD4^+$ (target) and $CD8^+$ (effector) cells (17) we conclude that the suppression represents classical, class 1-restricted CTL-mediated lysis of HTLV-1-infected cells. The rate of this $CD8^+$ cell-mediated lysis can be quantified, as previously described (16). Confirming and extending the previous results (16), there was a significant inverse correlation between the lytic efficiency and both the proviral load ($p<0.05$; Spearman rank test, n=16) and the frequency of spontaneous Tax protein by freshly isolated, naturally-infected $CD4^+$ T cells after overnight incubation (Figure 1B $p<0.05$; Spearman rank test, n=16). These observations suggest that an efficient $CD8^+$ cell-mediated response to HTLV-1 (high rate of lysis) reduced the proviral load by efficient killing of HTLV-1 infected T cells in vivo.

The efficiency of HTLV-1 suppression depends on the frequency and the avidity of HTLV-1-specific $CD8^+$ cells

To quantify the contribution of $CD8^+$ T cell avidity to the rate of $CD8^+$ cell-mediated lysis, we performed ELISpot experiments using samples of fresh unstimulated PBMCs from HTLV-1-infected patients. Tax is the immunodominant antigen recognized by HTLV-1-specific $CD8^+$ cells (8) (9) (10). We used a pool of overlapping synthetic peptides from the Tax sequence to

stimulate HTLV-1 Tax-specific cells in an IFN-γ ELISpot assay. We measured the frequency and avidity of responding Tax-specific $CD8^+$ cells by performing the ELISpots with a gradient of Tax peptide concentrations. Figure 2A shows a representative result for two patients, with or without stimulation by Tax peptides. Figure 2B shows an example of the calculation of $CD8^+$ cell avidity. The results show a significant positive correlation between the rate of lysis and the frequency of the IFN-γ^+ Tax-specific $CD8^+$ cells (Figure 3A $p<0.05$; Spearman rank test, n=16) and between the rate of lysis and the $CD8^+$ cell avidity of recognition of Tax peptides (Figure 3B $p=0.0017$; Spearman rank test, n=15). The frequency of the $CD8^+$ cells and the avidity were also positively correlated ($p<0.05$; Spearman rank test, n=19).

We then wished to quantify the respective contributions of the avidity and the frequency of Tax-specific CTLs to the $CD8^+$ lytic efficiency. We performed multiple linear regression, with the rate of lysis parameter as the dependent variable and frequency and avidity as the predictors. This analysis showed that $CD8^+$ T-cell avidity remained a significant predictor of lytic efficiency even after the specific $CD8^+$ T-cell frequency was taken into account ($p=0.0005$), whereas frequency lost significance ($p=0.85$). To test the robustness of this result, we repeated the analysis using transformed data, taking logarithms of the data and taking ranks of the data. In each case, the conclusion remained the same; avidity remained a significant predictor of lytic efficiency, while frequency lost significance. We conclude that avidity is a significant independent predictor of the quality of the CTL response.

There was a significant inverse correlation (Figure 4 $p=0.0039$; Spearman rank test, n=19) between $CD8^+$ T-cell avidity and the frequency of spontaneous HTLV-1 Tax protein expression in PBMCs after overnight incubation in vitro. This observation suggests that $CD8^+$

T cells from an individual with an "inefficient" $CD8^+$ T cell response to HTLV-1 (low avidity; low lytic efficiency) require a significantly greater antigen concentration to elicit an effector T cell response. If true, this implies that HTLV-1-infected $CD4^+$ T cells that express high levels of HTLV-1 antigen will be eliminated significantly more rapidly than those that express low antigen levels. To test this hypothesis directly we modified our existing assay of HTLV-1-specific $CD8^+$ T cell activity in samples of fresh PBMCs from infected individuals.

Tax^{high} cells were killed more rapidly than Tax^{low} cells by CTLs

Flow cytometric analysis was used to divide the Tax-expressing population into high Tax-expressing and low Tax-expressing cells. Figure 5A shows a representative example of the distributions of Tax staining intensity corresponding respectively to the lowest frequency ('depleted', left panel) and the highest frequency ('enriched', right panel) of $CD8^+$ cells used in the $CD8^+$ cell lytic efficiency assay. In all samples tested, enrichment of $CD8^+$ cells was associated with a significant decrease in the mean fluorescence intensity of Tax staining ($p<0.01$; Mann-Whitney test, n=22).

We then examined the correlation between the Tax staining intensity and the rate of lysis over a number of patients. There was a significant inverse correlation between the lytic efficiency and the percentage of cells present in the Tax^{high} gate (Figure 5B; $p<0.05$; Spearman rank test, n=17,).

Time-course analysis revealed a progressive increase in both the frequency and intensity of Tax staining during 18 hours' incubation in vitro (TK and CRMB, unpublished observations). That is, there was a progressive movement of Tax^+ cells from the Tax^{low} gate to the Tax^{high}

gate, and these two populations cannot be considered to be independent of each other. The lysis rate equation (equation 1, Material and Methods section) was therefore modified to take into account this continuous increase in Tax expression. To quantify the rates of lysis of Tax^{high} and Tax^{low} cells respectively, the modified lysis rate equations (equations 6 and 7) were then solved and fitted to the experimental data. The results (Figure 5C) show a statistically significantly higher rate of $CD8^+$ cell-mediated lysis of the Tax^{high} cells than that of the Tax^{low} cells ($p<0.01$; Mann-Whitney test; $n=14$).

