

**STUDY OF CYTOKINE PROFILE (IL-2, IL-4, IL-10, IFN- γ
TNF- α) IN PULMONARY TUBERCULOSIS PATIENTS
CO-INFECTED WITH HIV**

**THESIS SUBMITTED
UNIVERSITY OF BOMBAY**

**FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MICROBIOLOGY**

**SUBMITTED BY
S SMITA S. PATIL**

**UNDER THE GUIDANCE OF
DR. (MRS.) R. A. DESHMUKH**

**DEPARTMENT OF VIROLOGY
HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
BOMBAY**

Disclaimer

This research work belongs to

Smita Patil

No content of this work can be
circulated or used for any
commercial purpose without the
consent of respective researcher

**STUDY OF CYTOKINE PROFILE
(IL-2, IL-4, IL-10, IFN- γ , TNF- α) IN PULMONARY
TUBERCULOSIS PATIENTS CO-INFECTED WITH HIV.**

A

Thesis

Submitted to

THE UNIVERSITY OF MUMBAI

For the degree of

**MASTER OF SCIENCE
IN MICROBIOLOGY**

430

HAFKINE INSTITUTE LIBRARY
MUMBAI

Call No.
Price

No.
Source indicated.
Donated.

by

***SMITA PATIL
DEPARTMENT OF VIROLOGY
HAFKINE INSTITUTE
PAREL, MUMBAI - 12.
1999.***

STATEMENT UNDER UNIVERSITY RULE # 136

As required by the University rule number #136, I state that the work presented in the thesis is original and has not been submitted for any degree of this or other University. Whenever reference had been made to the work of others, it has been clearly stated along with the source of information in "Bibliography".



**Signature of the Research Guide
Dr. (Mrs) R. A. Deshmukh
M.D. Ph.D.**

**Officer In Charge,
Department of Virology,
Haffkine Institute for Research
Training and Testing.
Parel, Mumbai 12.**



**Signature of the Candidate
Miss Smita Patil**

To

**Aai, Baba
Jyoti & Avadhoot**

Acknowledgement

I take this opportunity to express my gratitude towards my guide Dr. (Mrs) R.A. Deshmukh, Officer In Charge, Department of Virology, Haffkine Institute. I wish to thank her for being kind and helpful. I really admire her patience and tolerance, especially with me. I am grateful to her for giving me freedom of planning my work and improving it from time to time with her invaluable suggestions.

I am thankful to Dr. V.L. Yemul, former Director of Haffkine Institute and Dr. B.G. Khadse, Director of Haffkine Institute for providing me excellent facilities to carry out the work.

The work would have not been possible without help of Dr. R.C. Padhi, Physician, Group Of TB Hospitals, Sewree and Dr. A. Deshpande, Head of Medicine department, JJ Group of hospitals, who permitted me to collect blood samples from patients in the respective hospitals.

Dr. S. V. Gadre, Assistant Director, Department of Bacteriology, Haffkine Institute is a great person to know, really approachable and ready to help in scientific as well as other matters. I cannot forget the innumerable times when he has helped me overcome problematic situations while working in the Institute as well as during my stay in the hostel.

I fall short of words to express my gratitude for Dr. (Mrs) S.V. Chiplunkar, Officer in Charge, Cellular Immunology, Cancer Research Institute. This work would have never come to its present form without her guidance. The CTLL cell line used for this work was a kind gift from her. I consider myself lucky to have not only the cell line and the training needed for my work but her invaluable guidance which helped me immensely in acquiring knowledge as well as discipline in the work and thoughts much needed for a student of Science. She made me understand the principles and importance of every small thing that is done during an experiment, also importance of planning the experiments. She helped me clear any confusion and her suggestions were of help to enable me to understand and interpret the data, otherwise I would have been just a glorified technician unable to think and understand what I was doing. I remain indebted to her.

I seek this opportunity to thank Dr. Partho Manna, Cellular Immunology, CRI for introducing me to the Techniques of cell culture.

I thank Mrs. M.V. Joshi, Librarian for providing me facilities as well as her timely suggestions which help me improve my presentations.

I thank Mr. J.V. Sawant, Instrument Engineer for providing attentive maintenance of the instruments and all the information about operating them.

I am thankful to Mr. A. Arekar, Biostatistician for statistical analysis of the data.

Mrs. Vidya and Mr. Loni, CRJ have been kind friends and teachers whose long caring vigils have help me learn and improve upon experimental techniques.

Mrs. Disha, Mrs Sharmila and Mrs Jyoti have been great friends, always willing to solve any difficulty that I had ever put across. I sincerely thank them for their suggestion which helped me avoid silly mistakes and clear confusions. I also appreciate their good will and support in the some difficult times during the work.

It gives me great pleasure to recall the initial days of the period spent in the Institute, associated with my senior Miss Sharmishtha Das and my colleague Mr. Arun Iyer. I will always remember their advises, encouragement and support when I did not know how to go about work. My association with them, although unfortunately short-lived, will always remain in the fondest memories.

Mr. Viraj Kulkarni has been a very kind senior and a friend in need. I am thankful to him for all the help, advises and care.

I cannot find words to express my feelings for dear friend Anuradha who has been with me during most trying times when every thing seemed to fail. I thank her for all the moral support that enabled me to complete my work.

Each member of the staff of the Virology department has contributed to the completion of work in some way. I am thankful to them for all the help extended to me.

The attempt of acknowledgements will remain incomplete without a mention of material help rendered by all the patients and healthy donors. I am grateful to them.

I thank my parents, sister and brother for all the love and faith bestowed on me. I wish I could be worthy of it.

I thank God for giving me opportunities to learn and improve.

Smita Patil

INDEX

CHAPTER	PAGE NO.
INTRODUCTION & REVIEW OF LITERATURE	1 - 44
AIMS & OBJECTIVES	45
MATERIALS AND METHODS	46 - 57
RESULTS	58- 91
DISCUSSION	93- 121
SUMMARY & CONCLUSION	122-123
APPENDIX I	124- 126
APPENDIX II	127-128
BIBLIOGRAPHY	129-172
SYNOPSIS	

INTRODUCTION
&
REVIEW OF LITERATURE

The acquired immune deficiency syndrome(AIDS) caused by the human immunodeficiency virus (HIV) and its related diseases have changed and shattered virtually every aspect of medicine and society at large. HIV is one of the most complex viruses known. Our ability to understand this virus and its replication in cell is likely to enhance profoundly our ability to understand cellular differentiation and proliferation in normal and disease states particularly within the immune network where HIV establishes itself and brings about dramatic dysfunction.

AIDS is just a name for cluster of diseases that immunodeficient people develop. The association between HIV and tuberculosis has emerged as a global threat with increasing number of co-infected patients all over the world. AIDS pandemic has stalled the elimination of tuberculosis and reversed many of the hard won gains in tuberculosis control in developing world. Tuberculosis being endemic in India is going to have devastating effect on the health care system.

HIV and tuberculosis have prolonged latency and dysfunction of cellular immune response is a common denominator in both the diseases. Thus both the pathogens play synergistic role when present together causing early damage of the immune system and accelerated progression to AIDS and death.

HIV and tuberculosis are the diseases of immune system and cytokines are the messengers of the immune system regulating a complex network of lymphoid cells, inflammatory cells and hematopoietic cells. Metchnikoff poked a rose throne into a starfish and observed a cellular reaction. A century later, this simple inflammatory reaction has been intensively studied and cytokines are the major components of it. Cytokinology is being extensively explored by generations of scientists and clinicians to scientifically characterise and clinically evaluate the potentials of these powerful molecules.

The present study was undertaken to evaluate the cytokine profiles in HIV-tuberculosis co-infected patients as altered cytokine profiles due to other infections need to be considered before designing vaccination therapy for AIDS in developing countries like India where diseases like tuberculosis, malaria, helminth infestations, which alter the immune responses inadvertently, are endemic.

The creator must have had an inordinate fondness for cytokines, having made so many that do so many things.

Agnus Thomson

The development of an effective immune response involves lymphoid cells, inflammatory cells and hematopoietic cells. The complex interactions among these cells are mediated by a group of low molecular weight regulatory proteins secreted by white blood cells and a variety of other cells in the body in response to a number of stimuli. These proteins are collectively known as cytokines to denote their role in cell to cell communication.

The term 'cytokine' encompasses those cytokine secreted by lymphocytes, formerly known as lymphokines and those secreted by monocytes and macrophages, formerly known as monokines. Although these two terms continue to be used in literature, they are misleading because many lymphokines and monokines are secreted by a broad spectrum of cells and not simply by lymphocytes and monocytes as their names would imply. Many of the cytokines are referred to as interleukins, a name indicating that they are secreted by some leukocytes and act upon other leukocytes. Presently, interleukins 1-18 have been identified. Other cytokines are known by their common names, these include the interferon and tumour necrosis factors. A group of low molecular weight cytokines including interleukin 8, is classified in the chemokine family. These molecules play an important role in inflammatory response.

Cytokines are best defined by not one but a set of characteristic features:

1. They are simple polypeptides or glycoproteins with a molecular weight $\leq 30\text{kDa}$ (some cytokines form higher molecular mass oligomers). Only IL-12 is a known heterodimer.
2. Constitutive production of cytokine is usually low or absent. Their production is regulated by various inducing stimuli at the level of transcription or translation.
3. Cytokine production is transient and action of radius is short.
4. Cytokines act by binding to specific high affinity cell surface receptors.
5. Most of the cytokine actions can be attributed to an altered pattern of gene expression in the target cells. Phenotypically, cytokine actions can lead to

an increase or decrease in the rate of cell proliferation, change in cell differentiation state and/or a change in expression of some differentiated functions.

Actions of cytokines can be profoundly influenced by the milieu in which they act, especially by the presence or absence of other biologically active agents with the resulting biological action reflecting various synergistic and antagonistic interactions of the agents present. Pleiotropy or ambiguity, redundancy, synergism/antagonism constitute the molecular philosophy of cytokine action. Cytokines may increase or decrease the production of other cytokines as well as expression of receptors for another cytokine. CD4⁺T helper cells exert most of their helper functions through secreted cytokines which either act on the cells that produced them in an autocrine fashion or modulate the responses of other cells through paracrine pathways [1].

Immune responses characterised by the production of distinct sets of lymphokines are optimally protective against different types of pathogens. In particular, responses dominated by the production of IFN- γ appear to provide defence against microorganisms that establish intracellular infection [2]. While responses dominated by the production of IL-4 and its congeners IL-5, IL-13 as well as IL-6 and IL-10 have been suggested to be most protective against infections with certain extracellular pathogens [3]. In addition, IL-4 and IL-10 may act to regulate the potential tissue damaging effects of responses dominated by IFN- γ production [4]. The seminal observation by Mossman *et al* [5] that repeated stimulation CD4⁺T helper (TH) lymphocytes *in vitro* with given antigens results in the development of a restricted and stereotyped pattern of lymphokine production that frequently falls in to one of the two phenotypes (TH1/TH2) has been amply confirmed in both murine and human systems [6,7]. They termed the two most readily discernible populations as TH1 and TH2 with TH1 clones being the ones that produced IL-2, IFN- γ , and TNF- β and The TH2 clones were those that produced IL-4, IL-5, IL-6, IL-13. Although IL-10 was originally described as the product of TH2 clones [8], it is now clear that IL-10 is also secreted by TH1 cells especially in human [9] and by activated macrophages.

Evidence rapidly accumulated showing that TH1 and TH2 clones differed in the types of antibody response they stimulated and that TH1 clones mediated DTH reactions, and that TH2 cells were more potent helpers for antibody production, thus providing simple explanation for observed dichotomy of immune response [10]. Soon after the identification of helper T cell subsets, it was proposed that TH1 cells were primarily responsible for cell-mediated immunity and DTH and TH2 cells for humoral immunity [10,11]. Nevertheless, it was clear from the early studies that IFN- γ , the signature of TH1 cells, induces the switching of B-lymphocytes to several IgG isotypes and was thus obligatory for some humoral responses [12,13].

