α1Antitrypsin therapy increases CD4+ lymphocytes to normal values in HIV-1 patients

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Abstract: Adult thymopoiesis is a multi-step process that in adult mice is highlighted by a 21-day cycle of coordinated journeying of progenitor cells between adult bone marrow and thymus. In the analogous human system, cell surface human leukocyte elastase (HLECS), the chemokine receptor CXCR4, and its ligand stromal-derived factor-1 (SDF-1, CXCL12) are required for progenitor cells to vacate bone marrow. We have recently observed that the number of circulating CD4+ lymphocytes is correlated with the HLECS ligand, α1antitrypsin (α1PI), proteinase inhibitor, α1PI). In HIV-1 disease, α1PI levels are deficient and rate limiting for CD4+ lymphocytes. We demonstrate herein that α1PI therapy increases the number of CD4+ lymphocytes in blood. In HIV-1 patients the number of CD4+ lymphocytes is increased to normal values after 2 weeks of therapy. Importantly, the 23-day periodicity of appearance of CD4+ lymphocytes suggests that α1PI regulates adult human thymopoiesis.

INTRODUCTION

The three predominant blood cell subtypes are erythrocytes, granulocytes, and CD4+ lymphocytes. Growth factors such as erythropoietin and G-CSF are currently used therapeutically to mobilize erythroid and myeloid progenitor cells. Emerging information related to stem cell mobilization suggested to us that a fundamental and previously unrecognized function of the blood protein α1PI is mobilization of CD4+ progenitor cells. Active α1PI binds to a receptor complex central to cell migration that includes the chemokine stromal-derived factor-1 (SDF-1, CXCL12), its receptor CXCR4, and the α1PI receptor, HLECS (1-3).

Blood cell migration occurs as the result of two discrete steps. First, the relevant receptors polarize at the leading edge of the cell, and second, these receptors are endocytosed at the trailing edge (4). When integrins are involved in the receptor complex, cells attach to the tissue matrix. Subsequent endocytosis of the receptor complex at the trailing edge releases the cells from the tissue matrix (4). Plasma membrane-associated proteinases at the attachment point do not act as proteinases, rather bind to their relevant proteinase inhibitors as receptor and ligand. When these proteinase receptors are in complex with their ligands, e.g. α1PI-complexed HLECS, conformational changes expose novel domains that attract nearby low-density lipoprotein (LDL) receptor complexes (4,5). Binding of the LDL receptor complex to the proteinase inhibitor complex induces their endocytosis which causes the cell to advance forward (4,5).

In earlier work, we established that antibodies reactive with HIV-1 gp120 also bind and inactivate human α1PI, producing IgG-α1PI immune complexes (6). IgG-α1PI immune complexes produce functional α1PI deficiency in HIV-1 infected individuals. A single amino acid differentiates chimpanzee α1PI from human α1PI, and this difference is in the HIV-1 gp120 homologous domain, perhaps explaining the lack of progression of HIV-1 infected chimpanzees to AIDS (7). Further, comparison of the amino acid sequences of human α1PI, HIV-1, HIV-2, SIV, HTLV-1, and HTLV-2 reveals that all share homology with the hydrophobic core of the fusion domain of HIV-1 gp41 (LFLGFL), but only HIV-1 gp120 shares homology with α1PI (6). We hypothesized that the insufficient α1PI that attends HIV-1 disease might secondarily cause CD4+ lymphocytes to become trapped in tissue, unable to complete the second step of cell migration and be released into blood. We demonstrate herein that α1PI
augmentation induces substantial increases in CD4+ lymphocytes that cycle with a 23±3.5 day periodicity.

**METHODS**

**Human Subjects.** It was determined using the empirical correlation between α1PI and CD4+ lymphocytes that a sample size of 2 HIV-1+ patients would be adequate two achieve a significance level with alpha = 0.05 and power of test = 0.8 between pre- and post-treatment CD4+ lymphocytes levels. Inclusion criteria for treatment were: i) active α1PI below 11 μM; ii) one year history with CD4+ lymphocytes at levels ranging between 150 and 300 cells/μl; iii) absence of symptoms suggestive of HIV-1 disease progression; iv) adequate suppression of virus (<50 HIV RNA/ml); and v) history of compliance with antiretroviral medication. Due to the small size of the study and to avoid other complications of pregnancy, only male HIV-1 patients were enrolled. The half-life of Zemaïra® (purified α1PI) after infusion is 4.5 days, reaching steady state after 3–4 wks therapy (18). CSL Behring contributed a sufficient quantity of Zemaïra® (lot# C405702) for administration of 8 weekly infusions at a dose of 120mg/kg.