Discussion

In the present study we quantified the contributions of $CD8^+$ T-cell quality and frequency to the efficiency of the specific CTL response in natural HTLV-1 infection in humans. As a measure of CTL quality, we quantified the concentration of viral peptides required to elicit a half-maximal response in a $CD8^+$ IFN-γ ELISpot assay. We refer to the reciprocal of this measure as CTL avidity. The major conclusions are that the efficiency of the CTL response correlates with the avidity of CTL recognition of viral antigen, and that this CTL efficiency correlates with the proviral load, which is in turn the strongest correlate of disease in HTLV-1 infection.

HTLV-1 infection is characterized by a strong cell-mediated immune response: the frequency of HTLV-1-specific CTLs is particularly high (4, 5). The proviral load, i.e. the fraction of PBMCs that carry a provirus, varies more than 1000-fold among infected individuals, and can exceed 20% of PBMCs (1). The high frequency of HTLV-1-specific CTLs, especially in individuals with HAM/TSP, has led to the suggestion that these cells fail to control HTLV-1 replication, and indeed that they may contribute to the inflammatory tissue damage seen in HAM/TSP and related inflammatory diseases (6) (23) (24) (25). However, we have argued from both experimental evidence (4) (26) and theoretical considerations (27) that the complex dynamics of the equilibrium between a host immune response and a persistently replicating pathogen makes the equilibrium frequency of the pathogen-specific CTLs an unreliable guide to their efficacy. The correlation between HTLV-1-specific $CD8^+$ cell frequency and HTLV-1 proviral load is either zero (26) or weakly positive (4, 5) (26). A similar problem is evident in HIV infection, in which there may be simultaneously both significant positive and significant

negative correlations between plasma viral load and the frequency of CTLs specific to different respective epitopes in the same patient cohort (28) (29). Recent studies of CTLs specific for single epitopes of HIV-1 suggested a correlation between CTL functional avidity and efficient control of the viral load (30) (31) and T-cell "quality" rather than "quantity" determines viral dynamics (32). The present results demonstrate that this correlation holds, in HTLV-1 infection, across all epitopes in the immunodominant antigen of HTLV-1 in functional assays of fresh, unstimulated peripheral blood T cells.

$CD8^+$ T cells have been shown in vitro to require only 10 complexes of MHC/peptide to elicit a lytic effector response (33) (34) (35). The detection of a significant difference in the rate of CTL-mediated lysis between cells with high Tax expression and those with low Tax expression was therefore surprising, since even the 'low'-Tax cells contain sufficient Tax protein to be readily detected by flow cytometry. Inefficient lysis might be caused by inefficient antigen processing, which would result in turn in few MHC/Tax peptide complexes being presented on the infected cell surface, despite the high level of intracellular Tax protein as indicated by intracellular staining. Alternatively, it is possible that there is not a uniformly high probability of CTL-mediated lysis when a low threshold of MHC/peptide density on the cell surface is exceeded, but rather that the probability of CTL-mediated lysis increases progressively with increasing density of MHC peptide complexes. Finally, it is possible that despite the immunogenicity of Tax protein in the $CD8^+$ T cell response (8), recognition of another HTLV-1 antigen by CTLs might be the factor that limits the rate of HTLV-1 replication in vivo (5) (36).

The conclusion that the immune control of HTLV-1 depends on the efficiency or 'quality' of virus-specific $CD8^+$ T cells is consistent both with previous theoretical analysis (27) (26) (20) and with recent experimental observations by Sabouri et al (37). These authors used CD107 LAMP-1) staining of $CD8^+$ T cells as a marker of the recent degranulation activity of HTLV-1-specific $CD8^+$ T cells in fresh PBMCs. They observed a higher frequency of HTLV-1 peptide-specific $CD8^+$ T cells in patients with HAM/TSP than in asymptomatic HTLV-1 carriers. However, the frequency of CD107 staining was lower – indicating lower recent lytic activity – in specific $CD8^+$ T cells from patients with HAM/TSP than in those from asymptomatic carriers. The present results demonstrate the role of the CTL response in determining the proviral load of HTLV-1 infection both in patients with HAM/TSP and in healthy HTLV-1 carriers. Previous evidence from host immunogenetics (38) and analysis of the dynamics of the immune response to HTLV-1 (38) suggest that additional factors, as yet unidentified, cause the emergence of HAM/TSP in susceptible individuals.

The results reported here suggest the following picture of chronic HTLV-1 infection. Efficient host control of HTLV-1 replication is associated with the presence of $CD8^+$ T cells of high avidity, i.e. $CD8^+$ T cells that respond to low antigen concentrations (low MHC/peptide density on the infected cell surface). In a host with such an "efficient" $CD8^+$ T cell response, an equilibrium is established between virus and host that is characterized by a low proviral load (1) and by high-avidity virus-specific $CD8^+$ cells (4) (26) (14) that efficiently kill autologous HTLV-1-infected cells (Figure 3B). Both the frequency and the per-cell intensity of HTLV-1 antigen expression are low in such individuals (39) (Figure 1B and 5B). The avidity of $CD8^+$ T cell antigen recognition is likely to be determined by the host genotype, in particular the MHC class 1 genotype (3) (40) (38).