The rather oversimplified classification of T effector functions into cell mediated and humoral immunity has been a source of considerable confusion and controversy. A logical functional classification of T cell subsets must be based on the known biological activities of cytokines produced by these T cells. The principal TH1 effector cytokine IFN- γ has two key functions:

1. It activates macrophages enhancing their microbicidal action.
2. IFN- γ stimulates the production of IgG antibodies, which bind to high affinity Fc γ receptors and complement proteins and therefore the principal antibodies involved in the opsonisation and phagocytosis of particulate microbes. IFN- γ dependent isotypes are best defined in mice and are mainly IgG2a and IgG3 [14]. Their human homologues are probably IgG1 and IgG3, but so far there is no good evidence that the production of complement fixing and opsonizing IgG antibodies in humans is TH1 or IFN- γ dependent.

Nevertheless, it is likely that the principal function of TH1 cells is to elicit phagocyte mediated defence against infections, because TH1 cytokines promote the ability of macrophages to both phagocytose and destroy microbes. TH1 dominant immune responses are often associated with inflammation and tissue injury, because two TH1 cytokines, TNF- β and IFN- γ , recruit and activate inflammatory leukocytes. The typical inflammatory reaction is delayed type hypersensitivity (DTH) and is often a price that is paid for protective immunity against microbes such as mycobacteria. Some TH1 cells acquire cytolytic capacity and the cytokines produced by TH1 cells, notably IL-2 and IFN- γ ,

promote the differentiation of CD8⁺T lymphocytes into active cytotoxic cells. These are the other ways in which TH1 subsets participate in the elimination of intracellular microbes.

The signature cytokine of TH2 cells are IL-4 and IL-5. IL-4 is the major inducer of B cell switching to IgE production and is therefore a key initiator of IgE dependent mast cell mediated reactions [15]. IL-5 is the principle eosinophil activating cytokine [16]. The production of these two cytokines by the same subset accounts for the frequent presence of both IgE and activated eosinophils in the TH2 dominated immune reactions such as allergies and helminthic infections.

IgE and eosinophil mediated defence is important for eliminating some but not all helminths [17] and it is still not clear how important TH2 induced phagocyte independent responses are in the defence against other infectious agents.

TH2 cells are excellent helpers for B lymphocytes and stimulate the production of high levels of IgM and non-complement fixing IgG isotypes, such as IgG1 in mice or its homologue, IgG4, in humans [14].

Several cytokines produced by TH2 cells have anti-inflammatory actions. IL-4 and IL-13 antagonise the macrophage activating action of IFN- γ . IL-10 suppresses numerous macrophage responses and TGF- β (which is produced by many other cell types besides TH2 cells) is anti-proliferative and inhibits leukocyte activation. The net result of TH2 activation is to inhibit acute and chronic inflammation, including DTH actions. This raises the possibility that an important physiological function of TH2 cells is not as effectors but as regulators of immune responses. In particular, TH2 cells may appear late in immune responses and serve to limit the injurious consequence of TH1 mediated protective immunity. Consistent with the hypothesis is the finding that during T cell activation *in vitro*, TH1 responses often develop early and TH2 cells accumulate as the response progresses [18]. A similar regulatory function has been proposed for some CD8⁺T cells that secrete 'Type 2' cytokines [19].

The TH1 and TH2 cells are not derived from distinct lineages that are committed before stimulation [20]. Experiments using limiting dilution analysis [21], and bulk culture experiments with homogenous population of cells from T cell receptor (TCR) transgenic mice [22,23] have confirmed the ability of a single cell

precursor to differentiate to either a TH1 or TH2 phenotype [23,24]. Using transgenic mice in which IL-4 producing cells expressed herpes simplex virus (HSV) type 1 thymidine kinase and therefore could be eliminated by gancyclovir, it was shown that activation of transgenic T cells in the presence of gancyclovir eliminates IL-4 and IFN- γ production [25]. This very elegant experiment demonstrated that effector cells producing either IL-4 or IFN- γ have a common precursor, which expresses the IL-4 gene. This precursor is a mature, naive CD4⁺T lymphocyte that produces mainly IL-2 upon initial encounter with antigen. They are referred to as 'precursors of T helper cells' or pTH cells. They are prevalent in thymus, lymph nodes and spleens of young immunologically naive animals. The pTH are small dense lymphocytes that are CD44^{lo}LECAM-1^{hi}, CD45Rb^{hi} when analysed by flow cytometry. They fail to make substantial amount of either IL-4 or IFN- γ . The pTH CD4⁺ T cells can develop into cells that principally produce IL-4 or IFN- γ upon restimulation. Swain has referred to recently differentiated cells that produce large amounts of lymphokine upon rechallenge as effector cells (eTH) [19]. The eTH cells appear uniformly large; their surface phenotype is CD44^{hi}LECAM-1^{lo} and CD45Rb^{lo} and can be induced to secrete IL-2, IL-3, IL-4 or IFN- γ rapidly (within 12-18 hours) following acute activation with polyclonal stimulators. Swain has referred to these cells as memory (mTH) cells [24].

TH1 and TH2 are not the only cytokine patterns possible T cells expressing cytokines of both patterns have been designated as TH0 [19]. TH0 cells probably represent a heterogeneous population of partially differentiated effector cells consisting of multiple discrete subsets, which can secrete both TH1 and TH2 cytokines. The cytokine response at effector level can remain mixed or can be induced to further differentiate into TH1 or TH2 pathway under the influence of signals received from the microenvironment [26]. T cells producing high amounts of transforming growth factor beta (TGF- β) have been termed TH3 [27].

Some studies however have demonstrated heterogeneity of cytokine synthesis at the single cell level even in polarised TH1 and TH2 responses [28,29]. Moreover, each of the cytokine genes seems to be under unique control, with distinct

tendencies for concordance (e.g. IL-4 and IL-5) or discordance (e.g. IL-4 and IFN- γ) [30].

Thus another possibility is that the cytokine profiles are largely random at the clonal level. Exogenous signals direct T cells to differentiate into TH1 or TH2 cells by increasing the probability of expression of certain cytokine genes at the population level and not by activation of expression of transcriptionally linked genes in the individual cell [29].

Although TH1 and TH2 cells are certainly not the result of a pre-existing functional dichotomy of CD4⁺ T cells they can be regarded as polarised forms of specific immune response that frequently occur under the combined action of genetic and environmental conditions.

The most potent stimuli inducing differentiation of naive cells into TH1 or TH2 types are cytokines themselves. Cytokines serve as autocrine growth factors for T cells which secreted them and later promote differentiation of naive T cells to that subset [31].

In both mice and humans, IL-12 and IL-4 are the most important cytokines in directing the development of TH1 and TH2 cells respectively. Whether TH1 cells develop in response to antigen is controlled by relative levels of T cell derived IL-4 versus APC (macrophage or dendritic cells) derived IL-12 [32].

IL-12 produced by activated macrophages and dendritic cells is the dominant factor in directing the development of the TH1 cells. Potent adjuvant which activate macrophages and many microbial products, including endotoxin, components of viruses, intracellular bacteria such as listeria and mycobacteria, and protozoa such as toxoplasma stimulate IL-12 production and induce TH1 dominated immune responses [33]. Functional receptors for IL-12 appear to be restricted to recently activated, uncommitted cells and to TH1 cells, and are lost on differentiated TH2 cells [34]. IL-12 activates three putative transcription factors, Stat 1, Stat 3, Stat 4 [35]. Of these Stat 4, appears to be activated by only IL-12 and no other cytokine. Knocking out either IL-12 [36] or Stat 4 [37] results in markedly reduced TH1 responses.

In vivo data from IL-12 deficient mice show that TH1 responses can be generated in absence of IL-12 although the magnitude of DTH is substantially decreased

and secretion of IL-4 is enhanced [38]. Thus although required; IL-12 is not essential for a TH1 response. In humans, IL-12 appears to be able to favour differentiation of TH1 cells. TH1 development depends, at least in part, on the production of endogenous IL-12. IL-12 not only induces strong augmentation in IFN- γ production but also inhibited the development of IL-4 producing T cells [39]. IL-12 directly affects the differentiation of both CD4 and CD8 human T cells by providing a stable inducing effect for IFN- γ production [40].

In murine model, it was demonstrated that the IL-12 production was dependent on IFN- γ , and TNF- α [41]. IFN- γ deficient animals display default to TH2 phenotype suggesting that IFN- γ is required for TH1 induction [42]. Also, activation of T cells in presence of IFN- γ unresponsive APCs fails to develop TH1 phenotype [43]. IFN- γ promotes TH1 development, partly by enhancing IL-12 secretion by macrophages [44] and partly by maintaining the expression of functional IL-12 receptors on CD4⁺T cells, rendering the cells more responsive to IL-12 [45]. IFN- γ may have a minor TH1 inducing effect that is independent of IL-12. Thus macrophages respond to many microbes by producing IL-12, which stimulates the development of T cell subset whose function is to enhance the microbicidal activities. There is an elegant symmetry in this sequence of events.

IFN- α can replace the requirement for IFN- γ . It augments IL-12 induced TH1 development or inhibition of subsequent IL-4 production even if it failed to significantly enhance IL-12 priming for subsequent IFN- γ production [46]. Role of IL-1 and TNF- α for development of TH1 cells producing maximal levels of IFN- γ was suggested [28]. TGF- β also may play a role in development of TH1 cells [47,48], but it also inhibits an ongoing TH1 type CMI [49] and also shown to have opposing effects on naive CD4⁺T cells from mouse developing into TH1 types [50] so the mechanism of action is not known. The role of IFN γ , IFN α and TGF- β in favouring the *in vitro* development of TH1 cells has been demonstrated in humans as well [51]. IFN- γ inducing factor (IGIF) that induces IFN γ production by NK cells and T cells more potently than IL-2 [52]. This may be involved in the development of TH1 cells as well as mechanism of tissue injury in inflammatory reaction.

IL-4 directs TH2 development. The addition of exogenous IL-4 to *in vitro* culture of naïve CD4⁺ murine T cells in presence of mitogen or antigen directs the naïve CD4⁺T cells to develop into a population which on restimulation produces high levels of IL-4, IL-5, IL-10 as well as IL-3 and GM-CSF (TH2 pattern). IL-4 functions not only to the effect of promoting TH2 pattern but also suppresses development of IL-2 and IFN γ secreting effector cells [53].

Presence of IL-4 during primary stimulation of naïve CD4⁺T cells results in T cell populations that secrete high levels of IL-4 [54]. Injection of anti IL-4 antibody at the time of immunisation strikingly diminished the magnitude of IL-4 production [55]. These models are suggestive of the IL-4 directed TH2 development. However, even in IL-4 knockout mice, the TH2 response was not completely absent [56]. In the presence of exogenous IL-4, human CD4 cells stimulated with PPD (an antigen that usually favours the development of TH1 like cells) were found to develop into effectors producing high amounts of IL-4 and IL-5 in addition to IFN γ or IL-2 (TH0) or even IL-4 and/or IL-5 alone (TH2). It also causes depressive effect on development of cytolytic potential.

IL-10 during primary stimulation of TCR transgenic T cells did neither upregulate IL-4 or IL-5 nor inhibits IL-2 production. However, IL-10 decreased IFN γ production [23]. Addition of antibody to IL-10 led to increased IFN γ production and decrease in IL-4 and IL-5 production. IL-10 only inhibits development of TH1 phenotype by inhibiting production of IL-12 by macrophages [57] but only IL-4 directly skews toward the TH2 phenotype.

IL-1 is a costimulator of TH2 but not TH1 cells. Competitive antagonist of IL-1 (IL-1RA) blocks the proliferative response of some TH2 clones [58]. Murine TH2 but not TH1 cells are able to produce IL-1 α in its active form and this cytokine acts as an autocrine growth factor for TH2 cells [59]. However, macrophage derived IL-4 is found to be required for the development of TH1 cells and IL-1 and TNF- α were required for production of maximal levels of IFN γ upon restimulation of TH1 cells [60]. In human system however, IL-1 is neither required for proliferation of TH1 and TH2 clones. However, IL-1 neutralisation in bulk culture favoured the development of T cell clones producing high concentration of IFN γ and no or low concentration of IL-4 [61]. Thus the role of IL-1 is controversial.

