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Mycobacterium avium infection in HIV-1-infected subjects increases monokine secretion and is associated with enhanced viral load and diminished immune response to viral antigens

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SUMMARY

The complex interaction between HIV-1 infection and *Mycobacterium avium* was studied. Viral burden was assessed, as well as immune response to HIV-1 in the context of *Myco. avium* infections. We also examined serum cytokine levels and cytokine release by blood mononuclear cells in HIV-1-infected subjects, infected or not with *Myco. avium*. Undetectable serum levels of IL-1, tumour necrosis factor-alpha (TNF-α) and IL-6 were found in normal controls and in groups I, II and III of HIV-1-infected subjects. Moderate levels of TNF-α, IL-1 and IL-6 were found in the sera of group IV patients. When group IV was subdivided into subjects with and without *Myco. avium* infections, subjects with *Myco. avium* infections were shown to have higher serum levels of TNF-α, IL-Iβ and IL-6 than those with other infections. Blood mononuclear cells from controls and HIV subjects were stimulated with bacterial lipopolysaccharide, and cytokine levels assessed. Cells from group II patients were shown to secrete normal levels of TNF-α and IL-6, and lower levels of IL-1β; group III subjects released higher levels of IL-6. Patients in group IV had blood cells that released elevated levels of IL-6 and TNF-α, and lower levels of IL-1β. Group IV subjects with *Myco. avium* infections had blood cells that released higher levels of TNF-α, IL-6 and IL-1 than group IV subjects with other infections. Assessment of viral burden in cells of HIV-1-infected subjects revealed that *Myco. avium*-infected subjects had a higher level of virus burden and a lower level of lymphoproliferative response to an inactivated gp120-depleted HIV-1 antigen than AIDS subjects with other infections. These data suggest that *Myco. avium* infections in HIV-1-infected subjects hasten the progression of viral disease, enhance cytokine release and contribute to the anergy to viral antigens.

Keywords *Mycobacterium avium* HIV-1 cytokines monocytes

INTRODUCTION

A decline in the number of CD4$^+$ T lymphocytes is the hallmark of AIDS, in subjects infected with HIV-1 [1,2]. Indeed, HIV-1 predominantly infects CD4$^+$ T lymphocytes, although there is good evidence that tissue macrophages constitute another important reservoir for the virus [3]. The development of AIDS determines a high sensitivity to opportunistic infections, lymphadenopathy, neurologic disorders, a progressive and relentless decrease in CD4$^+$ T cells and a polyclonal B cell activation [1,4]. The decline in CD4$^+$ T lymphocytes is associated with a decline in various immune functions, including the synthesis and release of cytokines crucial to host resistance, such as interferon-gamma (IFN-γ) [5].

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HIV-1-infected subjects have a very high incidence of disseminated infections with *Mycobacterium avium* [6], an opportunistic pathogen that primarily infects cells of the macrophage/monocyte lineage *in vivo*. *Mycobacterium avium* infections in the context of AIDS significantly contribute to the morbidity and mortality of infected subjects, notably by inducing a severe anaemia [7]. *Mycobacterium avium* infections occur in a large proportion of HIV-1-infected subjects, and a recent study has shown that the median survival of AIDS patients was 134 days after a positive blood culture for *Myco. avium*, with only 13% of patients alive at 1 year [8]. These *in vivo* observations suggest that *Myco. avium* infections may hasten the progression of AIDS. The exact relationship between the various opportunistic pathogens that infect AIDS patients and the progression of viral disease is still unclear. Some authors have raised the possibility that mycobacterial infections may enhance the replication of HIV *in vivo* by recruiting cytokines

(tumour necrosis factor-alpha (TNF-α), IL-1, IL-6) which induce viral replication, or contribute to the pathophysiology of HIV-1 infection by excess TNF-α release [9].

One hallmark of mycobacterial infections is the very high production of cytokines such as TNF-α, IL-1 and IL-6 [10], which, although they may increase resistance to microbial infections, may actually promote HIV replication in monocytes [11–16]. Moreover, the chronic and exuberant release of these proinflammatory cytokines will contribute to immune dysfunction and tissue damage [12]. In addition, mycobacterial infections in humans and experimental animals are associated with a number of suppressive events, linked to the development of T cell- or macrophage-suppressive populations [17,18].