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Footnotes

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Abbreviations used in this paper: HTLV-1, human T cell lymphotropic virus; ATLL, adult T-cell leukaemia/lymphoma; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; AC, asymptomatic carrier; SFC, spot forming cells.

Figure legends

Figure 1 Rate of lysis of HTLV-1-infected T cells by autologous CTLs is inversely correlated with the frequency of spontaneous proviral expression

(A) Rate of lysis of Tax^+ cells by CTLs. $CD8^+$ cell-depleted PBMCs from a patient with HAM/TSP were incubated for 18h in the presence of increasing numbers of autologous $CD8^+$ T cells. The cells are gated on lymphocytes in 4 dot plots with progressively increasing numbers of CTLs. The figure in each quadrant denotes the percentage of $CD4^+$ lymphocytes that express HTLV-1 Tax protein. The quadrant "normal" represents the normal frequency of $CD8^+$ cells for that patient.

(B) Negative correlation between rate of $CD8^+$-mediated lysis and proviral expression. Proviral expression and lytic efficiency (rate of lysis: see Materials and Methods) were assayed in a cohort of HTLV-infected patients (AC, △; HAM/TSP,▲). The rate of spontaneous proviral expression in the absence of CTLs was quantified by incubating $CD8^+$ cell-depleted PBMCs from HTLV-1-infected individuals in vitro for 18 hours. Tax protein expression by infected $CD4^+$ T cells was detected by flow cytometry. P values were calculated using Spearman's rank-sum test, and r^2 values by linear regression of log-transformed data.

Figure 2 Determination of frequency and avidity of Tax-specific $CD8^+$ T cells by IFN-γ ELISpot

Repesentative data and curve-fitting for two HTLV-1 infected patients: one with high avidity CTL responses, and one with low avidity CTL responses. $CD4^+$ T cell-depleted PBMCs were incubated for 6 hours in the presence of a range of concentrations of two pools of peptides spanning the entire Tax protein, or in the presence of medium alone. IFN-γ production by Tax-specific cells was detected by ELISpot (A). The frequency of Tax-specific T cells was calculated as follows. For each peptide concentration, the peptide-specific SFC was calculated by subtracting the mean SFC in no-peptide control wells from the SFC detected in the presence of peptide. The peptide-specific SFC was then normalized to the total frequency of $CD8^+$ T cells in the $CD4^+$ cell-depleted PBMCs. (B) To calculate the peptide concentration that gave half maximal SFC – the effective concentration at 50% (EC50), a sigmoidal curve (see Materials and Methods) was fitted to the experimental data (dashed line). The predicted EC50 is shown on the graph (solid line) with 95% confidence intervals (dotted lines) as calculated by the curve-fitting software. The functional avidity of the HTLV-1-specific $CD8^+$ T cells is defined as the reciprocal of the EC50.

Figure 3 Rate of lysis of HTLV-1 infected $CD4^+$ T cells is proportional to both the frequency and avidity of Tax-specific $CD8^+$ T cells

(A) Significant positive correlation between lytic efficiency and the frequency of Tax-specific $CD8^+$ T cells measured in the IFN-γ ELISpot assay.

(B) Rate of lysis was also significantly correlated with the avidity (10^6M^{-1}) of IFN-γ^+ Tax-specific $CD8^+$ T cells in a mixed cohort of HTLV-1-infected patients (AC, △; HAM/TSP,▲). P values were calculated using Spearman's rank-sum test, and r^2 values by linear regression of log-transformed data.

Figure 4 $CD8^+$ T-cell avidity is inversely correlated with proviral expression

Negative correlation between IFN-γ^+ $CD8^+$ T cell avidity (10^6M^{-1}) and spontaneous HTLV-1 proviral expression by infected $CD4^+$ cells in unfractionated PBMCs cultured for 18h. P values were calculated using Spearman's rank-sum test and r^2 values by linear regression of log-transformed data.