CROSSREGULATORY ACTIVITY OF CYTOKINES

Crossregulation by TH2 cells:

IL-4 prevents priming of naive T helper cells to become IFN γ producers. IL-4 is able to strikingly inhibit IL-2 production by naive T cells in response to anti-CD3 antibody and APC [62], but has no effect on T cell lines or T cells that have been already primed *in vitro*. The suppressive effects of IL-4 on IFN γ are markedly diminished in presence of IL-12 [63]. However, IL-4 inhibits the IFN γ mediated positive feedback stimulation of IL-12 production by macrophages [64].

IL-10 is selectively produced by murine TH2 cells [8], but common to all types of human T helper cells [65] inhibits lymphokine production by TH1 but not by TH2 [66]. The inhibition is somewhat selective and depends on the time for lymphokine produced secretion of IFN γ and IL-3 (secreted mainly at later times after stimulation, is substantially inhibited while secretion of GM-CSF, TNF- β (lymphokine secreted at early times) is inhibited slightly. Accordingly effect on IL-2 production is often less than the effect on IFN γ production [67]. IL-10 inhibits IFN γ mediated positive feedback stimulation of IL-12 production by macrophages [64]. It also suppresses costimulatory molecules like TNF α and IL-1 and thereby inhibits the stimulation of NK cells and T cells by IL-12. This results in downregulation of TH1 cells [68]. IL-10 also inhibits synthesis of IFN γ by CD8 CTL clones, but the extent of inhibition is somewhat lower as compared to that in TH1 clones [69]. IL-10 inhibits secretion of IL-2 by TH1 cells while IL-2 causes clonal proliferation of CD8⁺T cells through a paracrine pathway; thus TH2 cells and other cells that secrete IL-10 regulate clonal expansion of those subsets that produce IFN γ .

IL-13 is a property of murine TH2 cells but is less restricted in human T helper cells. It dose not exhibit direct crossregulatory activity on TH1 cells [70], but modulates several macrophage functions:

1. It enhances the expression of integrin, CD23 and MHC Class II antigens.
2. It downregulates the production of nitric oxide.
3. It downregulates the production of IL-1, IL-6, TNF α , IL-12, IFN α and chemokines such as IL-8 and MIP-1 α [71]. Thus, the indirect downregulation of TH1 cells is

brought about by IL-13 through its inhibitory activity on IL-12 and IFN- α production that is important in differentiation of TH1 cells.

Crossregulation by TH1 cytokines:

IFN- γ is shown to inhibit proliferation of TH2 clones exposed to IL-2 or IL-4, in murine models [72]. However, it does not affect the lymphokine secretion including IL-4 by TH2 clones [69]. While IFN- γ does not inhibit lymphokine secretion by stimulated TH2 cells, IFN- γ inhibits many of the agonist effects of those secreted cytokines; e.g. IFN- γ inhibits proliferation of murine bone marrow cells stimulated with IL-3, IL-4, or GM-CSF [73]. IFN- γ can inhibit IL-4 dependent B cell differentiation [70] in addition to inhibition of proliferation of TH2 clones [65].

IFN- α plays a negative regulatory role in the development of TH2 cells. It is found to suppress IL-4 mRNA induction [75]. IFN- α augments IL-12 effects on inhibition of IL-4 production [76]. IFN- α inhibited development of allergen specific T cells into TH2 like cells [77]. This effect is due to selective antiproliferative activity on TH2 like cells, as addition of IFN- α in bulk culture before cloning result in T cell clones showing TH1 or TH0 profile [78].

IL-12 primes TH1 cells to produce IFN- γ and therefore develop into TH1 cells [79]. It inhibits differentiation of T cells into IL-4 secreting cells i.e. into TH2 cells [39]. This inhibitory effect is partly mediated by APCs [80]. IL-12 also suppresses switching to IgE secretion [81]. IL-12 however, is not capable of suppressing a TH2 recall response [82]. IL-12 also induces IL-10 that downregulates both IL-12 production and IL-12 induced IFN- γ production [83]. Thus may also limit its own effects. When IL-4 and IL-12 are present together, IL-4 suppresses the IL-12 induced priming for IL-10 production, but only minimally decreases the priming for high IFN- γ production [84].

Other factors which play role in determination of type of effector response:

1. Site of antigen presentation
2. Type of presenting cell
3. Costimulatory molecules
4. Properties of the immunogen

LIBRARY
HAFKINE INSTITUTE
MUMBAI

- a Physical form of immunogen and type of adjuvant
- b Dose of antigen
- c Peptide density and binding affinity

5. Hormones [1].

*For too long we have closed our eyes as a nation, hoping the truth was not so real...
For too long we have allowed the human immunodeficiency virus to spread.. At times we
did not know that we were burying people who had died from AIDS. At other times we
knew, but chose to remain silent ...
Now, we face the danger that half of our youth will not reach adulthood. Their education
will be wasted. There will be large number of sick people whom the healthy will not be able
to maintain.
Our dreams as people will be shattered...*

Thabo Mbeki

Deputy President of South Africa

A televised appeal to the nation, October 1998

The epidemic of HIV infection and AIDS emerged in the last quarter of the 20th Century and within less than two decades has affected over 190 countries [85]. As of the end of 1997; an estimated 30.6 million people worldwide, 29.5 million adults and 1.1 million children younger than 15 years - were living with HIV/AIDS [86]. Since the beginning of HIV/AIDS epidemic approximately 11.7 million (9 million adults and 2.7 million children) died of HIV/AIDS resulting in approximately 8.2 million unfortunate children younger than 15 years of age being orphaned world wide because of the premature deaths of their HIV infected parents [86]. By the year 2000, an estimated 40 million people worldwide will be HIV infected [86,87]. More than 90% of these cases will occur in developing countries [86]. These figures are the testimony of the tragedy unfolding for the last 19 years.

Natural history of the disease

The course of HIV infection varies among individual patients. However, a common pattern of development has been recognised. Primary infection with HIV followed by the development of detectable humoral and CMI responses to the virus and a prolonged period (median 10 years) of clinical latency during which patient is usually asymptomatic followed by the appearance of constitutional signs and symptoms [88].

Acute seroconversion syndrome: What is known is that **acute infection** is associated with high levels of HIV-1 replication, manifest by both cellular and plasma viremia and sometimes accompanied by a mononucleosis like illness. Onset of symptoms occurs within 3-6 weeks after exposure. The most common symptom reported is fever, adenopathy, pharyngitis, and skin rash.

Approximately, 1-3 months after initial infection, seroconversion occurs accompanied by a resolution of symptoms, rapid decline of circulating virus and development of multiple humoral and cell mediated immune responses, including both neutralising antibodies and cytotoxic T lymphocytes. Virus titres may also be higher in lymphocyte-rich tissues, e.g. lymphnodes, than in peripheral blood. Acute seroconversion is characterised by marked lymphocytopenia with depletion of both CD4⁺ and CD8⁺ lymphocytes. Atypical lymphocytes are commonly seen in the peripheral blood and may be seen at the levels of 20-30%. The initial lymphocytopenia is followed by a period of relative lymphocytosis, predominantly of CD8⁺ lymphocytes. After clinical symptoms of the acute seroconversion syndrome resolve, the CD8⁺ lymphocytosis normalises, however, the CD4⁺ cell count although increases does not return to baseline values [89]. Within 10-21 days after the onset of symptoms, a detectable antibody response is noted. The initial antibodies detected are typically immunoglobulin M (IgM) antibodies, which reach a peak within 2-5 weeks after the onset of clinical symptoms and are undetectable within 3 months [90]. The IgG response occurs after appearance of detectable IgM antibodies, with anti-gp 160 and anti-gp24 antibodies being detected first, followed by antibodies to gp 120 and gp41 [91].

Early disease: Early in the course of disease, when the CD4 count is >500 cells/mm, majority of individuals with high CD4 count generally has no symptoms related to HIV infection, with the exception of fever, mild to moderate lymphadenopathy, pharyngitis and skin rash. Reversal of lymphadenopathy is a potential sign of disease progression [92]. When symptoms are present, the most common HIV associated findings in patients with early disease are dermatological in nature e.g. seborrheic dermatitis, psoriasis, pruritic folliculitis (itchy red bumps). A slow progressive decline in CD4⁺ cells is typically seen during this time. Although the rate of decline may vary considerably and is difficult to predict, a decrease of 40-80 cells/mm³/year on an average is generally expected [93].

Middle stage of the disease when CD4 count is between 200 and 500 cells/ mm³, the majority of individuals remain asymptomatic or have only mild disease manifestations. Many of the skin and oral infections persist or worsen. Other disorders previously referred to as ARC (AIDS Related Complex) begin to appear.

These symptoms include recurrent *Herpes simplex* disease, *Varicella zoster* virus disease (shingles), mild oropharyngeal or vaginal candidiasis, oral hairy leukoplakia, recurrent seborrheic dermatitis, pruritic folliculitis, recurrent diarrhoea, intermittent fever and unexpected weight loss (≥ 10 pounds over 6-8 weeks). Other constitutional symptoms such as myalgias, arthralgias, headache and fatigue can also be seen in patients at this stage. Bacterial infections most frequently presenting as sinusitis, bronchitis or pneumonia are commonly seen in a number of patients. The aetiology of bacterial infections is generally associated with community acquired organisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, *Rochalimaea quintana*, salmonella, syphilis, *Neisseria gonorrhoeae*.

Late Disease: During late disease (CD4 counts are between 50 and 200 cells/ mm³), the risk of developing AIDS defining conditions rises substantially when CD4 counts drop below 200 [94]. Patients at late stage of the disease are at risk of developing opportunistic infections including *Pneumocystis carinii* pneumonia, *Toxoplasma gondii*, encephalitis, cryptosporidiosis, isosporiasis, tuberculosis, lymphoma, Kaposi's sarcoma, and esophageal candidiasis. Neurological disease processes become especially prevalent at this stage of infection. Some patients develop primary nervous system lymphoma, progressive multifocal leukoencephalopathy, HIV associated dementia characterised by cerebral atrophy and diminished cognitive function. HIV wasting syndrome defined as involuntary weight loss of ≥ 10 pounds of usual body weight is seen [93].

The course and rate of HIV disease progression is quite variable. Although, the median time between initial infection and the development of AIDS defining illness is 10-12 (slow progressors) years, a few individuals may progress to AIDS within 18 months (rapid progressors) while some individuals may not progress to the disease at all (Long term non progressors) [95].

Immunopathogenesis

HIV outsmarts the host with an ingenious strategy of infecting a cell, which is essential for its elimination. The CD4⁺ lymphocytes play an important role in the immune network [96]. The CD4⁺ lymphocytes are crucial for the maintenance of appropriate immunological responses against a wide range of pathogens as well as

malignant tumours. The interaction of CD4⁺ cells with B cells, natural killer cells, cytotoxic T cells, and monocyte/macrophages is effected primarily by the release of cytokines or other soluble factors and to a lesser extent, by direct cell to cell contact. CD4⁺T cells secrete factors that affect the growth and differentiation of nonlymphoid cells as well. As a result, it is clear that either quantitative or qualitative abnormalities in the CD4⁺T cell population can have profound effects on human immune system function [97].

The precise mechanism whereby HIV brings about destruction of its target cells is not completely understood, but a number of potential mechanisms have been delineated:

1. High levels of viral RNA and aberrant RNAs accumulate in the cytoplasm of HIV infected cells *in vitro* [98,99]. These RNA species may interrupt the normal cellular RNA processing, leading to cell destruction and death.
2. Massive virion budding from cell membrane of infected cell producing HIV may weaken the integrity of cell membrane and cause lysis of cell [100,101].
3. Several studies have shown that intracellular complexing of CD4 and gp 120 during assembly of virion plays an important role in HIV mediated cytopathicity [102,103,104].
4. Formation of multinucleated giant cells or syncytia that are formed from fusion of uninfected CD4⁺T cells with infected cells. Syncytia formation has been invoked as a potential pathway for CD4⁺T cell destruction, even in the presence of relatively few HIV infected cells [105,106]. HIV infected monocyte derived macrophages express gp120 and can fuse with uninfected CD4⁺T cells *in vitro* [107]. However, the role of syncytia formation in the immunopathogenesis of HIV infection *in vivo* remains unknown.

