

**Fig. 1.** Different patterns of Tax-specific T-cell responses in various diseases associated with human T-cell leukemia virus type-1 (HTLV-1) infection. (a) Peripheral blood mononuclear cells (PBMCs) ( $2.0 \times 10^5$  cells/well) from two post-hematopoietic stem cell transplantation (HSCT) adult T-cell leukemia (ATL) patients in long-term remission (#37, #253), two HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients (#254, #259), two chronic-type ATL (cATL) patients (#227, #249), and two seronegative uninfected subjects (SN) were cultured alone (open bars), with glutathione-S-transferase (GST) (gray bars) or with a mixture of GST-Tax proteins (black bars) in a total volume of 200  $\mu$ L of medium/well for 4 days. Interferon (IFN)- $\gamma$  in the supernatants was measured by enzyme-linked immunosorbent assay. The results represent the mean  $\pm$  SD of duplicate wells. (b) Uncultured PBMCs from an uninfected subject, a HAM/TSP patient (#255), and a cATL patient (#227) were stained with carboxyfluorescein-conjugated CCR4 monoclonal antibody (mAb) together with phycoerythrin (PE)-Cy5-labeled CD4 (left) or CD8 (right) mAbs. Values indicate the percentages of positive cells in each quadrant analyzed by flow cytometry for a total of 10 000 gated lymphocytes.

PBMCs of an uninfected individual contained 44.8% CD4<sup>+</sup> cells and 6.5% CD4<sup>+</sup>CCR4<sup>+</sup> cells. In the cATL patient (#227), the proportion of CD4<sup>+</sup>CCR4<sup>+</sup> cells was markedly elevated, consistent with previous reports.<sup>(13)</sup> PBMCs from this cATL patient contained 86.5% CD4<sup>+</sup> cells and 82.5% CD4<sup>+</sup>CCR4<sup>+</sup> cells. In contrast, the proportion of CD4<sup>+</sup>CCR4<sup>+</sup> cells in the PBMCs from the HAM/TSP patient (#255) was comparable with that in the uninfected individual.

Since the proportion of HTLV-1-infected cells in the peripheral lymphocytes was elevated in cATL, the poor T-cell responses in these samples may be partly explained by the relative scarcity of normal lymphocytes and antigen presenting cells. Such leukemic PBMCs are not suitable for evaluation with a protein-based assay, and we only used PBMCs with lymphocyte numbers within the normal range hereafter.

#### Diversity in Tax-specific T-cell responses in ACs and sATL patients.

Using the GST-Tax protein-based assay system, we next examined the T-cell responses of 21 ACs and four sATL patients. One of the sATL patients (#213) had polyclonal HTLV-1-infected cells but was categorized as sATL because of the number of abnormal lymphocytes was >5%. The hematologic and virologic profiles of the donors tested are summarized in Table 1. The numbers of peripheral lymphocytes of all ACs and sATL patients were within the normal range.

The IFN- $\gamma$  production levels by whole PBMCs from the ACs and sATL patients in the absence or presence of GST or GST-Tax proteins are shown in Fig. 2(a). The levels and patterns of IFN- $\gamma$  production varied widely among individuals. We divided the samples into two groups, according to Tax-specificity of the responses. This was assessed by calculating the ratio of IFN- $\gamma$  produced in response to GST-Tax proteins divided by IFN- $\gamma$  produced in response to GST alone for each sample (Tax/GST ratios). Tax-specific patterns were observed in samples from seven ACs (#228, #232, #238, #251, #258, #264, #277) and one sATL patient (#265) who had a localized skin lesion but was without apparent abnormal lymphocytes in the peripheral blood. The remaining samples showed less-specific patterns of IFN- $\gamma$  production due to increased non-specific IFN- $\gamma$  production or low IFN- $\gamma$  production to any stimulation.

We compared the individual proviral loads between the two groups with Tax-specific and less-specific T-cell responses (Fig. 2b,c). The group with clear Tax-specific T-cell response possessed significantly lower proviral loads than the less-specific response group either in the total of ACs and sATL patients ( $P = 0.0107$ ) or in ACs alone ( $P = 0.0260$ ). The three sATL patients (#213, #252, #220) with highest proviral loads among those tested (>100 copies/1000 PBMCs), and several ACs with moderate levels of proviral loads (10–100 copies/1000 PBMCs) were in the less-specific response group. However there were also some ACs who exhibited low T-cell responses and low proviral loads (<10 copies/1000 PBMCs).