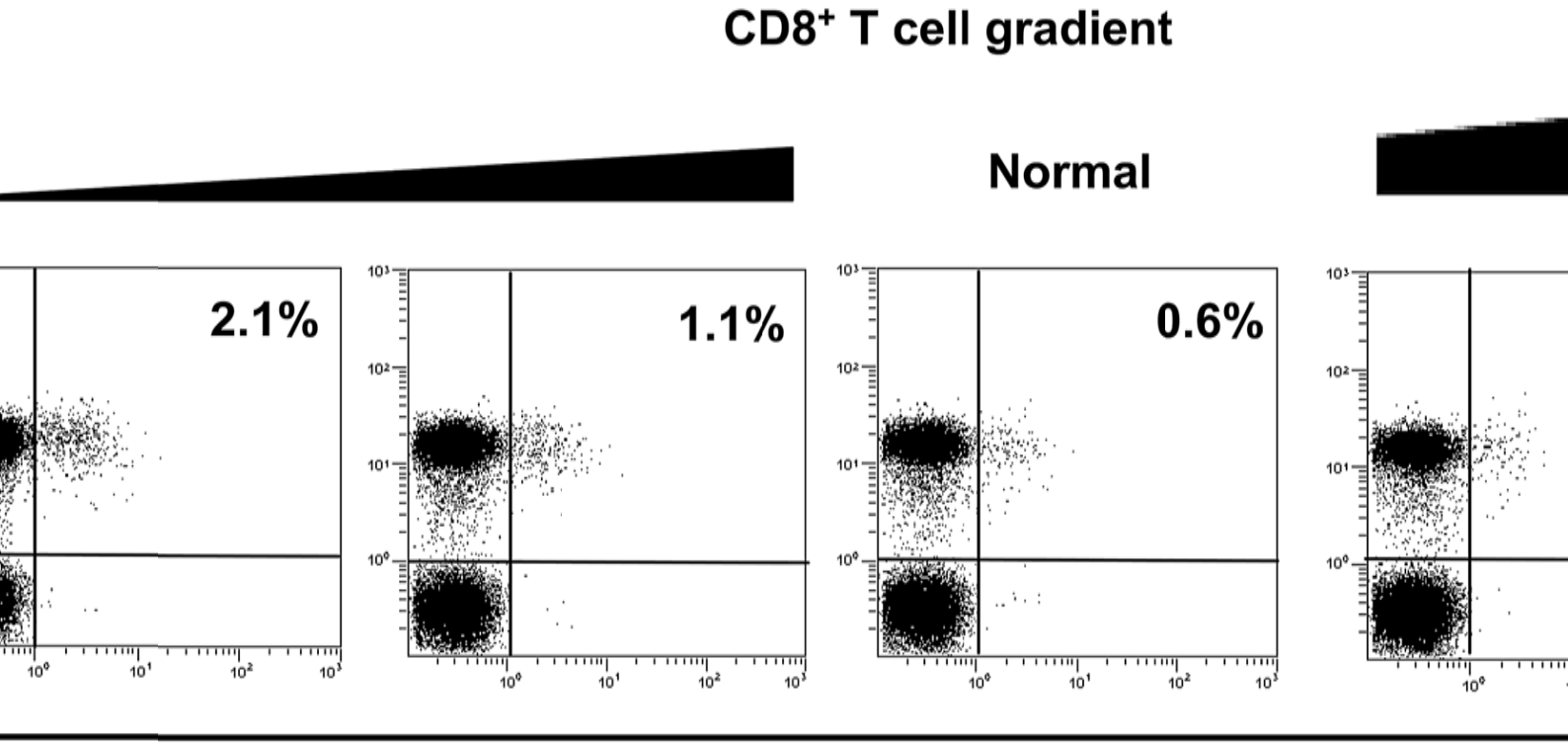
Figure 5: Tax^{high} cells are killed more rapidly than Tax^{low} cells

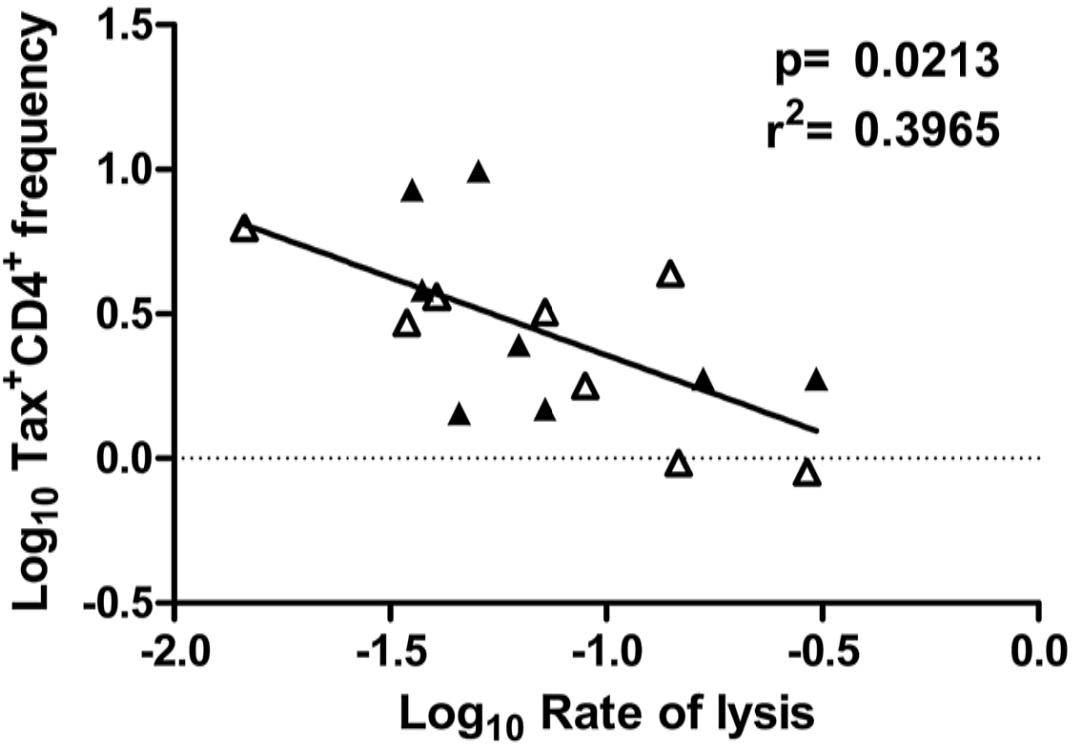
(A) Tax expression by $CD4^+$ T cells was detected by intracellular flow cytometry after incubation for 18h of $CD8^+$ depleted or $CD8^+$ enriched PBMC. Dot plots are gated on $CD4^+Tax^+$lymphocytes. The Tax^+ cells were divided into Tax^{low} and Tax^{high} cells; the line dividing the two was arbitrarily defined, and the same definition was used throughout this study. The results show a greater decrease in the frequency of Tax expression in the presence of enriched $CD8^+$ cells.