HIV replicated poorly and mechanisms other than direct HIV-mediated cytopathicity must be responsible for the slow but persistent CD4⁺T cell decline [108]. In addition to the direct HIV mediated cell killing, indirect mechanisms may play a role:

Autoimmune reactions: Non polymorphic determinants of MHC class II particularly HLA-DR and HLA-DQ share homology with gp120 and gp41 proteins of HIV [109] and antibodies to gp120 and gp41 may cross-react with MHC class II molecules. Antibodies that react with MHC class II molecules have been found in patients with

HIV infection. The antibodies could prevent interaction between CD4 and Class II molecules, thus impairing cellular interaction required for efficient antigen presentation and inhibiting antigen specific functions mediated by helper CD4⁺T cells [109,110].

Presence of autoantibodies has been demonstrated. These autoantibodies have potential to react with normal cells including lymphocytes [111,112,113], platelets and neutrophils [114], CD4 molecules [115,116] and CD43 molecules on the surface of thymic lymphocytes [117] and IL-2 [118]. In addition antibodies that react to peptides derived from regions of homology between HIV gp41 and MHC class II have been reported in HIV infected individual [119]. Autoantibodies bound to T cells have been reported in HIV infected individual [114]. Cytotoxic T lymphocytes (CTL) that can lyse uninfected CD4⁺T cells have also been reported [120].

Anergy: The binding of the gp120 molecules produced by infected cells to CD4 receptors on uninfected cells may inadvertently render these latter cells targets for HIV specific CTLs or antibody dependent cell-mediated cytotoxicity (ADCC) [121]. CTLs specific for gp120-CD4 complex can kill uninfected CD4⁺T cells that have taken up and processed bound gp120 molecules during the process of antigen presentation [81,82]. It has been also shown that natural killer cells (NK) also mediate ADCC against uninfected cells that are coated with HIV antigen [122].

Superantigens are presented on the Class II MHC and only need to interact with V β subunit of TCR (T cell receptor). As a result, a superantigen can stimulate a much higher proportion of T cells than any of the conventional antigens. After superantigen stimulation the stimulated cells undergo expansion and may be either deleted or rendered anergic. The superantigen hypothesis regarding HIV infection stems from the observations that endogenous or exogenous retroviral encoded superantigens stimulate murine CD4⁺T cells *in vivo*, leading to the anergy and deletion of a substantial percentage of CD4⁺T cells that have the specific variable - β region. However, rather than causing deletion of specific subgroups of T cells, it is more likely that superantigens, if present in HIV infection serve as potent activators of T cell, rendering them more susceptible to infection with the virus [125].

Apoptosis: Inappropriate induction of apoptosis in mature CD4⁺T cells may be a direct consequence of HIV replication [126]. In HIV infection, crosslinking of the CD4 receptor could occur as a result of gp120 binding alone or in conjunction with anti-gp120 antibodies [127]; with the second stage of the pathway being effected through stimulation of the TCR by any number of different antigens. In this regard, the presence of gp120/antigp120 antibody complexes on PBMNCs from HIV infected patients has been reported [128]. Picomolar concentrations of gp120 prime T cells for apoptosis [129].

Apoptosis has been shown to occur *in vitro* after acute HIV infection of lymphoblastoid cells or activated PBMNCs [130]. In addition, *in vitro* exposure of CD4⁺T cells from asymptomatic HIV infected individuals to superantigens or pokeweed mitogen resulted in the induction of apoptosis [127]. Presence of high levels of autoantibodies to double stranded DNA and histones in HIV infected individual is consistent with the nuclear antigens by apoptosis [131]. CD4⁺ and CD8⁺T cells from these individuals have also been reported to undergo spontaneous apoptosis in culture [132].

HIV infection of the precursor cells

Hematopoietic suppression is observed in HIV disease in the form of thrombocytopenia, granulocytopenia, anaemia, lymphopenia, and pancytopenia [133,134]. There is a significant loss or decrease in colony forming potential of granulocytes, erythrocytes, monocytes, megakaryocytes and erythroid cells [135]. Some of the haematological manifestations may be the result of opportunistic infections or therapies for HIV or for associated opportunistic diseases. But a certain proportion appears to be the direct result of HIV related effects.

Purified bone marrow cells can be infected by HIV *in vitro* [136,137]. Infection of precursor cells in thymus is proposed to be one possible explanation for the lack of regeneration of mature CD4⁺T lymphocytes during HIV infection.

Immunological defects in other types of immune cells:

A number of deficiencies in chemotaxis, monocyte dependent T cell proliferation, Fc receptor function, C3 receptor mediated clearance, and oxidative burst response have been reported in HIV infection.

Infection of monocytes/macrophages has been shown *in vitro* as well as *in vivo* which may cause impairment of antigen dependent cell cytotoxicity (ADCC), intracellular antimicrobial activity [138] and induction of IFN- α secretion [139].

Elevated numbers of B cells spontaneously secreting antibody and their refractoriness to signals for T cell independent B cell activation are characteristic of HIV infection [140]. Circulating B cell levels decrease early in the course of disease and the decrease becomes more pronounced as the disease progresses [141]. B cells from HIV infected individuals secrete higher levels of cytokines in response to gp120 and HIV [142,143]. High percentage of antibody producing circulating B cells from HIV infected individuals at all stages of infection are found [144].

The mechanisms of HIV pathogenesis hypothesised above may be epiphenomena of a more general mechanism such as immune activation [145,146,147,148]. Since HIV is not completely eliminated by the immune response, its persistence over time induces a chronic activation of immune system. This chronic activation of immune system reflects continuous activation of different effector cells involved in the ongoing immune response to HIV and /or other pathogens. However, at the same time, certain components of this general state of immune activation may be detrimental and contribute to HIV pathogenesis and disease progression

The chronic immune activation may potentiate virus replication either by stimulating secretion of certain cytokines, which induce a large pool of activated target cells for HIV that may efficiently support virus replication. Several of the pathogenic mechanisms discussed above may be triggered by this general state of immune activation and may progressively weaken HIV specific immune responses. The progressive response favours virus dissemination. Clearance of newly produced HIV will be less efficient and the vicious cycle of pathogenic events will be perpetuated. After prolonged period, the balance tilts towards the detrimental counterpart of the chronic state of immune activation and the outcome is the severe deterioration of the immune system.

HIV AND CYTOKINES

It has been well established that activation of CD4⁺T cells facilitates HIV replication and production of HIV *in vitro*. Activation of T cells enhance HIV

entry into the cell, triggering the completion of reverse transcription and viral DNA integration or by stimulating viral transcription from proviral DNA. [149] Activation of T cells by certain mitogens and IL-2 requires induction of NF- κ B proteins. NF- κ B proteins bind to the target cell genome and stimulate transcription of specific genes that is essential for T cell response [149]. NF- κ B binding sequences are also present in the HIV genome [150]. Thus activation of CD4⁺T cells results in simultaneous stimulation of HIV transcription.

The fact that activated CD4⁺T cells are major contributors to the production of cytokines and the cytokines exhibit either activation or suppresser effect on the cells that produced them, led to the investigation of these factors in the regulation of HIV expression.

It was observed that supernatants from PHA stimulated PBMNCs or Lipopolysaccharide (LPS) stimulated macrophage culture, could upregulate HIV replication from a state of latent or chronic infection to that of active virus expression in chronically infected T cell and monocyte/ macrophage- derived cultures. It was suggested that cytokines released from the PHA or LPS stimulated cultures may be responsible for the effects on HIV expression [151, 152].

On the basis of their ability to modulate virus expression and replication, cytokines may be divided in to three groups:

1. Inducers of virus expression.
2. Suppressers of virus expression.
3. Bifunctional modulators which mediate the induction or suppression of virus replication depending on the experimental system.

The pre-treatment of the supernatants with antibodies to specific cytokines, revealed that TNF- α was responsible for most of the viral induction [151].

Recombinant TNF- α had been shown to upregulate HIV expression in a variety of acutely and chronically infected T and monocytic cell lines and primary macrophages [152, 153]

TNF- α potentiates viral replication *via* the induction of NF- κ B protein that act as transcriptional activators of HIV [154]

In parallel with its physiological mode of action *in vivo* during immune regulation, TNF- α functions *in vitro* in an autocrine and paracrine manner to upregulate HIV expression [155]. IL-6, granulocyte macrophage colony stimulating factor (GM-CSF), upregulate HIV expression independently as well as in synergy with TNF- α [156].

The activation of cellular genes for cytokine production does not appear to require active virus replication. Binding of gp120 to CD4 molecule or binding of anti CD4 monoclonal antibody that specifically recognises the gp 120 binding site on the CD4 molecule can mimic the same effect [157].

Some studies have found substantially increased secretion of TNF- α by PBMNCs from AIDS patients as compared with controls both spontaneously and in response to cell activation [158]. Alveolar macrophages of HIV patients without opportunistic infections showed similar increase in cytokine production as compared to controls [159].

PBMNCs from blood of HIV infected individuals showed increased levels of IL-6 production [160]. CD4⁺T cells from HIV infected individuals have been found to secrete abnormal levels of IFN- γ [161]. Abnormally high levels of TNF- α and IL-6 are found in blood of HIV infected individuals [162]. B cells from HIV infected individuals constitutively secrete TNF- α and IL-6 that are capable of upregulating chronically infected cell lines. Cocultivation of these B cells with autologous T cells resulted in activation of virus expression. Also, B cells from HIV infected individuals but not the controls secrete markedly increased levels of cytokines in response to exposure to HIV and gp120 [163].

Thus abnormal cytokine secretion by T cells, B cells and macrophages in lymphoid organs of an HIV infected individual play an important role in the propagation of HIV infection. Recent data showed that TNF- α preferentially induces lysis of HIV infected cells [164]. Thus, in addition to its role as an inducer of HIV expression, TNF- α may also function in the continuous destruction of CD4⁺T cells *in vivo*.

In contrast to the inducer effects of these cytokines, IFN- α , IFN- β exert only suppressive effect on HIV expression. They act on both T cells and

macrophages. Acutely HIV infected PHA stimulated PBMCs exhibit a decrease in HIV protein expression and virus replication after treatment with IFN- α or IFN- β [165]. IFN- α is thought to downregulate HIV by blocking the reverse transcription of HIV genomic RNA into DNA [166]. It inhibits release of mature virions from cell membrane [167].

IFN- γ , TGF- β and IL-4 are bifunctional in this respect. IFN- γ and TGF- β are active on both macrophages and T cells. IL-4 is active only on macrophages. The inductive effect of these cytokines was found to be dependent on a number of factors: including the type of infected cell (T cell, monocyte/macrophages or thymocytes), whether the cells were primary or established cultures and the timing of cytokine treatment relative to HIV infection; e.g. IFN- γ upregulates HIV virus expression in T cells in an autocrine manner [155] in primary monocyte/ macrophage culture treated before HIV infection [168] and in chronically infected monocytoid cell line [169]. However IFN- γ downregulated HIV expression in monocyte/macrophage cultures if the cultures were infected before IFN- γ treatment [170].

TGF- β has been reported to upregulate HIV replication in primary T cell cultures [171] with dichotomous effects in monocyte/macrophage cultures depending on the timing of infection [172].

The upregulation of HIV by IL-4 is restricted to mature thymocytes and freshly isolated monocytes but not T cells and in some cases, 5 day-old monocyte/macrophage cultures [173].

Although the regulatory effect of cytokines on HIV replication is limited to *in vitro* observations, the fact that several of these cytokines may be found in increased levels in plasma, cerebrospinal fluid and lymphoid tissue of HIV infected individual suggests that they probably mediate similar effects *in vivo*. It is possible that the cytokines play an important role in maintaining expression and replication, particularly in lymphoid tissue.