Written informed consent was received from 4 HIV-1 patients designated Alpha, Beta, Gamma, and Delta. For comparison, blood was collected from 2 non-HIV-1 patients, both female, with a diagnosis of emphysema in the context of genetic α1PI deficiency (Plzz-1 and Plzz-2, ages 52 and 53, respectively). In all cases, patients had never before received α1PI augmentation therapy. After initiating therapy, an assessment of dosage and the quantity of donated Zemaïra® allowed for the extended treatment of patient Alpha for a total of 12 wks.

Due to an insufficient number of serum samples from patient Plzz-2, only functional analyses of CD4+ lymphocytes are presented. Patient Gamma who was PPD positive and elderly had become PPD negative 2 yrs prior to the treatment presented here which is clinically interpreted as a loss of immune function. Patient Delta reported to the first infusion stating that due to unforeseen circumstances, his antiretroviral medication was interrupted for 4 days. Although there was no fever present or other indication of infection at the time of the first infusion, in follow-up analysis, this patient was found to have pre-treatment serum IL-2 levels of 51 pg/ml (normal is undetectable) and other atypical baseline measures indicative of an inflammatory response and exceeding study inclusion criteria including 454 CD4+ cells/μl, 205 HIV RNA copies/ml, and 14 μM α1PI. Thus, blood from patient Delta was analyzed for the purpose of assessing treatment response in the presence of systemic inflammation. Only pre- and post-treatment NFxB activation, cytokine release, and lymphocyte phenotype were determined for this patient.

Blood was collected at each session and was sent to a contractor medical laboratory which provided independent measurement of the complete blood cell count (CBC) with differential, lipid panel, blood chemistry, lymphocyte panel, and HIV RNA. Periodically, kidney (BUN and creatinine) and liver function tests (ALT, AST) were monitored for potential immune complex disease, and all measurements were within the normal range. Lymphocyte function and phenotype analysis were performed by our laboratory. The study protocol was approved by CSL Behring and by the institutional review board of Cabrini Medical Center. No adverse effects were reported by any patient.

**Serum α1PI levels.** Active α1PI was determined in once-thawed serum samples by our laboratory as previously described with the modification that endpoint, rather than kinetic, analysis was measured (19). Briefly, the inhibition of porcine pancreatic elastase (PPE, Sigma, St. Louis, MO) that was specifically attributable to serum α1PI was quantitated in the context of the serum concentration of α1-macroglobulin and its higher affinity for PPE relative to α1PI.

**Lymphocyte phenotype analysis.** Surface staining on whole blood was performed by incubating for 15 min at 23 °C with ASR type, fluorescently conjugated antibodies recognizing CD4, CD3, CD8, CD45RA, CD45RO, CXCR4, CCR5, CD34, CD25, and isotype controls (BD Biosciences). Cells were subsequently stained to detect HLECs by incubating whole blood for an additional 15 min at 23 °C with rabbit anti-HLE (Biodesign, Kennebunkport, ME) or negative control rabbit IgG (Chemicon, Temecula, CA) which had been conjugated to Alexa Fluor 647 (Molecular Probes). At least 10,000 cells from each sample were acquired using a FACSCalibur flow cytometer. Markers on cells in the lymphocyte gate were quantitated, and CD4+ cells in the lymphocyte gate were validated using a contractor medical laboratory. Cell staining was analyzed using CellQuest (BD Biosciences) or FlowJo software (Tree Star, Inc., Ashland, OR).