(B) Negative correlation between the rate of lysis and the percentage of Tax^{high} $CD4^+$ cells in the Tax^+ $CD4^+$ gate ($p<0.01$; Spearman rank test, n=17).

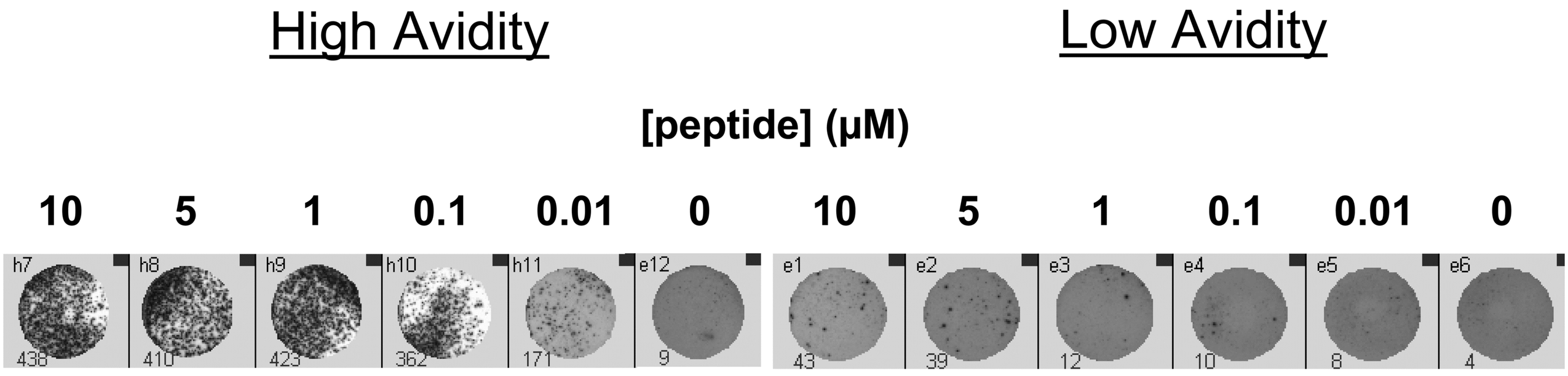
(C) Rate of lysis of Tax^{high} and Tax^{low} $CD4^+$ cells was calculated using a modified version of our original mathematical model, which accounts for the transition of Tax^+ cells from the

Tax^{low} to the Tax^{high} gate. Tax^{high} cells were killed faster than Tax^{low} cells in the same individual ($p<0.01$; Mann Whitney, n=14).

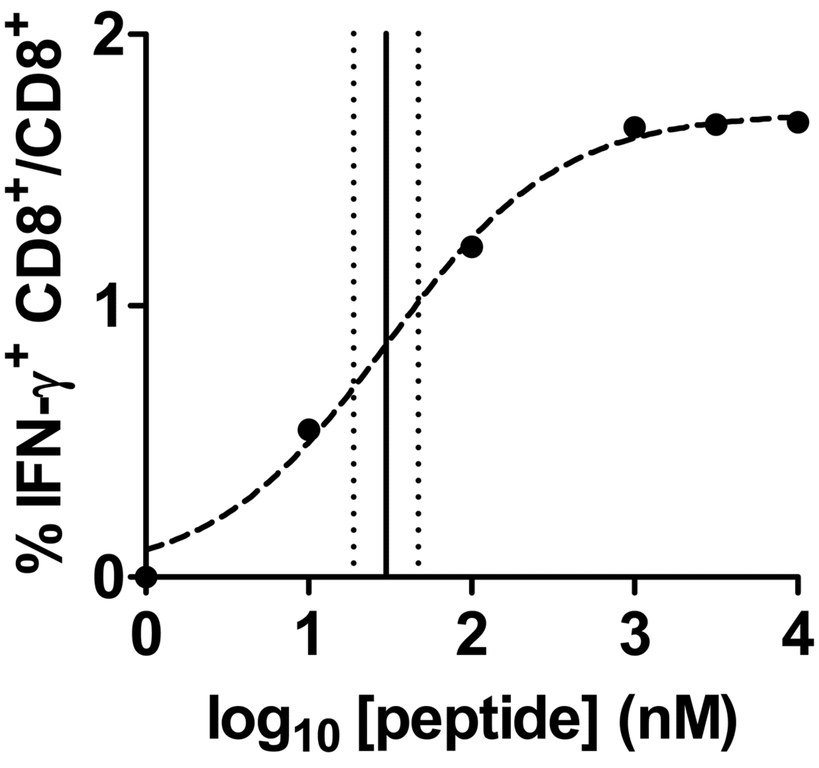

CD8+ T cell gradient
Normal
2.1%
1.1%
0.6%


p= 0.0213
r2= 0.3965
Log10 Tax+CD4+ frequency
Log10 Rate of lysis
1.5
1.0
0.5
0.0
-0.5
-2.0
-1.5
-1.0
-0.5
0.0