TH1/TH2 shift in HIV infection

Clerici M *et al* (1992) observed 50-75% of patients who are on AIDS therapeutic protocols, showed improvement in T helper (T_H) functions which was independent of $CD4^+$ T cell number. Furthermore improved T_H function correlated with infection free follow up periods where as changes in $CD4^+$ T cell number did not [174].

On the basis of these findings, it is clear that loss or recovery of T_H Function can occur in HIV positive individuals and AIDS patients independently of $CD4^+$ T cell count.

The possible mechanisms responsible for this phenomenon can be

1. The selective loss of memory cells [175].
2. A defect in antigen presenting cells [176].
3. The production of antibodies that block T cell APC interactions [120].
4. Immunosuppressive products of HIV like the protein gp120 [177] and tat [178].
5. The induction of suppressor cells and factors [179].
6. The elaboration of cytokines that exhibit immunoregulatory properties [180].

Because immunedysregulation in the progression to AIDS involves loss of helper function (as assessed by proliferation and IL-2 production), B cell activation and hypergammaglobulinemia, immunoregulatory cytokines can be considered as useful marker of disease progression [180].

Various experiments in order to study proliferation and IL-2 production by peripheral blood mononuclear cells (PBMNCs) of asymptomatic HIV seropositive individuals showed progressive sequential loss of T_H function such that T_H cell response to recall antigens is the first to be lost followed by loss of T_H function to allo-major histocompatibility complex (all-MHC) and finally by loss of response to PHA [181].

The findings that in HIV positive individuals IL-2 production declines while B cell activity increases, suggested that TH1 to TH2 switch occurs in the progression to AIDS. Also, loss of recall and MHC- stimulated IL-2 production was associated with a concomitant loss of PHA stimulated $IFN-\gamma$ [182].

Distinct observations were provided to substantiate the hypothesis of a TH1/TH2 switch in HIV infection:

1. HIV seronegative individuals who remained seronegative in spite of exposure to the virus showed production of IL-2 in cultures of PBMNCs stimulated with HIV envelope peptides [183].
2. Decrease of IL-2 responses to soluble antigens or HIV peptides was associated with increased IL-4 and IL-10 production in response to mitogens [184].
3. Defective T cell proliferation and IL-2 production in response to stimulation with influenza virus and HIV envelope peptides could be restored by addition in culture of IL-12 [185].
4. Increased expression of m-RNA for and production of IL-10 upon mitogen stimulation was found in virtually all HIV infected individuals, with a direct relationship between IL-10 levels and the severity of immunodeficiency [186].
5. Recently it has been suggested that HIV related *nef* gene expression may be responsible for the downregulation of TH1 cytokines IL-2 and IFN- γ without affecting the production of TH2 cytokines (IL-4, IL-10, IL-13) [187].

These observations suggest the possibility of a shift in the cytokine balance as a part of the progression of HIV disease [182]. Several attempts have been made to prove or disprove the TH1/TH2 switch hypothesis in HIV infection:

Short term culture

1. Short term culture of PBMNCs stimulated *in vitro* for 3 days with polyclonal activators such as PHA or PMA plus anti-CD3 monoclonal antibody produced decreased but not significantly affected IFN- γ and secretion of IL-4 and IL-10 was not increased but rather decreased as compared to HIV seronegative donors [188].
2. HIV infected individuals with reduced CD4⁺T cell counts produced lower amounts of IL-4, IL-5, IL-10 in comparison with HIV infected individuals showing quite normal or only slightly decreased CD4⁺T cell count [188].

3. In contrast to the above results higher levels of PHA induced IL-4 and IL-5 and lower levels of IL-2 and IFN- γ were found in PBMNCs derived from perinatally HIV infected children [189].

Cytokine production in long term cultures:

The proportion of CD4⁺T cells cultured were comparable in HIV seropositive where as the proportions of clones inducible to IL-4 and IL-5 production were significantly lower in HIV infected individuals due to their selective reduction in patients with low CD4⁺T cell counts [188].

Cytokine profile of antigen specific clones:

Forty percent of CD4⁺T cell clones specific for *Toxoplasma gondii* generated from HIV seronegative subjects showed TH1 profile (IFN- γ , TNF- β but no IL-4 or IL-5). Remaining 60% exhibited a TH0 phenotype. Virtually all *Toxoplasma gondii* specific CD4⁺T cell clones generated from peripheral blood of HIV seropositive donors, produced not only IFN- γ and TNF- β , but also IL-4, IL-5 IL-10.

PPD specific clones derived from controls showed TH1 profile in majority (80%) and remaining was TH0 type. 71% of PPD specific clones from seropositive exhibited a TH0 phenotype and a minority (29%) was TH1, suggesting a general shift at the level of memory T cells to wards TH0 type [188].

Increased proportion of TH0 like clones in the clonal progenies of PHA stimulated peripheral blood T cell suspensions enriched for CD45RO⁺ (memory) T cells have been found in HIV infected individuals. Meyaard *et al* have also found that cloning of CD45RO⁺ (memory) T cells obtained from the same donor at different time intervals after serodiagnosis and stored in a frozen state, revealed shifting towards the TH0 secretion profile with the progression of infection [190].

Defective production of TH1 inducers [IL-12 and IFN- γ]:

The possibility that defective production of TH1 inducing cytokines is involved in the shift of CD8⁺ T cells and possibly also of CD4⁺ T cells, towards the production of TH2 type cytokines is substantiated by several experimental data.

1. Macrophages from HIV infected individuals and normal macrophages infected *in vitro* with HIV usually produce normal or increased levels of IL-1 β , TNF- α , IL-6, GM-CSF and IL-10 [190], but production of IL-12 in response to pathogenic bacteria or parasites is strongly defective [191].
2. IL-12 induced irreversible priming for high IFN- γ production in CD4⁺ and CD8⁺ T cell clones derived from HIV infected patients [192].

This is the most important mechanism by which IL-12 induces TH1 differentiation [193]. IFN- γ has been shown to play an ancillary role in the IL-12 mediated *in vivo* development of murine TH1 cells [194], and a major role in the *in vitro* development of human TH1 cells [195].

TH2 responses favour HIV replication and disease progression:

CD4⁺ T cell clones derived from peripheral blood of HIV seronegative individuals infected *in vitro* with HIV and 2-3 week later were assessed for presence of provirus by PCR and for viral replication by measurement of p24 antigen in supernatant. In spite of the presence of DNA provirus, none of the TH1 clones produced p24 antigen in their supernatant whereas all TH2 and 2/3 of TH0 clones showed viral replication as indicated by both positive PCR and p24 antigen production [187].

In another studies where T cell clones specific for HIV gag 24 were assessed, only TH2 clones showed HIV replication, whereas TH1 clones did not replicate the virus and even showed the ability to inhibit HIV replication in other HIV infected cells [196].

The studies mentioned above suggest that HIV infection may result in enhanced TH2 type cytokine production which in turn allows higher virus replication. They also indicate that a preferential killing of TH2 type CD4⁺ T cells can occur and this facilitates further spread of HIV.

This hypothesis is supported by *in vivo* observations such as elevated IgE levels in serum at the time of infection indicate poor prognosis in terms of faster depletion of CD4⁺ T cells and rapid progression to full-blown AIDS [197].

HIV infected individuals with helminth infestations, which shift the balance from TH1 to predominant TH2 type progress more rapidly to AIDS [198].

Role of CD30 in HIV expression:

CD30 is a member of tumor necrosis factor (TNF- α)/nerve growth factor (NGF) receptor superfamily. It is preferentially expressed on and released in its soluble form (sCD30) by both CD4⁺ and CD8⁺ T cell clones producing TH2 type cytokines [199].

CD8⁺ T cell clones, which usually exhibit a TH1 like cytokine profiles do not express membrane CD30 [199]. A large number of CD8⁺ clones, exhibiting a TH2 cytokine secretion could be generated from the peripheral blood or the skin of the patients with AIDS [200]. These clones showed consistent expression of membrane CD30 and released substantial amounts of sCD30 into their supernatants [200]. Elevated levels of sCD30 have been detected in the serum of many HIV infected cases [201].

Biswas *et al* have shown that crosslinking of CD30, which is constitutively expressed on the chronically HIV infected human T cell line ACH-2, results in HIV expression. This is mediated by NF- κ B activation which leads to enhanced HIV transcription [202]. CD30 ligation induced NF- κ B in human T cell lines, as well as in human CD30 expressing TH0 and TH2 CD4⁺ T cell clones [203].

Anti CD30 antibody exerted a suppressive effect on the spontaneous HIV replication occurring in lymph node cells freshly derived from HIV seropositive patient showing CD30 expression in B cells and in proportion of CD8⁺ T lymphocytes [204].

In vitro infection with HIV of CD4⁺ T cell clones enhanced expression of CD30, which was often found to be associated with the death of clonal T cells [205].

Thus, CD30 is mainly and persistently expressed by TH0 and TH2 cells and their triggering may play an important role in HIV replication and death of HIV infected CD4 cells [206].

HIV and Apoptosis

Inadequate stimulation of naive T cell can result in apoptosis or programmed cell death. Activation induced cell death (AICD) occurs with superantigens, activated anti-CD3 antibody; it requires the presence of APC and is mediated by Fas/Fas Ligand (Fas-L) interaction. Fas and Fas-L are members of the TNF/nerve growth factor receptor family and of the TNF family respectively [207].

There is now convincing evidence to suggest that enhanced apoptosis in HIV infection is mediated by Fas/FasL interaction [208].

It is most likely that Fas-L in human is also predominantly expressed by or operating in, the TH1 subset. The fact that TH1 cells possess both FasL and Fas, whereas, TH2 cells express Fas but low Fas-L, suggests that TH2 cells are susceptible to AICD in the presence of TH1 cells,

Since TH1 express both Fas and Fas-L, whereas TH2 express Fas but little or no Fas- L [209]; TH2 cells may undergo apoptosis by activated CD4⁺TH 1 cells but not by TH2 cells themselves [208], whereas TH1 cells can be killed by interacting among themselves and with TH2 cells. This competitive disadvantage of TH1 cells has been envisaged to lead to a preferential loss of TH1 cells and to the proposed skewing of the immune system in favour of TH2 responses in HIV infection. TH2 cytokines IL-4 and IL-10 have a favouring effect on apoptosis [210,211]. The production of protective cytokines is defective in HIV infected patients. In absence of IL-2 (which occurs as a result of CD4 crosslinking) contributes to Fas upregulation and apoptosis induction [212].

Not only the CD4⁺ but also CD8⁺ T cells undergo accelerated apoptosis in HIV infection and it might be a major contributor to depletion of CD4⁺T cells[128,213,214,215].

Il bacillo non e ancora tutta la tubercolosis
[The bacillus not yet all there is to tuberculosis]

G Bacelli, ca. 1882.

Tuberculosis which had been controlled to a significant extent during the nineteen sixties and seventies, has re-emerged in recent years as one of the leading causes of death in the world. Nearly 3 million people die every year due to tuberculosis. There are 8.8 million new cases every year meaning about 1000 new cases every hour. This works out to 52000 death per week or 7000 death each day. [216]

Mycobacterium tuberculosis has since early days been exacting a substantial toll of mortality and morbidity on humanity. Suspected tuberculosis disease has been found in the bony remains of a stone age individual who lived about 6000 years ago [217,218].

On March 24, 1882, Robert Koch's presentation to the Berlin Physiological Society, that can be thought of as establishing the science of Microbiology. He described *M. tuberculosis*, the tubercle bacillus (still known as Koch's bacillus) and convincingly demonstrated it to be the cause of tuberculosis [219]. Paul Ehrlich, one of those who heard Koch's initial presentation, developed staining techniques, Zeihl and Neelsen quickly made further refinements, upon which diagnostic sputum smears are still based today.