**Involvement of CCR4<sup>+</sup> cells in high background IFN- $\gamma$  production by PBMCs from HAM/TSP patients.** We next assessed what cells in the PBMCs were responsible for the high background IFN- $\gamma$  production observed in HAM/TSP patients by depleting CD4<sup>+</sup>, CD8<sup>+</sup>, or CCR4<sup>+</sup> cells from PBMCs prior to the assay. In order to see the effects of cell depletion, the cell concentrations were equalized before cell depletion, and the resulting cell fractions were resuspended in the same volume, irrespective of the final cell numbers. The results are shown in Fig. 3(a). Whole PBMCs from a HAM/TSP patient (#255) showed high levels of non-specific IFN- $\gamma$  production with or without GST-Tax proteins. When the CD4<sup>+</sup> cells were depleted, decreased but significant levels of IFN- $\gamma$  were only produced in response to GST-Tax proteins. CD8<sup>+</sup> cell depletion did not markedly alter the non-specific responses. CCR4<sup>+</sup> cell depletion reduced the non-specific responses, but retained Tax-specific IFN- $\gamma$  production at a level comparable to that of whole PBMCs. These results indicated that CCR4<sup>+</sup> PBMC population from this patient mainly contributed to the non-specific responses in the assay, and that the effector of the Tax-specific responses was included in the CCR4<sup>+</sup> population that contained CD4<sup>+</sup> and CD8<sup>+</sup> cells.

We also measured the HTLV-1 p19 levels in the supernatants of these PBMC cultures after 7 days, and found that HTLV-1 p19 became detectable only in the CD8<sup>+</sup> cell-depleted fraction but not in the whole or CD4<sup>+</sup> cell-depleted or CCR4<sup>+</sup> cell-depleted fractions (Fig. 3b). Since the starting PBMC number before cell-depletion in each fraction was equivalent, the increase in p19 production in the CD8<sup>+</sup> cell-depleted fraction indicated that CD8<sup>+</sup> cells served as suppressors of viral expression in culture.

Reduction in the non-specific IFN- $\gamma$  production by CCR4<sup>+</sup> cell-depletion and enhancement in p19 production by CD8<sup>+</sup>

**Table 1. Blood samples from asymptomatic HTLV-1 carriers (ACs) and smoldering ATL (sATL) patients tested**

| ID   | Age | Sex | Clinical status   | WBC/ $\mu$ L | Lymphocyte (%) | Abnormal lymphocyte (%) | Provirus DNA copies/1000 PBMCs | Plasma HTLV-1 antibody titer <sup>†</sup> |
|------|-----|-----|-------------------|--------------|----------------|-------------------------|--------------------------------|---|
| #211 | 20s | F   | AC                | 4500         | 34             | 0                       | 33                             | >8192                                     |
| #213 | 50s | F   | sATL <sup>‡</sup> | 7500         | 36             | 8                       | 200                            | 4096                                      |
| #215 | 20s | M   | AC                | 8000         | 55             | 0                       | 13                             | 2048                                      |
| #216 | 70s | F   | AC                | 4200         | 31             | 3                       | 39                             | >8192                                     |
| #217 | 70s | F   | AC                | 6800         | 51             | 0                       | 14                             | >8192                                     |
| #218 | 50s | F   | AC                | 7500         | 55             | 0                       | <1                             | 1024                                      |
| #219 | 60s | F   | AC                | 7200         | 43             | 0                       | <1                             | 1024                                      |
| #220 | 50s | M   | sATL              | 4800         | 28             | 13                      | 277                            | >8192                                     |
| #223 | 60s | M   | AC                | 4200         | 26             | 0                       | <1                             | 1024                                      |
| #226 | 50s | M   | AC                | 5700         | 38             | 0                       | 28                             | 2048                                      |
| #228 | 60s | M   | AC                | 5900         | 59             | 0                       | <1                             | 256                                       |
| #232 | 30s | F   | AC                | 5800         | 46             | 0                       | <1                             | >8192                                     |
| #236 | 30s | F   | AC                | 6500         | 39             | 0                       | 22                             | >8192                                     |
| #238 | 60s | F   | AC                | 5700         | 51             | 0                       | 2                              | 1024                                      |
| #243 | 50s | F   | AC                | 4100         | 58             | 0                       | 3                              | 2048                                      |
| #244 | 50s | F   | AC                | 4900         | 27             | 3                       | 63                             | 1024                                      |
| #245 | 40s | F   | AC                | 5000         | 46             | 1                       | 58                             | 1024                                      |
| #246 | 50s | M   | AC                | 4600         | 37             | 0                       | 2                              | >8192                                     |
| #251 | 60s | M   | AC                | 4800         | 50             | 0                       | 2                              | 2048                                      |
| #252 | 50s | F   | sATL              | 4100         | 38             | 11                      | 207                            | >8192                                     |
| #258 | 60s | M   | AC                | 6400         | 30             | 3                       | 8                              | 256                                       |
| #263 | 60s | M   | AC                | 3900         | 49             | 2                       | 1                              | 2048                                      |
| #264 | 50s | F   | AC                | 3000         | 37             | 0                       | <1                             | 256                                       |
| #265 | 60s | F   | sATL <sup>§</sup> | 4700         | 38             | 1                       | 2                              | 2048                                      |
| #277 | 50s | F   | AC                | 4100         | 29             | 0                       | <1                             | 256                                       |

<sup>†</sup>Measured by a particle agglutination method.

<sup>‡</sup>Diagnosed as sATL because of increased abnormal lymphocyte number, although the infected cells were polyclonal.

<sup>§</sup>Diagnosed as sATL because of the presence of skin lesions.