**CD4+ lymphocyte functional analysis.** CD4+ lymphocytes were negatively selected from PBMC using magnetic cell sorting as recommended by the manufacturer (Miltenyi Biotec, Auburn, CA). Isolated cells (1x10⁶ cells/ml) were cultured in medium containing 10% FBS in 24-well tissue culture plates for 3 days at 37 °C, 5% CO₂, in the presence or absence of stimulation antibodies reactive with CD2, CD3, and CD28 as recommended by the manufacturer (Miltenyi Biotec). Culture supernatants were measured by ELISA as recommended by the manufacturer (R&D Systems, Minneapolis, MN) for IL-2, IL-4, IL-10, and IFNγ.

Harvested CD4+ lymphocytes were examined for NFκB phospho-epitope staining by flow cytometry as previously described (20). Briefly, 1x10⁶ cells/well in 96 well plates were fixed using 1.5%
paraformaldehyde, washed with PBS containing 1% BSA, and incubated at 4 °C for 10 min in 100μl ice-cold methanol. Cells were washed and incubated at 23 °C for 20 min with phosphoprotein-specific antibodies (BD Pharmingen and BD PhosFlow, San Diego, CA) directly conjugated with Alexa Fluor 647 (Molecular Probes Invitrogen, Carlsbad, CA).

RESULTS

CD4+ lymphocytes increase and CD8+ lymphocytes decrease in response to α1PI replacement therapy.

To examine the interrelationship between α1PI concentration and CD4+ lymphocyte numbers, data were examined from a blinded study conducted to monitor hematologic changes following weekly infusions of α1PI (60 mg/kg) in 11 individuals with genetic α1PI deficiency (Plzz) who had never before received α1PI therapy. Treatment with α1PI caused an increase in lymphocytes in 10 of these individuals (data not shown), suggesting that such treatment might benefit HIV-1+ patients.

Two HIV-1 patients, Alpha and Beta at different stages of disease (Table 1) received weekly infusions of 120 mg/kg α1PI augmentation. Two additional HIV-1 patients, Gamma and Delta, with a priori evidence of abnormal immune status received the same therapy. The ability of Gamma to respond to antigen was impaired (positive PPD followed by negative PPD), and Delta exhibited systemic inflammation. Finally, 2 non-HIV-1 patients were included in the study, Plzz-1 and Plzz-2, who manifested normal numbers of CD4+ lymphocytes and a diagnosis of emphysema in the context of genetic α1PI deficiency. The Plzz patients received half-dose weekly infusions of 60mg/kg α1PI augmentation. Patients Delta and Plzz-2 were included only in CD4+ lymphocyte functional analyses (see Methods).

HIV-1+ patients Alpha, Beta, and Gamma (Figure 1) and both Plzz patients (not depicted) responded to therapy with an initial burst of lymphocytes. After 2 wks of therapy, patients Alpha and Beta achieved normal numbers of CD4+ lymphocytes with increases from 297 to 710 and from 276 to 393 cells/μl, respectively. Patients Plzz-1 and Plzz-2 increased from 743 to 954 and from 899 to 1024 cells/μl, respectively. Patient Beta, who had never exhibited CD4+ lymphocytes in the normal range in more than 20 years, even continued to exhibit the normal range of CD4+ lymphocytes 2 wks after treatment stopped with 382 cells/μl. Using regression analysis, this duration of benefit was attributed to α1PI therapy (Figure 2). Patient Alpha who was first infected 5 years prior to the study had not exhibited CD4+ lymphocytes within the normal range in 2 years. At 5 wks and 14 wks after treatment stopped, patient Alpha continued to be in the normal range with 470 cells/μl. By regression analysis, this duration of benefit appeared to be related to antiretroviral medication as well as α1PI therapy (Figure 2). Patient Gamma, who was known to have lost immune function, showed an increase from 148 to 167 cells/μl and never achieved normal numbers of CD4+ lymphocytes.

CD4+ lymphocyte cycling is sinusoidal.

All patients exhibited cyclic changes in CD4+ lymphocytes (Figure 3). Patients Alpha, Beta, and Plzz-1 exhibited sinusoidal changes in CD4+ lymphocytes with periodicity 23±3.5 days (Figure 3). Patient Gamma exhibited sinusoidal changes in CD4+ lymphocytes with periodicity of 15 days. Of important note, patient Plzz-1, but none of the HIV-1 patients, exhibited sinusoidal changes in CD8+ lymphocytes. In patient Alpha, the sinusoidal wave was damped exhibiting decreased amplitude. In patients Beta and Gamma, the oscillations sloped downward. The 5 week treatment period for patient Plzz-2 was insufficient for determining the occurrence of periodicity.