PATHOGENESIS OF PULMONARY TUBERCULOSIS:

The principle risk of acquiring infection with *M tuberculosis* is breathing [220]. The first stage begins following the inhalation of tubercle bacilli in to alveolus. Alveolar macrophages ingest the bacillus. If the macrophage fails to destroy the inhaled unit of one to three bacilli, the bacilli multiply until that macrophage (or its progeny) bursts. The bacillary load is then readily ingested by other alveolar macrophages and by non-activated macrophages i.e. monocytes emigrating from he bloodstream. Both types of macrophages are attracted to the site by released bacilli, cellular debris and a variety of chemotactic factors of the host origin, e.g. the complement component C5a

and the cytokine monocyte chemoattractant protein (MCP-1). The immature blood macrophages readily ingest the released bacilli. The macrophages have not been yet activated hence cannot inhibit or destroy the bacilli. More and more macrophages and bacilli accumulate at the lesion. The alveolar macrophages in such lesions remain peripheral, rather far from bacilli, which are almost always located centrally. Hence alveolar macrophages rarely participate in the response.

In the second stage called symbiotic stage: Tubercle bacillus grows slowly, dividing every 18-24 hours. The organisms grow unimpeded until CMI develops after 4 to 8 weeks. The stage of logarithmic growth may be a better name than the symbiotic stage which was used by Lurie, since the uncontrolled growth of bacilli during this stage, The bacilli producing antigen stimulus necessary to trigger delayed type hypersensitivity (DTH) and cell mediated immunity (CMI). Once the CMI develops, activated T cells in the inflammatory areas and macrophages form the granulomas, which wall off the organisms. Granulomatous inflammation is a non-specific host response in which collections of T cells and macrophages in various stages of activation come together in a loosely organised lesion (caseum). CD4+T lymphocytes and monocytes recruited from the blood surround the lesion. Monokines and lymphokines are released that activate the surrounding tissue macrophages derived from monocytes further transform into epithelioid cells and fuse to form multinucleated giant cells [221,222]. A granuloma within the lung and draining lymphnodes is called a primary or Ghon's complex.

The third stage of the disease begins when the host becomes tuberculin positive (develops DTH) and the lesions undergo caseous necrosis in their centres. The logarithmic phase of bacillary multiplication stops as soon as caseous necrosis develops. *M tuberculosis* appears to be arrested due to CMI. Although tubercle bacilli survive for many years in solid caseous material, they are unable to, multiply owing to anoxic conditions, reduced pH and the presence of inhibitory fatty acids [223]. Although primary complex can sometimes be seen on chest radiograph, the majority of pulmonary tuberculosis infections are inapparent [224]. A positive skin test is the only

apparent vestige of *M. tuberculosis* infection. Individuals with tuberculosis but not active disease are not infectious. They are at risk of developing active disease if not treated. Approximately, 10% of individuals with normal immunity will develop active tuberculosis within lifetime, 5% within 2 years of infection [225].

The forth stage of Liquefaction and cavity formation:

Later in the course of tuberculosis, DTH may be more harmful to the host than helpful causing the tissue damage characteristic of tuberculosis, especially lung cavitation. The large number of bacilli estimated at greater than 10^8 , also increase probability of mutations to drug resistance and also transmission.

The factors that cause liquefaction are largely unknown, but in experimental animals, liquefaction is associated with high levels of tuberculin reactivity [226] and elevated hydrolytic enzymes (probably proteinases, nucleases and lipases [224].

The breakdown products of caseous material are osmotically active, so that water is absorbed from the surrounding tissue and an excellent culture medium for tubercle bacilli is created.

Because the host is now highly sensitive to tuberculin like products of the bacillus, this large antigenic load is quite toxic to the cells. The walls of nearby bronchi often become necrotic and rupture, forming a cavity. The bacilli and the liquefied caseous material are discharged into the airways. The walls of cavities consist of external zone of collagen, a cavity capsule and caseous liquefying internal zone where the high oxygen content from the ambient air nurtures growth of bacilli [221,222]. Within the cavities host defences are ineffectual and in this unique extracellular milieu, tubercle bacilli multiply to great numbers. The patient, which has cavitary tuberculosis characteristically, has persistent coughing and may have hemoptysis. Systemic symptoms of fever weight loss and anorexia are also associated with cavitary disease. Macrophages do not survive in liquefied (or even solid) caseous material. Possibly they have been passively sensitised to tuberculin

and other antigens that are present and they are in high (lethal) concentrations. Possibly the macrophage entering the liquefied caseum are killed by toxic fatty acids originating from host cells and/or the bacilli [223,224].

T CELL RESPONSES AND CYTOKINES IN *M TUBERCULOSIS* INFECTION:

The clinical outcomes of infection with *M tuberculosis* depend on the efficacy of cell mediated immunity. Antimycobacterial immune defences are primarily mediated by T lymphocytes and macrophages. Adoptive transfer of resistance against tuberculosis in animal models is mediated by T cells [228]. Although neutrophils and natural killer cells can exhibit mycobacteriostatic effects *in vitro* [229,230], and eosinophils have been shown to ingest mycobacteria [231], their role in immune defences *in vivo* is uncertain.

The role of mononuclear phagocytes:

Macrophages produce a characteristic pattern of soluble mediators, cytokines in response to *M tuberculosis*. These include interleukin 1 (IL-1), IL-6, IL-10, tumor necrosis factor alpha (TNF- α) and transforming growth factor beta (TGF- β) [232,233,234]. These cytokines mediate many of the clinical manifestations of tuberculosis through their immunoregulatory effects.

IL-1 is an endogenous pyrogen that contributes to fever that is characteristic of tuberculosis [235]. IL-6 enhances immunoglobulin production by activated B cells [236] and may mediate hyperglobulinemia common in tuberculosis patients. TNF- α synergises with gamma interferon to increase production of nitric oxide metabolites [237] and mycobacterial killing [238]. TNF- α is essential for granuloma formation to contain mycobacterial infection [239]. TNF- α may cause the immunopathogenic effects such as fever, weight loss and tissue necrosis that are typical of tuberculosis [240]. IL-10 inhibits cytokine production by monocytes and lymphocytes [241]. TGF- β suppresses T cell proliferation and inhibits macrophage effector function [242]. These two

cytokines may prevent excessive inflammation and tissue damage from an uncontrolled inflammatory response.

The presentation of mycobacterial antigens on macrophages in association with MHC induces expansion of specific CD4⁺T cells. The CD4⁺T cell population is central to acquired resistance to *M. tuberculosis* antigen [243]. The observed defect in lymphocyte proliferation and IL-2 production in response to PPD (purified protein derivative) has been shown to be partly reversed by depletion of monocytes [243]. Patients with depressed lymphocyte proliferation in response to *M. tuberculosis* have elevated percentages of circulating monocytes that are abnormally activated in terms of expression of class II MHC determinants [244] and the monocytes have been shown to produce large amounts of IL-1 in response to *M. tuberculosis* PPD [245]. Monocytes from such patients constitutively express IL-2 receptors [246]. Thus the monocytes may exert their suppressive effects partially through consumption of IL-2 which is a critical T cell growth factor. Monocytes in tuberculosis patient produce increased amounts of TNF- α and IL-6 [247,248].

Production of immunosuppressive cytokines has been shown to be increased in tuberculosis infection. IL-10 mRNA is selectively increased at the site of disease and is reported to be expressed predominantly by macrophages rather than T cells [249]. Lipoarabinomannan, a complex heteropolysaccharide from mycobacterial cell membrane has been shown to suppress proliferative response to *M. tuberculosis* [250]. This effect is thought to be due to induction of macrophages to release immunosuppressive cytokines such as IL-10 [232].

TGF- β is produced constitutively by monocytes from patients with tuberculosis and production is increased in response to PPD. Langhans giant cells and epitheloid cells in tuberculous granulomas also express mRNA for TGF- β [233]. This suggests that local production of TGF- β may result in deactivation of macrophages and immunopathology.

The critical role of IFN- γ as a macrophage-activating factor in mycobacterial disease has been demonstrated in murine models of tuberculosis. IFN- γ

inhibits intracellular growth of *M. tuberculosis* in murine macrophages through increased production of reactive NO metabolites [251]. It stimulates human macrophages to produce TNF- α and 1,2,5- dihydroxy vitamin D, both of which facilitate mycobacterial killing [252,253]. However, in human studies, IFN- γ fails to inhibit mycobacterial replication consistently in monocytes or monocyte derived macrophages unless combined with other cytokines or monocyte products [254].

Some investigators have suggested that IFN- γ may promote rather than inhibit mycobacterial growth *in vitro* [255]. LAM inhibits IFN- γ mediated activation of macrophages and scavenges oxygen free radicals, inhibiting a major pathway of destruction of intracellular pathogen [256]. LAM from virulent *M. tuberculosis* does not induce macrophage early activation genes and does not elicit production of large quantity of TNF- α [257]. Thus avoiding macrophage activation may be a strategy that allows the virulent *M. tuberculosis* to evade the cytokine-mediated mechanism of elimination. Secretory proteins of *M. tuberculosis* can also induce cytokine production by macrophages and may thereby contribute to systemic immunosuppression and local immunopathology. Both the 30 KDa α antigen and 58 KDa constituent of *M. tuberculosis* culture filtrates induce production of TNF- α by monocytes [258].

CD4⁺T cells dominate the defences against tuberculosis. It has been shown that mice depleted of CD4 cells prior to infection with *M. bovis* are unable to control mycobacterial growth [259]. In humans, the CD4⁺T cells are selectively expanded at the site of the infection in patients with a resistant immune response, such as in those with tuberculosis pleuritis [260,261]. CD4⁺ memory T cells are selectively concentrated at the pleural spaces of patients with tuberculosis pleuritis whereas naïve cells are not, and they proliferate and produce IFN- γ in response to *M. tuberculosis* antigens [256]. Depletion of CD4⁺T cells by HIV infection markedly increases susceptibility to primary and reactivation tuberculosis [262]. Extrapulmonary involvement, mycobacteremia, and negative acid-fast smears become more common in HIV infected patients as CD4⁺ count deteriorates. Thus the progression to

disease is worsen in favour of bacilli as CD4⁺ T cells deplete e.g. The frequency of mycobacteremia rises from 4% in patients with more than 200 CD4⁺ cells/ μ l to 49% in those with 100 or fewer CD4 cells/ μ l [263].

Published data on cytokine production by human T cells in response to *M tuberculosis* are conflicting. Some studies indicate that most *M tuberculosis* reactive CD4⁺ T cell clones propagated *in vitro* are TH1 like, producing high concentration of IFN- γ and low concentration of IL-4 and IL-5 [264]. Some clone in the above study, expressed m-RNA for IL-4 although IL-4 was not detectable in supernatants. These disparate results may reflect culture selection of T cell subpopulations by culture conditions *in vitro* and may not reflect events *in vivo*, therefore, the cytokine response at the site of mycobacterial disease are more important. In tuberculosis pleutitis, the expression of m-RNA for TH1 cytokines, IFN- γ and IL-2 is greater in pleural fluid mononuclear cells than in the blood mononuclear cells and concentration of IFN- γ is 15 fold higher than that in serum. In contrast, expression of IL-4 m-RNA is lower in pleural than in blood lymphocytes. Also pleural fluid lymphocytes stimulated with *M tuberculosis* produce 6 fold more IFN- γ and 40 fold more IL-2 than do peripheral blood lymphocytes. This gives a strong evidence for active accumulation of TH1 cells at the site of disease in a person with resistant immune response and suggests that TH1 cells play an important role in human antimycobacterial defences [249].

Alternative mechanism by which T cells contribute to immune defence is through direct cytotoxicity of macrophages and non-phagocytic cells infected with *M. tuberculosis*. CD4⁺T cell lines and clones express class II MHC restricted cytotoxicity for mononuclear phagocytes pulsed with killed *M. tuberculosis*, PPD or the purified 65-kDa antigen. *M. tuberculosis* specific cytolytic activity of CD4 is greatly enhanced at the site of the disease as compared to that of peripheral blood cells [265]. This can be a means for freeing up mycobacteria from overburdened macrophages so that they can be released into an extracellular environment where they replicate poorly or can be ingested by more potent effector cells such as freshly recruited and activated macrophages [266]. Alternatively this can be a scavenger

mechanism where by dead macrophages containing large numbers of dead bacilli are killed so that they can be catabolized by surrounding mononuclear cells [267]. This phenomenon of killing of infected macrophages which results in release of toxic products may cause autotoxicity contributing to the genesis of caseation necrosis or autoimmune phenomenon [268].