HTLV-1, human T-cell leukemia virus type-1; ACs, asymptomatic HTLV-1 carriers; ATL, adult T-cell leukemia; sATL, smoldering-type ATL; PMBC, peripheral blood mononuclear cell; WBC, white blood cell.

cell-depletion were also observed in the samples from two other HAM/TSP patients (#288 and #290) (Fig. 3c,d).

The prevalence of HTLV-1-infected cells in the CCR4<sup>+</sup> cell fraction was further confirmed by a real-time PCR method in uncultured PBMCs from subjects #255 (HAM/TSP) and #211 (AC), although there were still detectable levels of proviruses left in the CCR4<sup>+</sup> cell-depleted fractions (Fig. 3e).

**Poor effects of CCR4<sup>+</sup> cell depletion on Tax-specific T-cell responses in PBMC cultures from some ACs and sATL patients.** We then assessed whether ACs and sATL samples with apparently less-specific responses actually possessed Tax-specific responses, similarly to the samples from HAM/TSP patients. We examined the effects of CCR4<sup>+</sup> cell-depletion on Tax-specific T-cell responses and the effects of CD8<sup>+</sup> cell-depletion on HTLV-1 p19 production in the PBMC samples from several available ACs and sATL patients with less-specific responses and elevated proviral loads (Fig. 4).

PBMCs from AC #238 were examined as a control that exhibited clear Tax-specific T-cell responses. As shown in Fig. 4, the Tax-specific pattern in this sample was not altered by CCR4<sup>+</sup> cell depletion. HTLV-1 p19 production in the PBMC culture of #238 was very low, consistent with the low proviral load in this subject.

In the sample from AC #211, which had a moderately elevated level of proviral load (10–100 copies/1000 PBMCs), whole PBMCs showed a less-specific response pattern with high background IFN- $\gamma$  production. This was improved to a Tax-specific pattern by depletion of CCR4<sup>+</sup> cells. HTLV-1 p19 production in CD8<sup>+</sup> cell-depleted fraction from this subject was 10 times higher than that in whole PBMCs, indicating that CD8<sup>+</sup> cells efficiently suppressed HTLV-1 p19 production in culture.

The whole PBMCs from AC #244, possessing a proviral load similar to that of subject #211, also showed a less-specific

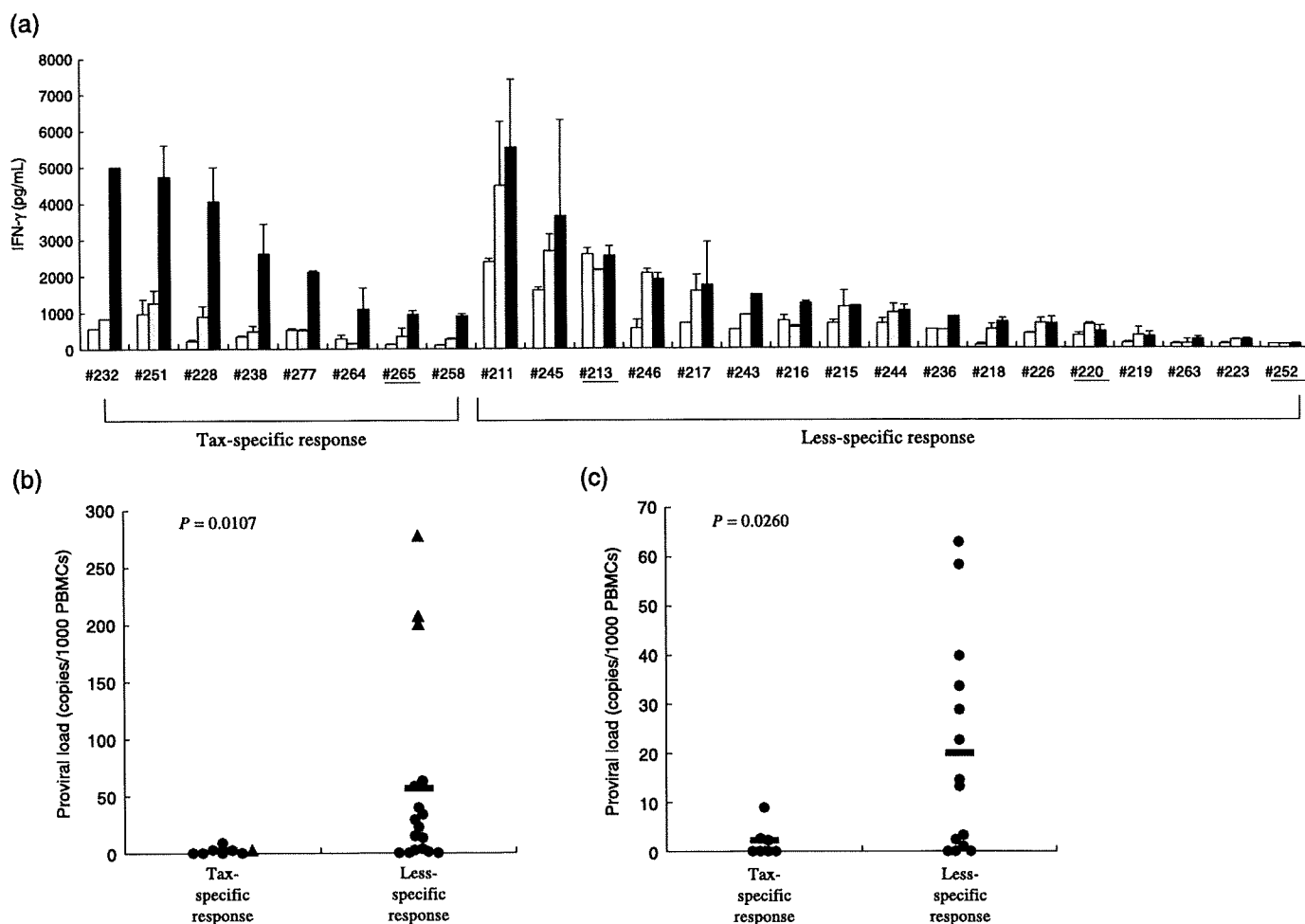
response pattern. CCR4<sup>+</sup> cell depletion reduced spontaneous IFN- $\gamma$  production but did not improve the difference between the responses to control GST and GST-Tax by much. Whole PBMCs from subject #244 spontaneously produced a substantial amount of HTLV-1 p19, which was slightly enhanced by depletion of CD8<sup>+</sup> cells.