The best approach to dissecting these problems may well be to examine the cellular events in HIV-1 subjects with *Myco. avium* infections. In this study, we compared a number of important immune parameters in HIV-1-infected subjects, with and without *Myco. avium* infections.

SUBJECTS AND METHODS

Subjects

HIV-1-infected individuals were divided into four groups according to the classification proposed by the Centres for Disease Control [4]. Patients of group II are seropositive carriers with asymptomatic infection ($n = 12$). Group III includes patients who have had palpable lymph nodes for more than 3 months in the absence of a concurrent illness or condition other than HIV infection to explain the findings ($n = 8$). Patients in group IV were subdivided into patients of category C.1 with *Myco. avium* infections ($n = 8$), patients of category C.1 with *Pneumocystis carinii* infections ($n = 9$), or patients with fungal infections (candidiasis, pulmonary or bronchial) ($n = 6$). Healthy age- and sex-matched controls were seronegative volunteers and did not belong to any known risk group for HIV-1 infection.

In group II, seven patients were not receiving any antiretroviral therapy, whereas five others were receiving azidothymidine (AZT; mean duration of treatment $2{\cdot}4 \pm 1{\cdot}8$ months). All patients in group III were receiving AZT (duration of treatment $8{\cdot}3 \pm 4{\cdot}6$ months). Patients in group IVC with *Myco. avium* infections were receiving AZT (duration of treatment $13{\cdot}4 \pm 8{\cdot}6$ months); none of these subjects was receiving a regimen of antibiotics at the time of blood sampling. Four patients in this group had recovered from disseminated cytomegalovirus infection at the time of sampling. Patients with *Pneumocystis* infections were receiving AZT (duration of treatment $14{\cdot}8 \pm 7{\cdot}9$ months); four of them were also receiving trimethoprime sulphamethoxazole at the time of blood sampling. Patients with candidiasis were receiving AZT (duration of treatment $17{\cdot}6 \pm 10{\cdot}4$ months).

Cell cultures

Mononuclear cells were separated from heparinized peripheral blood by sedimentation on Histopaque (Sigma, St Louis, MO). Cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics (penicillin/streptomycin) and 1% pooled AB serum (all from Gibco, Grand Island, NY) at 10^6 cells/ml in polypropylene tubes. Cells were stimulated with 10 μg/ml of lipopolysaccharide (LPS; from *Escherichia coli* 0111:B4; Sigma) for 18 h. Supernatants were obtained by filtration with a 0·45-μm filter, and frozen at −70°C before cytokine analysis.

Cytokine levels

Levels of antigenic TNF-α, IL-1β and IL-6 were determined using commercial ELISA kits from R&D Diagnostics (Minneapolis, MN). The sensitivities of the assays were 30, 54 and 40 pg/ml, respectively. Since these assays use a mouse coating antibody to capture the cytokines, all positive samples were re-tested in the presence of 50 μl of normal mouse serum, to avoid the possibility of false reactivity due to heterophilic antibodies [19].

T cell proliferation assay

Peripheral blood mononuclear cells (PBMC) from either HIV-1$^+$ or HIV-1$^-$ individuals were seeded in round-bottomed 96-well plates (Costar, Toronto, Canada) at 2×10^5 cells/well in 100 μl of Neuman–Tytell serumless medium (Gibco) with 5% pooled human AB serum. The response to HIV-1 was assessed using a density gradient purified HIV-1 virus preparation (a kind gift of Immunocorp Inc., Montreal, Canada). This preparation was obtained as described elsewhere [20]. The virus was inactivated with β-propiolactone at 37°C for 12 h, followed by irradiation with 45 kGy. The gp120 content of the final preparation was less than 1 ng/mg. Pilot studies showed that 5 mg/2×10^5 cells was the optimum HIV-1 antigen concentration for proliferation.

After a 6-day incubation, cells were labelled with 1 μCi of ^{3}H-thymidine (Amersham, Arlington Heights IL; 44·5 Ci/μmol). Eighteen hours later, cells were harvested on filters, and processed for scintillation counting. Results are expressed as a 'stimulation index' (SI), which is the geometric mean ct/min of the cells plus antigen divided by the geometric mean ct/min of the cells without antigen. The mean SI for the seronegative samples was $1{\cdot}03 \pm 0{\cdot}07$ (s.e.m.), with a range of 0·74–1·37. An SI of 1·7 or greater was considered a positive response ($P < 0{\cdot}01$, Dunnett's test).