CD8⁺ T lymphocytes constitute the major cytolytic T cell population in defences against many intracellular pathogens. Murine CD8⁺T cells can directly lyse *M. tuberculosis* infected cells in an antigen specific manner *in vitro* and depletion of CD8 cells increases severity of tuberculosis [266]. Mice that lack functional CD8 T cells exhibit severe manifestations of tuberculosis [269]. Human *M. tuberculosis* specific cytolytic T cells evaluate to date are not CD8⁺ [270]. CD8⁺ T cells are not found to be selectively concentrated at the site of disease in tuberculosis patients [271] and severity of tuberculosis in HIV infected patients is unaffected by CD8⁺ cell count [263].

The γ/δ T cell subsets have unknown protective role against tuberculosis. A large proportion of human peripheral blood γ/δ T cells proliferate in response to mycobacteria [272]. *In vitro* γ/δ T cells secrete a pattern of cytokines similar to the secretion pattern of TH1 cells and are cytotoxic [273,274]. There is an evidence supporting early involvement of γ/δ cells in establishing immunity to tuberculosis [275].

Expansion of *M tuberculosis* reactive γ/δ T cells is greater in healthy tuberculin reactors and in patients with tuberculosis plueritis than in those with advanced pulmonary and milliary tuberculosis suggesting that γ/δ T cells contribute to immune resistance [275]. It is believed that they contribute to control of primary infection before α/β T cell response has become established. *M tuberculosis* reactive γ/δ produce IFN γ , TNF α , IL-2, IL-4, IL-5, IL-10 [276] and lyse target cells infected with *M tuberculosis*. γ/δ T cells recognise mycobacterial protease resistant antigens distinct from the proteins recognised by the α/β T cells [277,278]. In addition, *M tuberculosis* stimulated

γ/δ T cells enhance macrophage aggregation and may therefore contribute to granuloma formation [279]. This supports role for γ/δ T cells during the early phase of immune response, such as in lungs and lymph nodes of persons recently infected with *M. tuberculosis*.

TH1/TH2 AND MYCOBACTERIA

Tuberculosis can manifest as reactive or non-reactive disease depending on the state of balance between protective immunity and various strategies of the surviving bacilli to evade the bactericidal mechanisms of their host cells.

TH1 cytokines, IFN- γ , TNF- α activate macrophages and blood monocytes and this can be considered as a central crucial step in elimination of mycobacteria [280]. IFN- γ plays a vital role. IFN- γ or IFN- γ receptor gene disruption mutant mice are highly susceptible to *M. bovis* BCG and *Mycobacterium tuberculosis* infections and develop disseminated disease [280]. Treatment of patients suffering from disseminated *M. avium* infections with recombinant IFN- γ in addition to chemotherapy caused clinical improvement. [281]. PPD specific CD4⁺T cells from healthy individuals usually develop into TH1 clones [282]. There is a preferential mRNA expression of TH1 type cells (IFN- γ ⁺, IL-2⁺) in classic DTH reactions in human skin [283].

When the kinetics and frequencies of cytokine producing cells were studied by immunofluorescent visualisation of intracellular cytokines in healthy individuals vaccinated with BCG, TH1 cytokines IL-2, IFN- γ and TNF- β were detected early at day 4 and 5 post vaccination. IL-4, IL-5 and IL-10 (TH0) was detected, late at day 10-12 when synthesis of TH1 cytokines and monokines declined [284]. Bioactive IL-12 was produced in supernatants of pleural fluid cells stimulated with *M. tuberculosis* [285]. Alveolar macrophages ingest mycobacteria and produce IL-12 favouring development of TH1 cells.

But TH2 cytokines have been detected during infection with *M. tuberculosis* and *M. bovis* although at low-level [286]. Mycobacterium specific T cells clones from healthy donors and PPD specific T cell clones from pleural fluid of patients with *M. tuberculosis* infection can exhibit TH0 like profile [287].

This suggests that although IFN- γ is the major cytokine induced by mycobacteria in healthy individuals and has protective role in controlling the infection, TH2 cytokines also participate in the response. PBMNCs from tuberculosis patients with active disease produce elevated levels of IL-4. Significantly higher levels of sCD30 were found in the sera of patients with unreactive in comparison with patients with reactive tuberculosis [288]. Other studies found that *M. tuberculosis* induction of TH1 cytokines IFN- γ and IL-2 is depressed in tuberculosis, as compared to healthy tuberculin reactors [288]. But tuberculosis patients showed no enhanced mRNA expression or production of TH2 cytokines IL-4, IL-10 and IL-13, suggesting that the reduced TH1 response is not mediated by strong TH2 response [288]. The TH1 response in tuberculosis may be depressed through mechanisms independent of TH2 cells, such as dysregulation of co-stimulatory molecules, anergised TH1 cells and enhanced production of immunosuppressive monokines including TGF- β [289].

So far, it is not clearly determined which parameters determine whether infection with mycobacteria results in protective immune response (TH1) or severe infection (TH2). Genetic predisposition or early immune response may be involved.

IL-12 seems to play critical role as it increases resistance of BALB/c mice to *M. avium* [290] and to *M. tuberculosis*, but only in presence of IFN- γ as its activity is abolished in mice in which IFN- γ gene has been disrupted [291]. *M. tuberculosis* and *M. leprae* can concomitantly activate both TH1 and TH2 reactions and at least with regard to *M. leprae*, the developed phenotype is stable and uninfluenced by related antigens of the microbe [292]. Thus, in these infections, host genetic differences in early innate immune system may act as a decisive control point of the ensuing specific immune response.

HIV -TB INTERACTION

Tuberculosis has been recognised to be more common among persons who were in ill health. Immunosuppressive diseases and conditions e.g. malnutrition, haematological and reticuloendothelial malignancies, solid tumours, diabetes mellitus, end stage renal disease and immunosuppressive therapies, cytotoxic drug therapy, seemed to increase the risk of tuberculosis [293,222]. Because of the epidemic spread of the HIV infection, large populations in developed and developing world are immunocompromised. Moreover, HIV infection is most common among populations in whom there is a high prevalence of tuberculosis. As a result of the superimposition of HIV infection and tuberculosis infection, rates of tuberculosis have increased dramatically. A study predicts that in the last decade of the century, there will be 30 million deaths from *M. tuberculosis*, of which 2.9 million will be attributable to HIV infection [294]. Clinical and epidemiological observations indicate that HIV infected persons have an increased likelihood of developing active tuberculosis following infection with *M. tuberculosis* [295]. In comparison with the 5-10% lifetime risk of developing a latent tuberculosis lesion incurred by HIV negative people with primary infections, HIV positive patients who were Mantoux skin test positive have 8% annual risk of tuberculosis [296]. So far, infection with HIV confers the greatest known risk for reactivation of latent tuberculosis infection and of progression of newly acquired infection to disease.

It is well established that HIV directly infects the blood monocytes and macrophages [138]. The effectiveness of alveolar macrophages in initial non-specific response to *M. tuberculosis* may be limited in persons with HIV infection. The antigen presentation function of macrophages may be impaired resulting in failure to initiate an effective immune response.

The immunologic deficit that characterise HIV disease and leads to profound life threatening immunodeficiency is a multifactorial process that commences at the point of infection and leads to dysfunction in addition to measures of depletion. Perturbations in a range of immunological responses, e.g. diminished cellular responses, as indicated by decreased T and B cell

proliferation in response to a number of mitogens, decreased IL-2 and IFN- γ elaboration, increased spontaneous immunoglobulin secretion and decreased response to new antigens even earlier than any substantial reduction in CD4⁺T cell count [181,297,298,299]. CD4⁺T lymphocytes are involved in many aspects of immune response of *M. tuberculosis* including binding to processed antigen, secreting cytokines, killing mycobacteria infected cells and persisting antigen specific memory cells [300,301]. Given the essential nature of these responses in the immunologic reaction to *M. tuberculosis*, it is likely that the effect of HIV infection on CD4⁺ lymphocytes is a major factor in predisposing to tuberculosis. Tuberculosis most commonly presents in HIV infected patients with CD4⁺T lymphocytes in the range of 150-350 per ml and are less immunocompromised than the patients with other AIDS defining opportunistic infection. However, tuberculosis, being the most virulent often the earliest infection to present, when the cellular immunity is relatively intact [302], with normal or slightly decreased CD4⁺T cell number. Tuberculosis precedes other AIDS defining illnesses by up to 2 years in about half the cases [303]. Functional lymphocyte defects and possible predilection of HIV to infect CD4⁺ memory T lymphocytes may lead to tuberculosis reactivation in person with normal CD4⁺T lymphocyte level. Tuberculosis provokes a more severe prognosis than that observed in seronegative subjects [304]. Nonetheless, mortality in HIV associated tuberculosis remains high despite appropriate and apparently effective therapy [305]. Death is infrequently due to active tuberculosis (13%), more often; it results from other AIDS-related causes. In United States, the mortality rate among dually infected patients at 9 months was 21%, even when the subjects with AIDS defining illnesses at the time of tuberculosis were excluded [305]. In other study, mean interval to an AIDS defining diagnosis after diagnosis of tuberculosis was only 6-9 months [306]. In Uganda, a mortality rate at 1 year after diagnosis of tuberculosis was found to be 30% despite exclusion of seriously ill subjects. Most of the fatal cases followed up in the above study showed partial or complete clearing of mycobacteria. A wasting syndrome was common among those who died [307].

These observations definitely suggest that:

1. HIV infected patients tended to develop opportunistic infections more rapidly after tuberculosis than did HIV infected patients without tuberculosis.
2. HIV infected patients with tuberculosis had a significantly reduced survival rate compared with that of HIV patients without tuberculosis but a similar degree of immunosuppression as measured by CD4⁺T cell count, even when controlling for baseline differences in risk behaviour (including history of intravenous drug use), presence of other opportunistic infections and therapy for HIV infection [305].

The observed difference in survival are not explained by deaths caused by tuberculosis as higher mortality occurs despite adequate response to anti-tuberculosis therapy [305], and therefore may be attributable to HIV infection or to one of its complications. Indeed, the previous epidemiological studies suggest that the tuberculosis and HIV are bi-directional and that tuberculosis acts as a cofactor accelerating the clinical course of HIV disease.

Role of Cytokines:

M. tuberculosis is found to upregulate HIV replication in both direct and indirect ways. *M tuberculosis* or PPD can increase HIV long terminal repeat (LTR) driven transcription in transiently transfected T and monocytic lines [308] and *M. tuberculosis* increases HIV production in chronically infected [309] or acutely infected monocyte cell lines [310]. *M tuberculosis* and its products can induce expression of mononuclear phagocyte cytokines such as TNF- α , IL-1 (IL-1 β or IL-1 α) and IL-6 in *in vitro* cell culture and in humans with active tuberculosis [246,276]. *M tuberculosis* upregulates the genes for these cytokines in peripheral blood monocytes *in vivo* from patients with active tuberculosis and release of these cytokines is enhanced when these cells are cultured *in vitro* [248,311]. A correlation between *M tuberculosis* induced HIV production and secretion of cytokines such as IL-1 β as well as TNF- α and IL-6 via NF- κ B [309] and NF-IL-6 induction [310] has also been observed.

The mechanism of stimulation of enhanced replication by the cytokines has been localised to the 5' LTR of HIV and specifically to the NF- κ B transcription enhancer site [150,154,155,312,313,314]. The combination of events may be detrimental to the host. Moreover, mycobacteria and mycobacterial products convert latent HIV infection of monocytes to a productive infection through a mechanism that depends, at least in part, upon the induction of TNF and IL-1 expression [315].