PBMCs from sATL patient #213, who had a high proviral load (>100 copies/1000 PBMCs), spontaneously produced significant levels of IFN- $\gamma$  irrespective of the stimulation. Depletion of CCR4<sup>+</sup> cells slightly reduced the general levels of IFN- $\gamma$  production, but did not improve the non-specific pattern. Unfractionated PBMCs from this subject produced high levels of HTLV-1 p19, and CD8<sup>+</sup> cell depletion did not alter the levels of HTLV-1 p19 at all.

PBMCs from sATL patient #252, who also had a high proviral load, showed very low levels of IFN- $\gamma$  production in response to GST or GST-Tax proteins. CCR4<sup>+</sup> cell depletion from these PBMCs did not improve the scale or specificity of the IFN- $\gamma$  production by these PBMCs. Whole PBMCs from subject #252 spontaneously produced a significant amount of HTLV-1 p19, which was slightly increased by CD8<sup>+</sup> cell depletion.

Thus, CCR4<sup>+</sup> cell depletion revealed Tax-specific T-cell responses in some, but not all, samples with less-specific responses. Samples that did not show a Tax-specific pattern after CCR4<sup>+</sup> cell depletion were associated with insufficient control of HTLV-1 production in culture.

**Unresponsiveness of Tax-specific CTLs in PBMCs from an AC.** Finally, we investigated whether the five subjects shown in Fig. 4 had Tax-specific CTLs, using a flow cytometric analysis with tetramers. As shown in Fig. 5(a), the PBMCs from AC #238 contained HLA-A\*1101/Tax88-96 tetramer-binding CTLs,



**Fig. 2.** Diversity in Tax-specific T-cell responses among asymptomatic human T-cell leukemia virus type-1 (HTLV-1) carriers (ACs) and smoldering-type adult T-cell leukemia (sATL) patients. (a) Peripheral blood mononuclear cells (PBMCs) isolated from 21 ACs and 4 sATL patients (underline) were incubated alone (open bars), with glutathione-S-transferase (GST) (gray bars) or with a mixture of GST-Tax proteins (black bars) for 4 days, and interferon (IFN)- $\gamma$  amounts in the supernatants were measured by enzyme-linked immunoabsorbent assay and a Cytokine Beads Assay in part. The results were divided into Tax-specific or less-specific response groups depending on the ratios of anti-GST-Tax/anti-GST IFN- $\gamma$  production were more than 2.5 or not, and then aligned in the order of absolute values of IFN- $\gamma$  production against GST-Tax proteins in each group. (b, c) HTLV-1 proviral loads of all the ACs and sATL patients tested (b) or ACs alone (c) were indicated. The mean values of proviral loads (bars) in Tax-specific and less-specific response groups were 2.0 and 56.9 copies/1000 PBMCs, respectively ( $P = 0.0107$ ) in (b), and 1.9 and 20.1 copies/1000 PBMCs, respectively ( $P = 0.0260$ ) in (c). AC, closed circle; sATL: closed triangle.

and ACs #211 and #244 were positive for HLA-A\*2402/Tax301-309 tetramer-binding CTLs. In the PBMCs from subjects #213 and #252 (sATL), tetramer-binding cells were not detectable, although these subjects were positive for HLA-A24 and A11, and HLA-A11, respectively (data not shown).