A

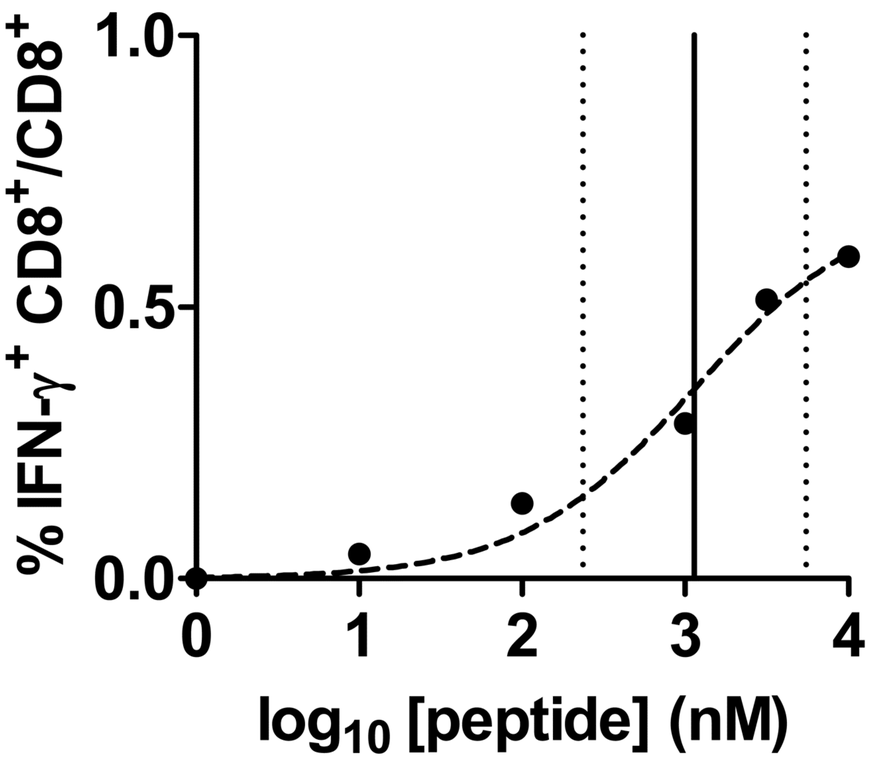


B



EC50=29nM
Avidity=$1.1 \times 10^7 M^{-1}$

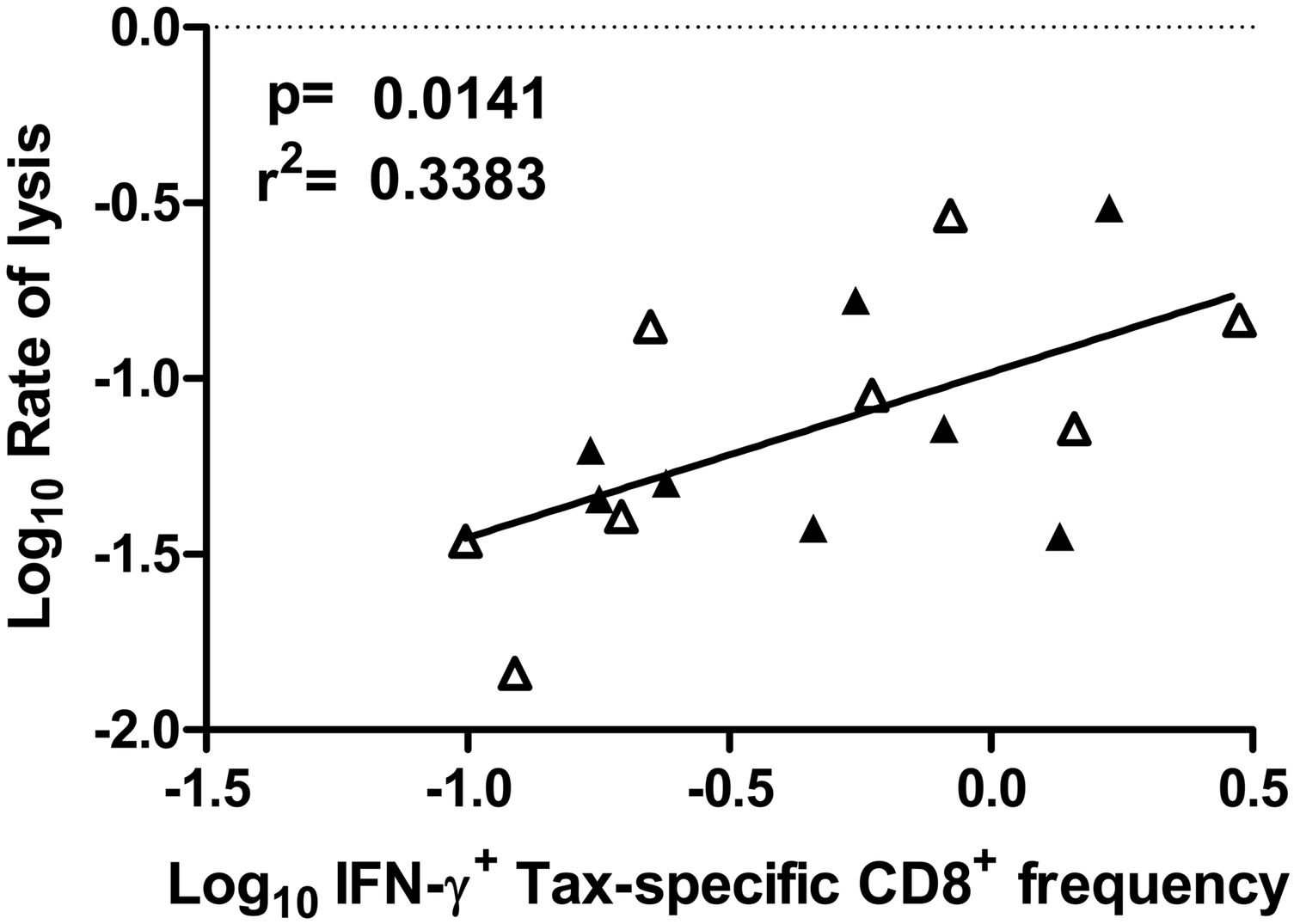
Maximum frequency = 1.68% $CD8^+$ T cells