Virus isolation

Virus burden in HIV-1-infected subjects was indirectly assessed via a co-cultivation method. Cultures were initiated by adding 10^7 PBMC from an HIV$^-$ subject to 5×10^6 PBMC from a seronegative subject with 15 μg/ml of phytohaemagglutinin (PHA). After a 7-day incubation, supernatants were obtained and p24 levels assessed via an ELISA kit (Coulter Immunology, Boston, MA).

β_2-microglobulin

Serum β_2-microglobulin was measured via a commercial ELISA kit (Immunocorp, Montreal, Canada).

Statistical analysis

Comparisons between multiple groups were made by simultaneous comparisons of means. Comparisons between pairs were made by a one-tailed non-parametric test using medians, or by χ^2 test. All statistical tests were done with computer software (Brainpower, Inc.).

RESULTS

Subject groups

The characteristics of the subjects are shown in Table 1. Patients in group IVC were divided into patients with or without *Myco. avium* infections; these subjects had similar lymphocyte and

Table 1. Characteristics of the subjects

Group	n	Total lymphocyte count/μl	CD4 count/μl	Treatment†
Controls	10	2153 ± 236	908 ± 158	
Group II	12	1713 ± 314	411 ± 113	AZT [5]
Group III	8	1411 ± 316	208 ± 97	AZT [8]
Group IV C.1 (*Mycobacterium avium*)	8	738 ± 311	101 ± 61	AZT‡ [8]
Group IV C.1* *Pneumocystis*	15	809 ± 257	148 ± 45	AZT [15] Trimethoprime Sulphamethoxazole [4]
Candida §				

* Nine subjects with *Pneumocystis*, six with bronchopulmonary candidiasis. No significant differences emerged in T cell or $CD4^+$ counts between subjects infected with *Pneumocystis* and those infected with *Candida*.

† Duration of treatments indicated in Subjects and Methods.

‡ Blood was obtained before administration of antimycobacterial antibiotics.

§ Treatment with Amphotericin B was begun after sampling for three of these patients.

$CD4^+$ counts. Subjects with *Candida* or *Pneumocystis* had active disease. Total lymphocyte counts and $CD4^+$ counts were within the range expected for the various groups.

Serum cytokine levels

Sera from the various subject groups were examined for the presence of IL-1β, IL-6 and TNF-α (Table 2). Sera from normal controls and from HIV-1-infected subjects in groups II and III contained undetectable levels of IL-1β, IL-6 and TNF-α (i.e. less than 20–40 pg/ml). However, sera from group IV subjects contained appreciable levels of all three cytokines. Group IV subjects with *Myco. avium* infections had TNF-α

Table 2. Serum cytokine levels in group IV (*Mycobacterium avium* and non-*Myco. avium* infected)

Group	Pathogen	Patient*	TNF-α†	IL-1β†	IL-6†
IV	*Pneumocystis*	1	76	208	336
Non-*Myco. avium*	*Pneumocystis*	2	84	246	512
infections	*Pneumocystis*	3	72	314	481
	Pneumocystis	4	95	317	517
	Pneumocystis	5	101	413	548
	Pneumocystis	6	178	93	612
	Pneumocystis	7	141	98	701
	Pneumocystis	8	133	149	214
	Pneumocystis	9	126	316	319
	Candida	10	99	349	349
	Candida	11	108	81	408
	Candida	12	114	372	308
	Candida	13	122	391	509
	Candida	14	130	241	432
	Candida	15	91	239	508
			111 ± 34	**217 ± 51**	**417 ± 96**
IV		1	211	311	839
Myco. avium		2	541	217	754
infections		3	326	481	859
		4	512	474	931
		5	236	361	630
		6	229	379	621
		7	287	418	786
		8	241	127	791
			323 ± 39‡	**346 ± 81‡**	**748 ± 118‡**

* All patients were receiving azidothymidine (AZT).

† All cytokine levels are expressed in pg/ml.

‡ Significantly different from the results of Group IV, non-*Myco. avium* infections.