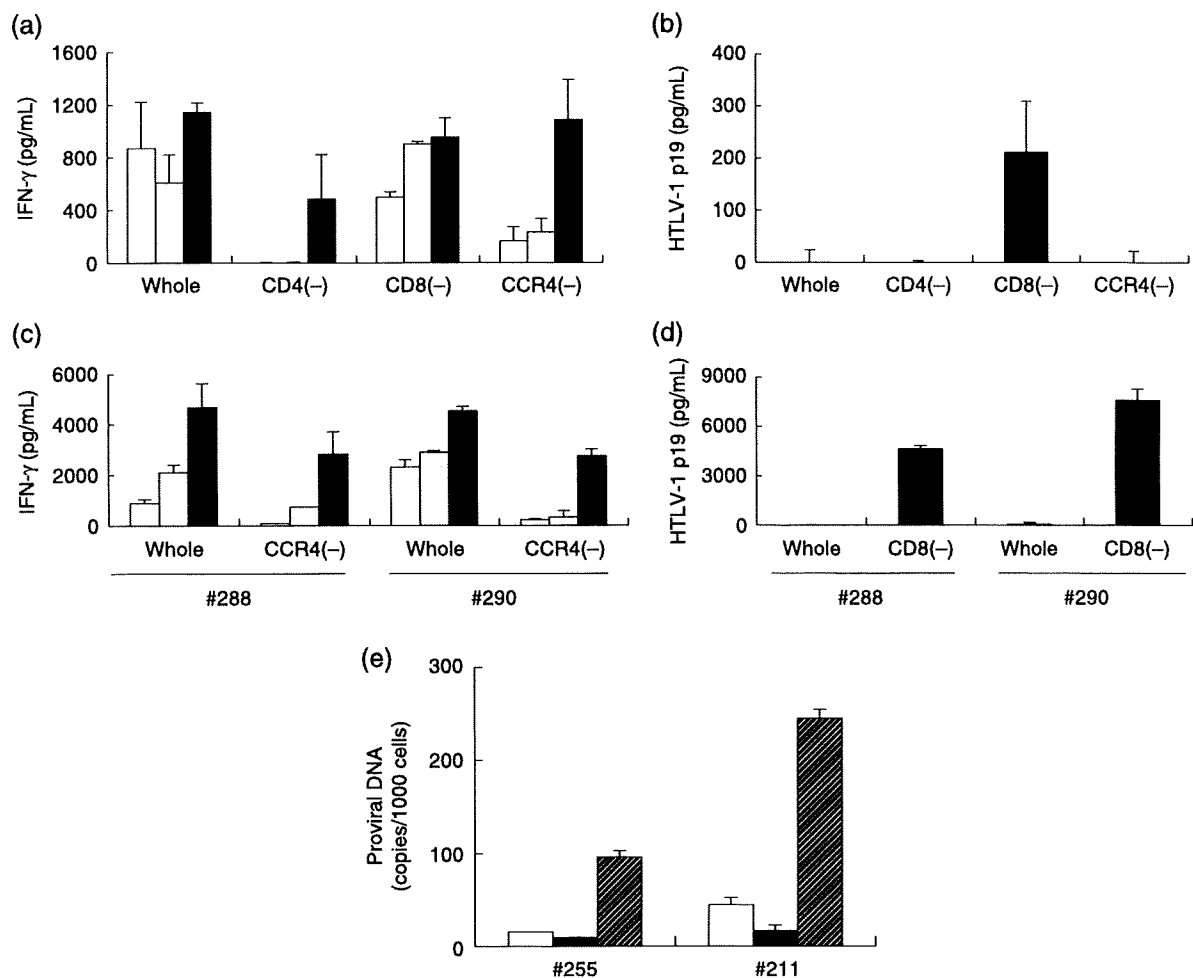
We examined the activity of Tax-specific CTLs by stimulating CCR4<sup>+</sup> cell-depleted PBMCs from three ACs, which contained tetramer-binding CTLs, with oligopeptides corresponding to the tetramers in culture. As shown in Fig. 5(b), CCR4<sup>+</sup> cell-depleted PBMCs from subjects #238 and #211 produced IFN- $\gamma$  in response to Tax88-96 and Tax301-309 peptides, respectively, whereas those from subject #244 did not respond to Tax 301-309 peptides. These results are consistent with the results of the assay using GST-Tax proteins and suggest that Tax-specific CTLs in subject #244 might be in an anergic state.

## Discussion

In the present study, we investigated HTLV-1-specific T-cell responses in unselected HTLV-1-infected individuals without any bias of HLAs by using a GST-Tax protein-based assay

system. This assay detected clear Tax-specific responses in ATL patients in complete remission, less-specific responses in HAM/TSP patients, and very weak responses in cATL patients. Interestingly, all of these patterns were observed in PBMCs from ACs and sATL patients, indicating wide diversity in Tax-specific T-cell responsiveness at these stages. Most importantly, some individuals at these stages exhibited impaired Tax-specific T-cell responses and elevated proviral loads, indicating that such conditions are present before an overt leukemia stage.