The CD4/CD8 ratio in patients Alpha, Beta, and Plzz-1 increased following α1PI therapy at a rate of 0.02±0.008 per week (Figure 1, 3). The degree of

<table>
<thead>
<tr>
<th>Patient</th>
<th>NRT/NRNT/PI</th>
<th>Age</th>
<th>HIV-1+ since (μM)</th>
<th>CD4 cells/μl</th>
<th>HIV RNA copies/ml</th>
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<tr>
<td>Alpha</td>
<td>Epivir/Sustiva/none</td>
<td>47</td>
<td>2001 9</td>
<td>297</td>
<td>&lt;400</td>
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<tr>
<td>Beta</td>
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<td>53</td>
<td>1982 7</td>
<td>276</td>
<td>&lt;400</td>
</tr>
<tr>
<td>Gamma</td>
<td>Combivir/Viramune/Kaletra</td>
<td>70</td>
<td>Unknown 4</td>
<td>148</td>
<td>&lt;400</td>
</tr>
<tr>
<td>Delta</td>
<td>Truvada/Sustiva/none</td>
<td>51</td>
<td>1982 14</td>
<td>445</td>
<td>205</td>
</tr>
</tbody>
</table>

1 All patients were at different stages of HIV-1 disease progression and were on antiretroviral medication with adequate suppression of virus. 2 Infected for many years, and first tested 01/03/2005. 3 Serum levels.

Table 1. HIV-1 population at baseline
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**Graph a:**
- **Alpha:** CD4⁰ cells/µl over months diagnosed.
- **Beta:** CD4⁰/CD8⁰ ratio.
- **Gamma:** CD4% vs. CD8%.

**Graph b:**
- **Alpha:** HIV RNA copies/ml.
- **Beta:** IL₁₀ (µM).
- **Gamma:** CD4⁰ cells/µl.
- **Gamma:** CD8⁰ cells/µl.
- **Gamma:** Granulocytes cells/µl.
- **Gamma:** Monocytes cells/µl.
- **Gamma:** Treatment Weeks.
increase in the CD4+ lymphocyte axis of oscillation in patients Alpha, Beta, Gamma, and PI Z2-1 was inversely related to baseline CD8+ lymphocyte percentage (n=4, r^2=0.99, p<0.008). This observation suggests that the improved CD4/CD8 ratio resulted from the increase in the horizontal axis of oscillation of CD4+ lymphocytes as well as the longitudinal decrease in CD8+ lymphocytes. There was no sustained increase or decrease in B cells (CD19+ lymphocytes), NK cells (CD16+/56+ lymphocytes), granulocytes, monocytes, eosinophils, basophils, or platelets (data not shown). However, there were cyclic changes in all cell types, as illustrated by the levels of monocytes and granulocytes (Figure 1, lower panels). Granulocytes, NK cells, basophils, eosinophils, and platelets, all derive from myeloid-lineage progenitors and cycled in tandem. Monocytes are of myeloid lineage, but cycled independently of lymphocytes and granulocytes.

Expanded CD4^+ lymphocytes are phenotypically mature and respond to stimulation.