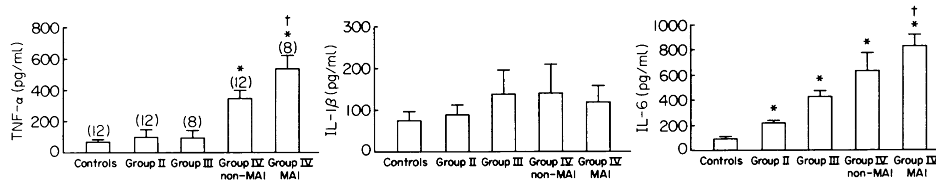

Fig. 1. Monokine secretion by peripheral blood mononuclear cells (PBMC) from subject groups. Cells were left untreated and supernatants were obtained for cytokine determinations by ELISA. MAI, *Mycobacterium avium-intracellulare*. Numbers of patients are indicated above columns. Results are means ± s.e.m. $^*P < 0{\cdot}05$ versus control values (Dunnett's *post-hoc* test). $^\dagger P < 0{\cdot}05$ versus all other groups.

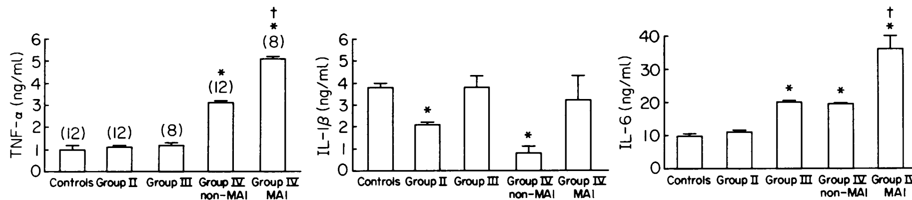

Fig. 2. Monokine secretion by peripheral blood mononuclear cells (PBMC). Cells were stimulated with 10 μg/ml of lipopolysaccharide (LPS) and supernatant cytokines were measured by ELISA. Legend as in Fig. 1.

levels ranging from 211 to 541 pg/ml, whereas group IV subjects with non-*Myco. avium* infections had levels ranging from 76 to 178 pg/ml ($P < 0{\cdot}001$). Similarly, subjects with *Myco. avium* infections had higher levels of IL-1β (346 ± 81 *versus* 217 ± 51 pg/ml; $P < 0{\cdot}05$) and IL-6 (748 ± 118 *versus* 417 ± 96 pg/ml; $P < 0{\cdot}005$).

Spontaneous cytokine release by PBMC from subject groups

Figure 1 shows spontaneous cytokine release by PBMC from subject groups. Cells from normal subjects produced low to undetectable levels of cytokines. HIV-1-infected subjects in stages II and III had cells that spontaneously released IL-6. Subjects in group IV infected or not with *Myco. avium* had cells that spontaneously released IL-6 and TNF-α, but levels were highest in AIDS subjects with *Myco. avium* infection.

Cytokine releasability of PBMC

Next, we assessed the capacity of blood mononuclear cells to release IL-1β, IL-6 and TNF-α in response to LPS (Fig. 2). HIV-1-infected subjects in group II produced normal levels of IL-6 and TNF-α, but lower levels of IL-1β than normal subjects. Group III subjects released normal levels of IL-1β and TNF-α, but elevated levels of IL-6. Group IV subjects with *Candida* or *Pneumocystis* infections released elevated levels of IL-6 and TNF-α and lower levels of IL-1β. Group IV subjects with *Myco. avium* infections released significantly higher levels of IL-6, IL-1β and TNF-α than group IV patients with non-*Myco. avium* infections.

Serum β_2-microglobulin

Serum β_2-microglobulin data are shown in Fig. 3. β_2-microglobulin levels were moderately elevated in HIV-1-infected subjects in groups II and III. Group IV subjects with non-*Myco. avium* infections had elevated levels of this factor, but *Myco. avium*-infected group IV subjects had the highest levels.

Viral antigenic burden

Next, we indirectly assessed the viral antigenic burden in the subject groups using the co-culture method described in Subjects and Methods. As shown in Fig. 4, p24 levels were

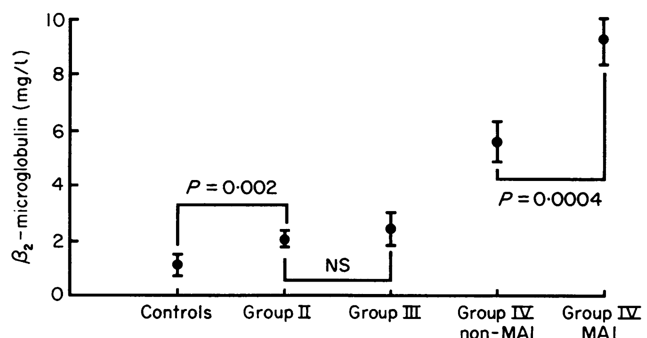

Fig. 3. Serum β_2-microglobulin from the different subject groups. Legend as in Fig. 1.