High background responses have been an obstacle to evaluate T-cell responses in HTLV-1-infected individuals. This may be similar to the phenomenon known as 'spontaneous proliferation' of PBMCs from HAM/TSP patients and ACs.<sup>(42-44)</sup> In our study, depletion of CCR4<sup>+</sup> cells containing HTLV-1-infected cells from the PBMCs reduced this spontaneous response and revealed a Tax-specific T-cell response pattern in the samples from several HAM/TSP patients and ACs with strong non-specific responses (Figs 3 and 4). The sources of spontaneous IFN- $\gamma$  production may be either HTLV-1-specific T-cells reacting with coexisting infected cells in culture, or HTLV-1-infected cells themselves, or both.<sup>(45,46)</sup> As CCR4<sup>+</sup> cell depletion from the PBMCs reduced the



**Fig. 3.** Involvement of CCR4<sup>+</sup> cells in high background interferon (IFN)- $\gamma$  production by peripheral blood mononuclear cells (PBMCs) from human T-cell leukemia virus type-1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients. PBMCs from a HAM/TSP patient (#255) were equally divided into four fractions. One fraction was used directly (whole) and the other three fractions were used after depletion of CD4<sup>+</sup> [CD4(-)], CD8<sup>+</sup> [CD8(-)], or CCR4<sup>+</sup> [CCR4(-)] cells. The cell numbers in each fraction were  $2.0 \times 10^5$ ,  $0.8 \times 10^5$ ,  $1.1 \times 10^5$ , and  $1.3 \times 10^5$  cells/well, respectively. (a) The fractions were cultured alone (open bars), with glutathione-S-transferase (GST) (gray bars), or with a mixture of GST-Tax proteins (black bars) for 4 days. IFN- $\gamma$  in the supernatants was measured by enzyme-linked immunosorbent assay (ELISA). (b) The levels of HTLV-1 p19 antigen in the culture supernatants of the PBMC fractions in the absence of any stimulation were measured by ELISA at 7 days after the initiation of culture. The results represent the mean  $\pm$  SD of duplicate wells. (c, d) Whole and fractionated PBMCs from two other HAM/TSP patients (#288 and #290) were similarly examined for IFN- $\gamma$  production for 4 days against medium (open bars), GST (gray bars), or GST-Tax proteins (black bars) (c), and HTLV-1 p19 production for 7 days of culture (d). The cell numbers of whole, CCR4(-), and CD8(-) PBMCs in each well were  $2.0 \times 10^5$ ,  $1.7 \times 10^5$ , and  $1.2 \times 10^5$  for patient #288, and  $2.1 \times 10^5$ ,  $0.9 \times 10^5$ , and  $0.9 \times 10^5$  for patient #290, respectively. (e) HTLV-1 provirus numbers (copies/1000 cells) in whole (open bars), CCR4<sup>+</sup> cell-depleted (closed bars), and CCR4<sup>+</sup> (hatched bars) PBMC fractions before culture from subjects #255 (HAM/TSP) and #211 (AC) were measured by real-time polymerase chain reaction methods. The results represent the mean  $\pm$  SD of duplicate samples.

number of HTLV-1-infected cells without losing CD8<sup>+</sup> cells, T-cell response against exogenously added Tax proteins became clearly detectable. CD8<sup>+</sup> cells in these PBMCs effectively suppressed HTLV-1 p19 production in culture, presumably by killing infected cells.

Although the number of samples available for detailed analysis was limited, PBMCs from at least two sATL patients (#213 and #252) with high proviral loads (>100 copies/1000 cells) exhibited impaired T-cell responses even after CCR4<sup>+</sup> cell depletion. CD8<sup>+</sup> cells of these subjects had poor effects on HTLV-1 p19 production (Fig. 4). This was not attributable to the scarcity of non-ATL cells, as these PBMCs contained 16.5% and 12.0% CD8<sup>+</sup> cells, respectively. HTLV-1-infected cells were the most likely source of IFN- $\gamma$  production in whole and CCR4<sup>-</sup> PBMC cultures from subject #213, as HTLV-1 p19 was detected even in the CCR4<sup>+</sup>

cell-depleted fraction during further culture (data not shown), presumably because of expansion of the remaining infected cells in the initially CCR4<sup>-</sup> cell culture, in the absence of any anti-Tax response. HTLV-1-infected cells from subject #252 expressed viral antigens but not IFN- $\gamma$ .

ACs with less-specific T-cell responses and moderate levels of proviral loads (10–100 copies/1000 PBMCs) seemed to be a mixed population in regards to Tax-specific response. CCR4<sup>+</sup> cell-depleted PBMCs showed Tax-specific T-cell response in subject #211, but did not markedly do so in subject #244. Similarly, CD8<sup>+</sup> cell-mediated control on HTLV-1 p19 production in #211 was more efficient than in #244. The results of peptide stimulation also indicated impaired status of Tax-specific response in #244 (Figs 4 and 5). However, the level of p19 production in the CD8<sup>+</sup> cell-depleted cell fraction was even higher