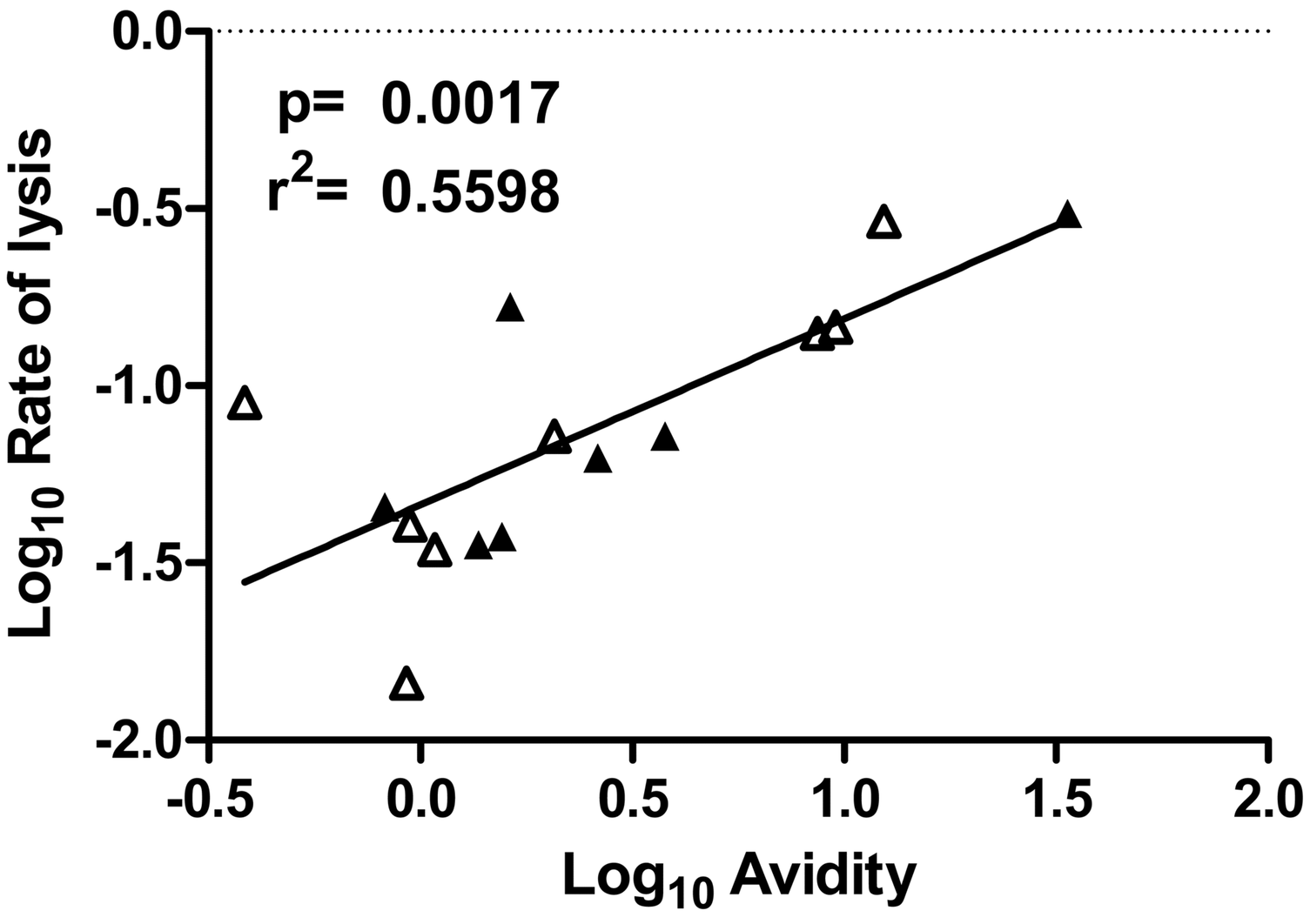
EC50=2596nM
Avidity=$3.9 \times 10^5 M^{-1}$