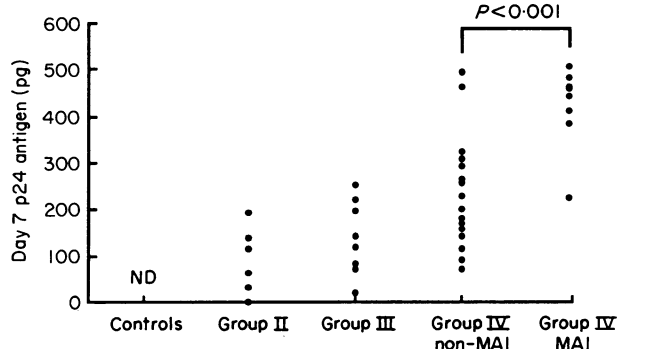

Fig. 4. Viral p24 antigen levels at day 7 of co-cultivation of HIV-1-infected subject peripheral blood mononuclear cells (PBMC) with seronegative feeder blood cells with phytohaemagglutinin A (PHA, 10 μg/ml). MAI as in Fig. 1.

relatively low in HIV-infected subjects in group II. Group III subjects had a greater viral burden. Patients in group IV had the most elevated viral burden, with *Myco. avium*-infected subjects having a significantly higher viral burden than those with non-*Myco. avium* infections.

Immune response to viral antigens
Lastly, we examined the proliferative response of PBMC to an HIV-antigenic preparation depleted of gp120 (Fig. 5). As expected, cells from normal subjects did not proliferate in response to the antigen (data not shown). Cells obtained from HIV-infected subjects in groups II and III proliferated significantly in response to viral antigens, with both SI greater than 2. Group IV subjects had cells that proliferated poorly in response to HIV antigens, and subjects with *Myco. avium* infections (SI=0·7) had a significantly lower response than subjects with non-*Myco. avium* infections (SI=1·8).

DISCUSSION

Disseminated *Myco. avium* infections occur as relatively late manifestations of HIV infection, frequently following the occurrence of other opportunistic diseases [21]. Of interest is the recent finding that a high proportion of young homosexual men, seronegative or seropositive for HIV, have evidence of a serologic response against *Myco. avium* [22]. This suggests that subjects in risk groups may be exposed early on to *Myco. avium*, before disseminated infections occur. There are considerable variations in time elapsed from initial HIV-1 infection to development of AIDS, and a number of investigators have hypothesized that co-factors such as opportunistic bacterial pathogens and certain viral agents are important in determining the progression of HIV infections to full-blown AIDS [1,9,23].

Disseminated infections with mycobacterial agents may modulate HIV infection, given the fact that mycobacteria and products thereof stimulate the production of cytokines [9,10]. These mediators (especially IL-6 and TNF-α) have been shown to be involved in the superinduction of HIV in T cells and/or monocytic cell lines [13]. Similarly, Wallis and co-workers reported that tuberculous HIV-1-infected subjects had greatly elevated levels of cytokines and circulating β_2-microglobulin [24]. These authors also suggested that enhanced TNF-α release could trigger accelerated HIV-1 replication; this remains controversial. Indeed, some reports have shown that interleukins and/or TNF-α did not significantly modulate HIV-1 replication in blood monocyte-derived macrophages [25]. Moreover, recent data show that activation of primary macrophages with interferons or bacterial products may decrease HIV-1 replication [25,26].

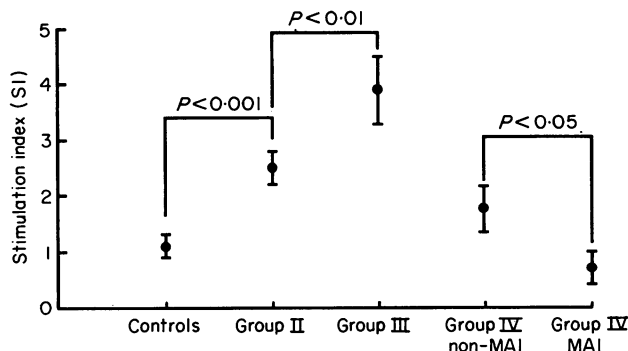

Fig. 5. Cellular immune response to HIV antigens. Mononuclear cells from the different subject groups were isolated, and the proliferation of cells to HIV antigens measured as described in Subjects and Methods. MAI as in Fig. 1.