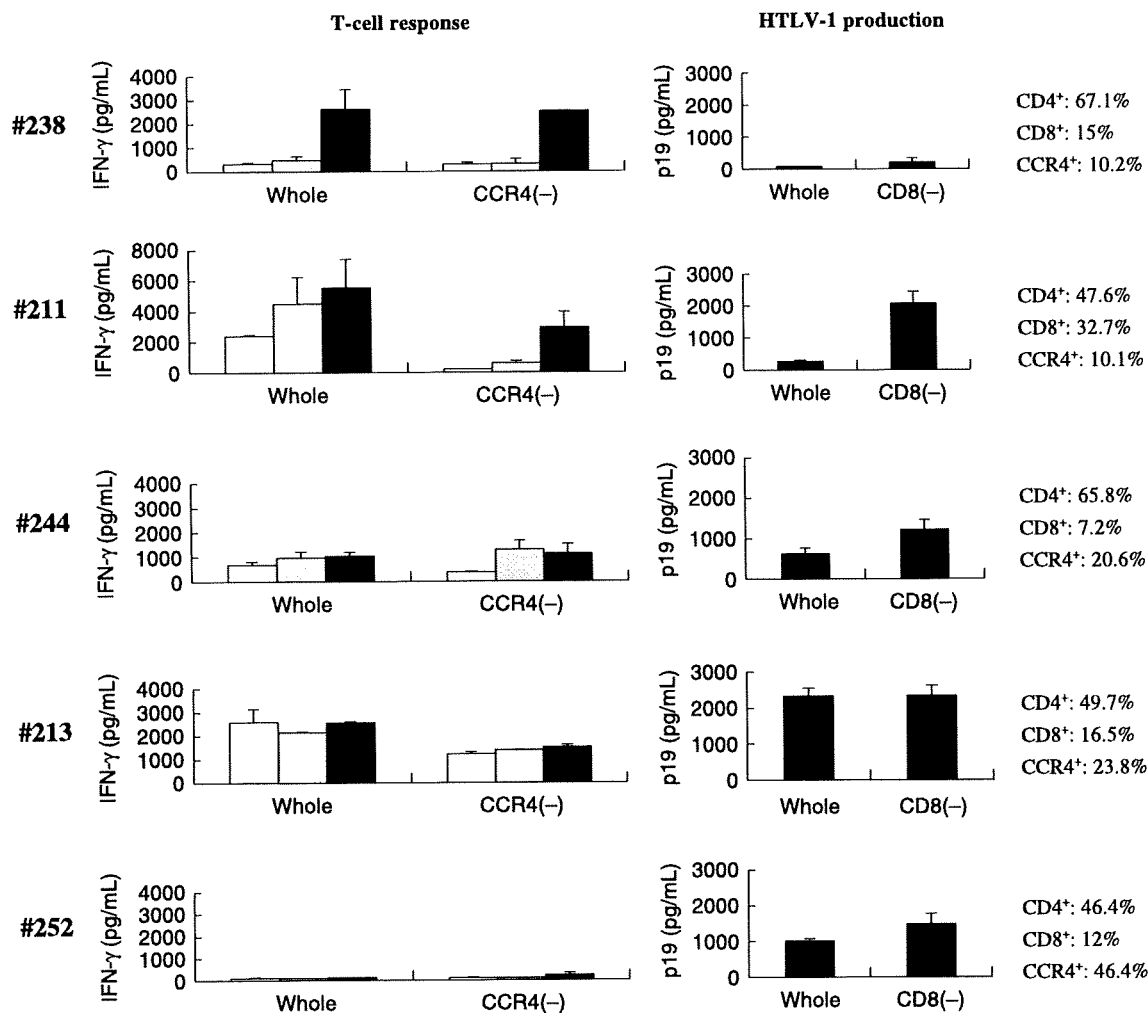


Fig. 4. Tax-specific T-cell response associated with CD8<sup>+</sup> cell-mediated control of human T-cell leukemia virus type-1 (HTLV-1) production in peripheral blood mononuclear cell (PBMC) cultures from asymptomatic HTLV-1 carriers (ACs) and smoldering-type adult T-cell leukemia (sATL) patients. Whole and CCR4<sup>+</sup> cell-depleted [CCR4(-)] PBMC fractions prepared from subjects #238 (AC), #211 (AC), #244 (AC), #213 (sATL), and #252 (sATL) were cultured alone (open bars), with glutathione-S-transferase (GST) (gray bars), or with a mixture of GST-Tax proteins (black bars) for 4 days. Interferon (IFN)- $\gamma$  produced in the supernatants was measured by enzyme-linked immunoabsorbent assay (left panels). The levels of HTLV-1 p19 production in whole and CD8<sup>+</sup> cell-depleted [CD8(-)] PBMC cultures from the same donors were measured on day 7 (right panels). The ratios of CD4<sup>+</sup>, CD8<sup>+</sup>, and CCR4<sup>+</sup> cells (%) in uncultured PBMCs from each subject were indicated at right, as determined by flow cytometry.

in #211. Nevertheless, #211 and #244 had comparable levels of proviral loads (33 and 63 copies/1000 PBMCs, respectively). These results suggested that proviral load might represent equilibrium between growth capabilities of infected cells and the T-cell response against them.