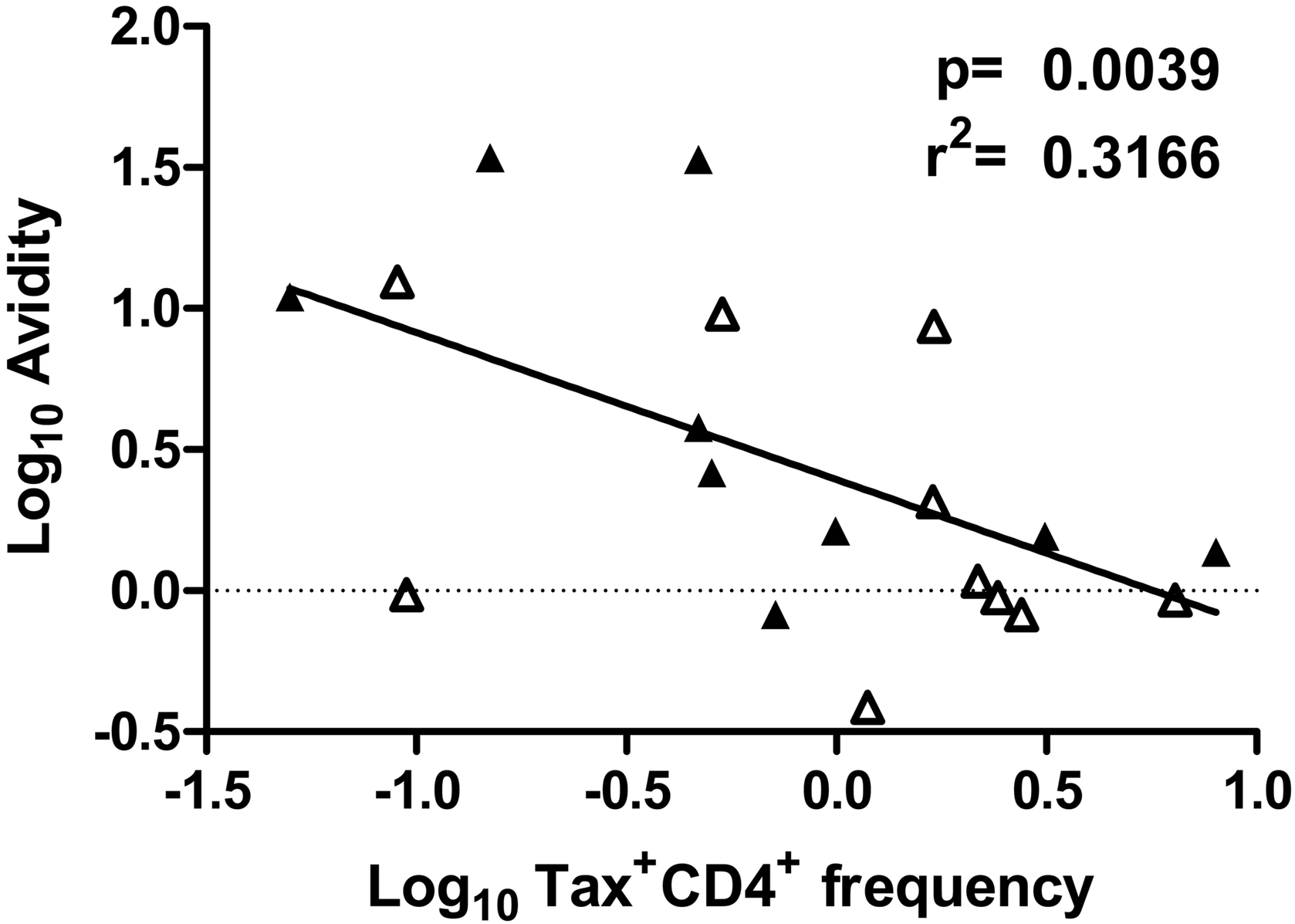
Maximum frequency = 0.59% $CD8^+$ T cells

A

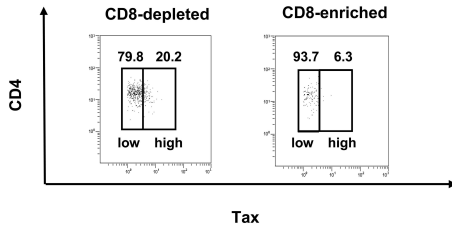

p= 0.0141
r2= 0.3383
0.0
-0.5
-1.0
-1.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
Log10 Rate of lysis
Log10 IFN-γ+ Tax-specific CD8+ frequency

B

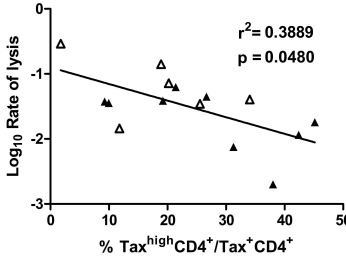

p= 0.0017
r2= 0.5598
0.0
-0.5
-1.0
-1.5
-2.0
-0.5
0.0
0.5
1.0
1.5
2.0
Log10 Rate of lysis
Log10 Avidity


p= 0.0039
r2= 0.3166
2.0
1.5
1.0
0.5
0.0
-0.5
-1.5
-1.0
-0.5
0.0
0.5
1.0
Log10 Avidity
Log10 Tax+CD4+ frequency

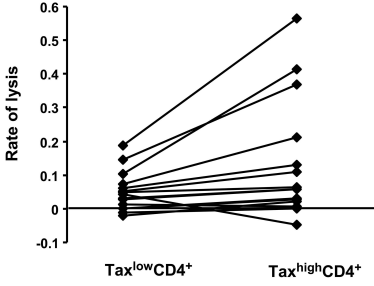
A


CD8-depleted
CD8-enriched
79.8 20.2
93.7 6.3
low high
low high
CD4
Tax

B


r2= 0.3889
p = 0.0480
Log10 Rate of lysis
0
-1
-2
-3
0
10
20
30
40
50
% Taxhigh CD4+/Tax+CD4+

C


Rate of lysis
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Taxlow CD4+
Taxhigh CD4+