Glucocorticoids cause the release of granulocytes and lymphocytes from tissue, but the peak increase occurs within 6 hrs and dissipates within 24 hrs (8). Glucocorticoid-induced demarginalized lymphocytes are unresponsive to stimulation. To determine whether α1PI-mobilized CD4^+ lymphocyte populations were functional, CD4^+ lymphocytes were isolated from blood and cultured in stimulation media. Harvested culture supernatants were quantitated for a panel of cytokines representing subpopulations of CD4^+ lymphocytes, specifically IL-2 (Th1), IL-4 (Th2 + NKT), IL-10 (Th2), and IFNγ (Th1 + NKT). Harvested cells were examined for NFκB activation. Whether isolated from HIV-1 uninfected volunteers or from HIV-1 patients pre-treatment or under treatment, CD4^+ lymphocyte populations were equivalently capable of being stimulated (Table 2, Figure 4).
CD4+ lymphocytes in whole blood were analyzed by FACS analysis for phenotypic markers characteristic of mature and immature, activated and quiescent cells including CD34 (stem cells), CD8 (double positive and double negative immature cells), CD45RA (naive cells), CD45RO (memory cells), CD25 (IL-2Rα activated cells and thymocytes), CXCR4 and CCR5 (HIV-1 tropism-determining chemokine receptors). Patients Alpha and PIzz-1 expressed significantly greater CD4+CD45RA+ naïve cells than did the HIV-1 uninfected volunteers (Figure 4b). In parallel, patient Alpha and PIzz-1 as well as PIZZ-2 expressed significantly lower CD4+CD45RO+ memory cells than the HIV-1 uninfected volunteers. However, these differences did not appear to be related to α1PI therapy since the percentage of naïve and memory cells remained steady in each patient throughout therapy. All other surface markers measured were normal (not depicted). These results suggest that the phenotypic profile of CD4+ lymphocytes unique to each individual was maintained within the new generation of lymphocytes.

**DISCUSSION**

The processes that renew the adult human CD4+ lymphocyte population are poorly understood and the sinusoidal changes we observed may offer insight into the renewal mechanisms. While cyclic variation in circulating CD4+ lymphocytes has not been previously described, cyclic neutropenia occurs with an average 21-28 day periodicity and is caused by mutations in the α1PI receptor, HLECS (9). The α1PI-induced in situ proliferation of CD4+ lymphocytes (24-48 h) (10) or the release of functional CD4+ lymphocytes from lymph tissue into circulation as occurs in an acute phase reaction (4-24 h) (11) may explain cycling, but neither of these explanations account for the 2 wk lag in appearance and 23 day periodicity. Rather, a multi-step process is implied.

In adult mice, thymopoiesis is a multi-step process defined by a 21-day cycle. The process involves the cyclic accumulation of progenitor cells in the adult bone marrow (3-5 wk), export of progenitor cells from the bone marrow thereby vacating the bone marrow niche (~1 wk), temporally coordinated

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**Figure 3. Sinusoidal analysis of CD4+ and CD8+ lymphocytes and the corresponding increase in CD4/CD8 ratio.** Sinusoidal curve fit analyses were performed using CD4 and CD8 data from patients Alpha (a), Beta (b), Gamma (c) and PIZZ-1 (d) over the course of Zemaira® therapy. CD4+ lymphocyte oscillation is defined by f(x) = y0+a* sin [(2πx/b)+c] where y0 = axis of oscillation, a = amplitude, x = days in treatment, b = wavelength, and c = phase shift from day 0. Each sine curve has a fit of r²>0.99, α=0.05, power of test = 1.0, and each variable contributed to the equation with p<0.02 with the exception of patient Gamma’s CD4 data where amplitude contributed to the equation with p<0.07. CD8 data for patients Beta and Gamma did not fit a sine curve. CD4 sine wave values determined for each patient (y0,a): Alpha (585,100), Beta (318,59), Gamma (145,15), PIZZ-1 (814,92). By sigmoidal regression, greater amplitude is correlated with a greater axis of oscillation, r²=0.999, and a greater pre-treatment CD4/CD8 ratio is correlated with a greater post-treatment axis of oscillation, r²=0.999.
importation of progenitor cells into the finite niche of the thymus, positive selection, release of mature T cells and progenitor cells into blood, and repopulation of the bone marrow niche at the end of the cycle (12). When positive selection of CD4+ thymocytes is impaired, the number of CD4+ lymphocytes diminishes by up to 80% (13), and as a consequence the number of CD8+ lymphocytes increases thereby producing a decreased CD4/CD8 ratio (13,14).