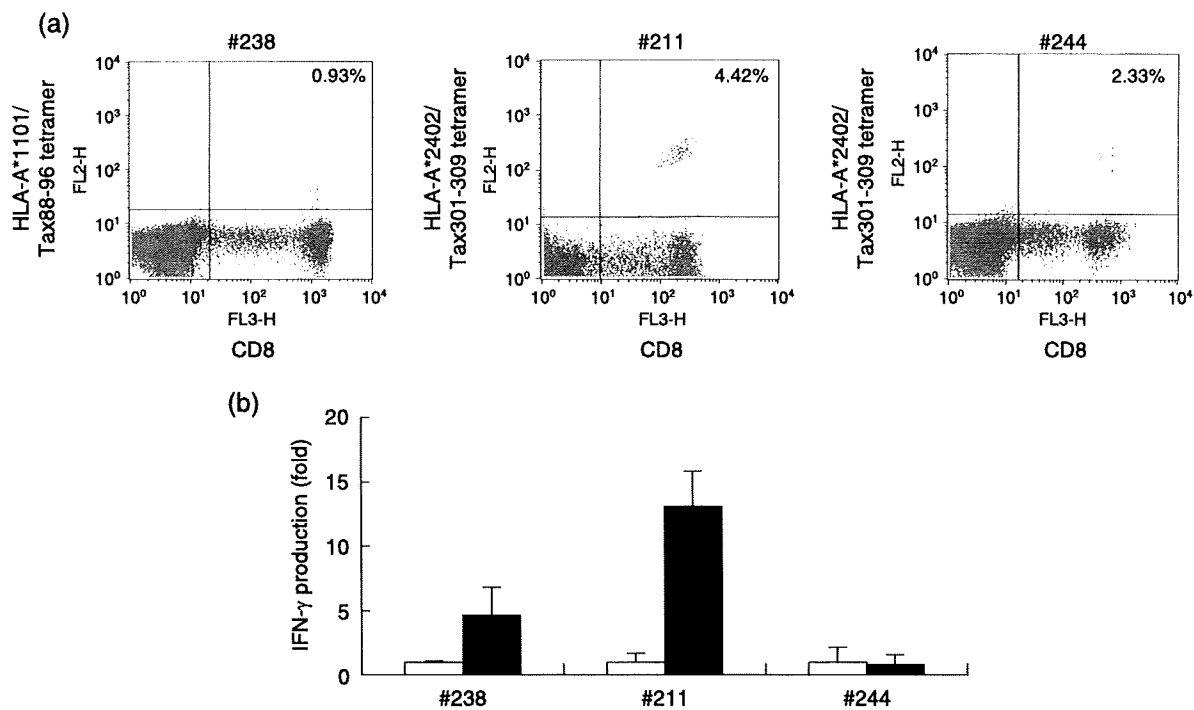
It is of note that some ACs (#219, #223, #263) exhibited very low anti-Tax T-cell responses that were not improved by CCR4<sup>+</sup> cell depletion, and also had low proviral load. HTLV-1-infected cells in these individuals might have low proliferative abilities. Since these subjects maintained considerable levels of anti-HTLV-1 antibodies in the plasma (1:1024–2048, Table 1), poor T-cell responsiveness cannot be explained merely by the scarcity of viral antigens. The mechanisms of general and/or HTLV-1-specific immune suppression in HTLV-1 infection remain to be clarified.

In conclusion, ACs and sATL patients consist of diverse subpopulations with different patterns of T-cell responses against HTLV-1, containing a subpopulation exhibiting impaired HTLV-1-specific T-cell responses and insufficient control of HTLV-1-infected cells. This strongly suggests that impairment

of HTLV-1-specific T-cell response is not a consequence of advanced ATL but present at early stages or even before the disease onset. Although there must be multiple steps towards ATL development, impaired HTLV-1-specific T-cell response could be one of the underlying conditions that allows expansion of HTLV-1 infected cells *in vivo*. Reactivation of HTLV-1-specific T-cell response by immunotherapeutic strategies such as vaccines in the subpopulation with insufficient T-cell response might contribute to the recovery of host control on HTLV-1-infected cells *in vivo*.

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**Fig. 5.** Discrepancy between the presence of Tax-specific cytotoxic T-lymphocytes (CTLs) and interferon (IFN)- $\gamma$  production in peripheral blood mononuclear cells (PBMCs) from asymptomatic human T-cell leukemia virus type-1 (HTLV-1) carriers (ACs). (a) Uncultured PBMCs from indicated subjects were stained with phycoerythrin (PE)-Cy5-conjugated CD8 monoclonal antibody (mAb) together with PE-conjugated HLA-A\*1101/Tax88-96 tetramers for subject #238, and HLA-A\*2402/Tax301-309 tetramers for subjects #211 and #244. The percentages of tetramer<sup>+</sup> cells among CD8<sup>+</sup> cells are indicated, as analyzed by flow cytometry. (b) CCR4<sup>+</sup> cell-depleted PBMCs from the same subjects were incubated with control dimethylsulfoxide (DMSO) (open bars) or 10  $\mu$ M of Tax peptides (closed bars) for 4 days, and the amounts of IFN- $\gamma$  in the supernatants were measured by enzyme-linked immunosorbent assay. Tax peptides used were Tax 88-96 for subject #238 and Tax 301-309 for subjects #211 and #244. The values represent folds of IFN- $\gamma$  production against control DMSO, and indicated as the mean  $\pm$  SD of duplicate samples.

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TEL 092 (541) 3 2 3 1  
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