The exuberant release of cytokines in the context of AIDS is likely to be linked to the progressive cachexia and related pathological consequences accompanying the development of opportunistic infections [12,27–29]. Our data suggest that *Myco. avium* infections in AIDS subjects are associated with very large increases in TNF-α, IL-1β and IL-6 release in serum, as well as an increase in the synthesis of TNF-α by PBMC. The levels of cytokines were much higher than in AIDS subjects infected with *Pneumocystis*, a pathogen that stimulates cytokine synthesis [30]. TNF-α production is likely to lead to enhanced activation of oxyradicals and other toxic metabolites by neutrophils and macrophages, as well as an increased adhesion of leucocytes to endothelial cells [31]. Elevated levels of TNF-α, IL-1β and IL-6 in the sera of AIDS subjects have already been reported, as well as an enhanced production of these factors by blood monocytes or alveolar macrophages [32, 33]. Viral products, notably gp120 (envelope protein) appear to modulate macrophage monokine and prostaglandin release [34], although HIV-1 infection of monocytes *per se* appears to impact only modestly on cytokine release [35]. We observed a diminished IL-1 production in HIV-1-infected subjects in group II, confirming the findings of Hober *et al.* [36].

β_2-microglobulin is not a specific marker for HIV-induced cell death. Indeed, as pointed out by Wallis *et al.*, it may represent a general marker of cell turnover [24]. However, in asymptomatic HIV-1^+ men there is a six-fold increased risk of progression to AIDS in subjects with serum β_2-microglobulin higher than 3·0 mg/*l*, compared with those with less than 1·89 mg/*l*, making serum β_2-microglobulin one of the most promising parameters for staging HIV disease [37]. Our data suggest that β_2-microglobulin is very significantly elevated in AIDS patients with *Myco. avium* infections, suggesting a hastening of the progression of AIDS following mycobacterial infections. In view of recent data which suggest that TNF-α may lyse HIV-1-infected T lymphocytes [38], the higher levels of β_2-microglobulin in *Myco. avium*-infected AIDS patients may be related to enhanced TNF-α levels which may lead to the lysis of HIV-1-infected cells.

There is increasing attention being given to cellular immune response to HIV antigens, and the subsequent cytokine production (i.e. IL-2) which may be related to the progression of viral disease [20,39–41]. As HIV-1-induced disease progresses, a remarkably specific suppression occurs that precedes the appearance of a suppressive activity against recall antigens or microbial antigens [41]. Cell-mediated responses to HIV antigens have been shown to decline surprisingly early in the course of HIV infection [1,21]. Our own data on cell-mediated immune response to HIV antigens show that patients in groups II and III have a significant response to gp120-depleted HIV, in relative accordance with results from other groups [20,39]. Individuals in group IV were shown to have a low level of proliferative activity to HIV antigens, with significantly lower proliferation in *Myco. avium*-infected patients; this lowered responsiveness was correlated with a higher viral load. Direct

assessment of p24 levels in the blood is not a very efficient method of estimating viral burden, owing to the presence of high levels of anti-p24 antibodies that confound the assay. New methods, based on acidification of the serum to dissociate the antibody–antigen complexes, have been suggested [42]. However, in our hands, this technique failed to allow us to detect significant p24 in the sera of most AIDS patients (data not shown).

Mycobacteria and products thereof will generate suppressor T cells, suppressor macrophages [18], and will induce suppressive soluble factors [43]. This may contribute substantially to HIV-1-induced immunosuppression which may occur via a combination of defective IL-2 responsiveness [44], suppressor cell activity mediated by enhanced prostaglandin synthesis [45], cytokine-mediated T cell destruction [38], as well as a direct suppression mediated by HIV-1 antigens [46]. Our data provide evidence for a relationship between burden of HIV-1 and cellular responsiveness to viral antigens; this suggests a potential usefulness for immune response enhancers in the treatment of AIDS [47]. In addition, AIDS progression could possibly be slowed by an aggressive prophylactic therapy against *Myco. avium* infections.

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