In the analogous human system, HLECS, CXCR4, and CXCL12 are required for progenitor cells to vacate bone marrow, (2,15). In the present study, a lower pre-treatment CD8 percentage was inversely correlated with α1PI–induced expansion of CD4+ lymphocytes as would be expected if treatment had re-established positive selection. Thus, we propose that the observed α1PI–induced changes in CD4+ and CD8+ lymphocyte numbers resulted from the binding of α1PI to the CXCR4/HLECS/CXCL12 complex during cell migration thereby facilitating adult thymopoiesis. In contrast to all other patients in the study who exhibited a 3-week cycle, patient Gamma exhibited a 2-week cycle suggesting some parts of the thymopoiesis cycle were intact, but that at least one step was impaired. This patient was documented to have lost the ability to respond to PPD which is a T lymphocyte-mediated response and is clinically interpreted to mean loss of immune function. In support of this hypothesis, there was no evidence of increased numbers of T lymphocytes in this patient suggesting that progenitor cells were being released from bone marrow, but not establishing residence in the stem cell niche of the thymus.

The phenomenon that PIZZ patients exhibit deficient hepatocyte-synthesized α1PI from birth, yet manifest normal numbers of CD4+ lymphocytes suggests there are additional considerations during fetal thymic selection, possibly the thymic or stromal supply of α1PI. Both myeloid and lymphoid cells are known to synthesize α1PI in bone marrow (16,17) Alternatively, the role of α1PI in hematopoiesis might be supplanted by other proteinase inhibitors during fetal development.

The duration of benefit for 2 wks, but not 5 wks, post-treatment suggests α1PI augmentation might be effective with less frequent than weekly administration. Our results predict that α1PI augmentation may overcome a localized pathologic system thereby allowing the immune system to recover and regain production of normal numbers of CD4+ lymphocytes in a subset of HIV-1+ patients who are on antiretroviral therapy and have functioning lymphatic and hematopoietic tissue.

Figure 4. Functional characteristics of expanded CD4+ lymphocytes. a) NFκB phosphorylation in response to stimulation of the T cell antigen receptor complex. Isolated CD4+ T cells were cultured at 10^6 cells/ml for 3 days in the presence of antibodies reactive with CD2, CD3, and CD28 as described in Methods. Cells from patient Alpha were isolated after 11 weeks of Zemaira® therapy. Cells from patient Delta were isolated at baseline. Culture supernatants were simultaneously measured for cytokine release (Table 2). b) Percentage of immature, naïve, and memory T cells. Analysis for the presence of naïve (CD4+CD45RA+) and memory (CD4+CD45RO+) T cells in whole blood was performed as described in Methods. Phenotypic analysis was performed at 3-8 different time points during therapy using blood collected from patients Alpha ( ), Beta ( ), Gamma ( ), Delta ( ), PI2Z-1 ( ), PI2Z-2 ( ), and non-HIV-1 controls ( ). Values represent mean and standard deviation. Stars (*) indicate statistical differences with respect to non-HIV-1 control values, p<0.001 and power of test α > 0.99.
Table 2. Stimulated T cell cytokine release

<table>
<thead>
<tr>
<th>Patient</th>
<th>Treatment</th>
<th>IL-2 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>INFγ (pg/ml)</th>
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<tr>
<td>Alpha</td>
<td>11</td>
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<td>148</td>
<td>&gt;500</td>
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<tr>
<td>Beta</td>
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<td>792</td>
<td>&lt;31.2</td>
<td>&lt;7.8</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Delta</td>
<td>untreated</td>
<td>&gt;800</td>
<td>&lt;31.2</td>
<td>n.d.²</td>
<td>&gt;500</td>
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<tr>
<td>non-HIV-1</td>
<td>untreated</td>
<td>&gt;800</td>
<td>&lt;31.2</td>
<td>239</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

¹ Cytokines were measured in culture supernatants harvested from isolated CD4⁺ T cells stimulated by antibodies reactive with CD2, CD3, and CD28. Harvested CD4⁺ T cells were simultaneously monitored for NFκB activation (Figure 4). In two attempts, the number of CD4⁺ lymphocytes isolated from Patient Gamma was insufficient for stimulation analysis. Cytokines were undetectable in serum or culture supernatants from unstimulated CD4⁺ T cells with the exception of Patient Delta who exhibited 51 pg IL-2/ml in his baseline serum sample, and Patient Beta who exhibited 14 pg IL-10/ml in serum at treatment week 8.

² not determined

REFERENCES


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