It has been shown that *M. tuberculosis* increases HIV replication both at local site [310] and at systemic level [316]. Tuberculosis is associated with an increase in cellular activation [317]. *M. tuberculosis* induced HIV activation is critically dependent on cellular and on the balance of several endogenous cytokines. Simultaneous inhibition of endogenous TNF- α and IL-1 β decreases *M. tuberculosis* induced HIV-LTR driven transcription dramatically [310]. Neutralisation of TNF- α leads to a partial decrease in *M. tuberculosis* induced HIV expression in chronically infected U1 cell [309]. Taken together these data suggest that *M. tuberculosis* induction of HIV at either transcriptional [310] or post-transcriptional level [309,316] is the result of activation events and cytokines acting in autocrine and paracrine loops.

It was confirmed in a model system of HIV infection, that without addition of exogenous mitogens or cytokines, an antigen specific response against *M. tuberculosis* induces cellular activation and thus viral replication [318]. Peripheral blood monocytes demonstrated an enhanced susceptibility to productive infection with HIV *in vitro*. Enhanced susceptibility may result from activation of monocytes by exposure to *M. tuberculosis* [319].

Once latent HIV is activated in the patient with dual infection, it replicates and produces new viral particles that can spread to infect other blood cells. Over time, this process may lead to greater viral load, a progressive decline in the number of CD4⁺T lymphocytes and increased risk of opportunistic infection and death. Additionally, *M. tuberculosis* increases secretion of IL-10, TGF- β which cause immune suppression often observed during tuberculosis [320,321] and to the replication of *M. tuberculosis in vitro* [322]. These anti-inflammatory cytokines have been reported to downregulate and up regulate

HIV replication [155,323,324]. Thus tuberculosis is associated with both immune activation [317] and immune suppression [320].

It has been postulated that HIV switches the balance from TH1 to TH2 type cytokine as it progresses. In tuberculosis patients, decrease in TH1 type of cytokines has been observed although without concomitant increase in TH2 cytokines.

In tuberculosis patients, production of IFN- γ and IL-2 by PBMC has been shown to be depressed but this was not associated with increased IL-4 or IL-10 [325,326]. In contrast, Surcel and colleagues [327] showed that the number of IFN- γ secreting cells did not differ between patients and controls, but IL-4 response was stronger in patients than controls. Cytokine profile during tuberculosis /HIV co-infection are also the subject of debate. HIV patients with tuberculosis, when compared with HIV positive individuals without tuberculosis showed increased IFN- γ , TNF- α and IL-2 when PBMC were stimulated with PPD [328]. However, Zang and colleagues [329] showed that coinfection with HIV in tuberculosis patients results in decreased type 1 (IFN- γ , IL-2) and it was not associated with increased production of type 2 cytokines (IL-4, IL-10).

Taken together, these studies suggest that the increase in HIV replication *in vivo* during tuberculosis is due to atleast in part by pro-inflammatory cytokines. A delicate balance between TH1 and TH2, pro-inflammatory and anti-inflammatory cytokines play an important role in determining the course of tuberculosis and HIV disease progression.

The department of Virology, Haffkine Institute, has reported a high mortality rate of 40% in dually infected patients [Dr. R.A. Deshmukh, Personal communication]. In a country like India, with 50% adult population already infected with tuberculosis, superimposition of the two epidemics is going to cause havoc. As per the experiences in other countries, active finding of tuberculosis cases and their treatment is necessary to check the mortality and morbidity caused by the two infections and to increase the life span of the HIV infected patients.

In this study, we aimed to evaluate the cellular immune response to mycobacterial antigen in tuberculosis patients and HIV patients and how these responses are affected by co-infection. This was in order to characterise our population and set a base line for further studies as the cytokine balance is going to be exploited in the future therapy for various diseases. As immunologic markers of antigen recognition we measured blastogenic responses, IL-2 and IFN- γ secretion in response to PHA and mycobacterial sonicate. To characterise further the role of cytokines during the dual infection, secretion of IL-4, IL-10 and TNF- α was also analysed.

AIMS AND OBJECTIVES :

1. Determination of HIV seroprevalence in clinically diagnosed pulmonary tuberculosis patients.
2. Quantitative estimation of IL-2, IFN-gamma (TH1) and IL-4 (TH2) cytokines in cell free supernatants of PHA and *Mycobacterium tuberculosis* whole sonicate (MTBs) stimulated peripheral blood mononuclear cells of pulmonary tuberculosis patients co-infected with HIV and comparison with cytokine secretion in healthy volunteers, AIDS patients without tuberculosis and asymptomatic HIV seropositive patients.
3. Quantitative assessment of production of TNF- α (pro-inflammatory) and IL-10 (anti-inflammatory) cytokines from PBMNCs stimulated with PHA and MTBs in HIV seropositive and HIV seronegative pulmonary tuberculosis patients and comparison with healthy volunteers and HIV seropositive cases without tuberculosis.
4. Study and comparison of ability of PBMNCs from patients in all the above mentioned groups, to proliferate in response to mitogen (PHA) and antigen (MTBs)



MATERIALS
&
METHODS

PATIENTS

In this study, 240 patients and 10 healthy volunteers were included.

Among the patients included in this study, 30 were admitted to or were visiting OPDs of JJ Hospital; Byculla, Mumbai and remaining 210 were pulmonary tuberculosis patients admitted to The Group of TB Hospitals, Sewree, Mumbai.

Of the 30 patients from JJ Hospital, 16 were HIV seropositive asymptomatic cases visiting the OPD and 14 patients admitted to the hospital were HIV seropositive, AIDS cases with at least one AIDS defining opportunistic infection.

Two hundred and ten patients suffering from active pulmonary tuberculosis admitted to the TB hospital, Sewree were screened for HIV seroprevalence. They were divided into two groups: HIV seropositive pulmonary tuberculosis patients and HIV seronegative pulmonary tuberculosis patients.

The healthy volunteers and the patients were categorised according to the infections they were suffering from and were designated a group name as follows:

Controls: The 8 healthy volunteers were either students or employees of the Haffkine Institute and of the remaining 2, one was a student and the other was an employee at Cancer Research Institute, Parel, Mumbai. All of them tested HIV seronegative by commercially available ELISA and had no apparent signs of infection with *M. tuberculosis* or any other pathogen.

Group A (HIV seropositive asymptomatic cases): Sixteen HIV seropositive (Antibodies for HIV I&II were detected in the serum by commercially available ELISA) and Western blot confirmed cases visiting the OPD of JJ Hospitals, Byculla were included in this group. These patients were apparently healthy and free from any other infection.

Group B (AIDS syndrome cases without tuberculosis): Fourteen patients admitted to the JJ Hospitals, Byculla, Mumbai were included in this group. The patients were HIV seropositive by commercially available ELISA and Western blot confirmed. All of them were suffering from at least one AIDS

defining opportunistic infection. However, they did not show any clinical or radiological evidence of tuberculosis.

Group C: Two hundred and ten clinically diagnosed and radiologically confirmed active pulmonary tuberculosis patients, admitted to the Group of TB hospitals, Sewree, Mumbai, were screened for presence of HIV I&II antibodies in the sera, using commercially available ELISA kits. Out of the 210 patients tested, 51 were seropositive and 159 were seronegative. The Group C is further divided into two groups according to the HIV serostatus of the pulmonary tuberculosis patients:

Group C1 (HIV seropositive pulmonary tuberculosis patients): Twenty five of the 51 pulmonary tuberculosis patients who tested positive for HIV I&II antibodies by commercially available ELISA were included in this group after well informed, written consent was taken.

Group C2 (HIV seronegative pulmonary tuberculosis patients): Twenty-five of the 159 active pulmonary tuberculosis cases that were HIV seronegative by ELISA donated blood for the *in vitro* cytokine profile study.

DETECTION OF HIV SEROSTATUS

A. Blood collection :

Informed written consent from all the patients and controls was taken before collecting blood. Five millilitre blood of all the patients as well as the controls was collected by vein puncture in sterile test tubes. Blood was allowed to clot in the tubes at 37°C. Separated serum was centrifuged at 2000 rpm for 20 minutes. The clear supernatant was collected and stored in labelled, 2 millilitre plastic vials (Laxbro) at -20°C till used for HIV I & II antibodies testing by ELISA.

B. ELISA: The presence of antibodies to HIV was detected using commercially available ELISA kits. Initial screening was performed using Gamma (Belgium) kits, later on reactive samples were retested in duplicate

using EIA kits from Labsystems. Samples that were reactive by both the assays were considered to be HIV positive in the present study [330].

Principle: Gamma (Belgium) ELISA [marketed by Merind Diagnostic] is a solid phase immunoassay, the wells being coated with synthetic and recombinant antigen, corresponding to the transmembrane proteins gp41, gp36, and p24 gag regions of HIV I & II respectively. Labsystems HIV EIA marketed by Sewa Medicals is also an indirect solid phase immunoassay, the wells being coated with combination of synthetic peptide derived from highly immunoreactive sites of HIV I & II structural proteins and HIV I core protein. ELISA was able to detect antibodies to HIV I gp120, gp41, and gp24, and HIV II gp36 in human sera.

When present in patient's sera, HIV antibodies combine with HIV peptides attached to the polystyrene surface. Residual patient sample was removed by washing and Horseradish peroxidase conjugated anti-human IgG (sheep) was added. Wells were washed and a colourless enzyme substrate (H_2O_2) and chromogen (TNB) were added. Enzyme chromogen substrate reaction was terminated with sulphuric acid (H_2SO_4). The intensity of the colour was read at 450nm using ELISA reader (Multiskan).

PREPARATION OF ANTIGEN

(WHOLE SONICATE OF *Mycobacterium tuberculosis*, MTBs.)

Mycobacterium tuberculosis H₃₇Rv (NCTC7416) was purchased from National Collection of Type Cultures, Collindale London. The strain was maintained on Lowenstein Jensen medium slants.

For preparation of antigen, the *Mycobacterium tuberculosis* strain was cultivated in Doubs and Yomans medium (Appendix I). Five to six weeks old surface growth was harvested and subjected to 2.5 M-rad gamma radiation in a cobalt 60 radiation source to kill the virulent cells. Irradiated cells were then washed in phosphate buffered saline P_H 7.2 (Appendix) and then subjected to disintegration in a sonicator (Branson Sonifier, B-30) with 50% duty cycle at 20 kHz for 45 minutes, keeping the tube constantly on ice. The sonicate was

then centrifuged at 50,000 g (Sorval OTD Combi-Ultra- Centrifuge) for 1 hour to remove cell debris [331]. Cell free supernatant was then filtered through 0.22µm membrane filter (Millipore, USA). The protein content was estimated by Lowry's method (Appendix II). Sterility of the sonicate was checked using sodium thyoglycolate liquid medium and the sterile sonicate was aliquoted and stored at -20°C and used as an antigen for lymphoproliferative assays and induction of cytokines response in PBMNCs cultures.

LYMPHOCYTE PROLIFERATION AND CYTOKINE PRODUCTION

A. Blood collection :

15-20 ml of venous blood was collected in a sterile tube containing preservative free heparin. Ten units of heparin were used as an anticoagulant per millilitre of blood.

B. Separation of peripheral blood mononuclear cells :

Peripheral blood mononuclear cells (PBMNCs) were separated from the heparinized venous blood by loading 10 ml of 1:1 diluted blood (diluted with RPMI 1640) gently on 2.5 ml of Ficoll Hypaque per tube in 2 tubes. The tubes were centrifuged at 1500 rpm for 20 minutes at room temperature.

The PBMNCs were carefully removed from the interface and washed thrice with RPMI 1640. The number of viable cells were detected using trypan blue [1:1 (v/v) of the cell suspension] and a haemocytometer [332].

C. Lymphocyte proliferation :

PBMNCs were suspended in CRPMI at the concentration of 2×10^6 cells/ml. 100µl of cells was added to each of the wells so that cells were dispensed as 10^5 cells/well in a 96 well round bottomed microplate (Nunc, Denmark). PHA-M was diluted to the concentration of 5µg/ml and 100µl each was added to 3 wells (each well contained 0.5µg of the PHA). MTBs was diluted to 10µg/ml and another 3 wells were added with 100µl each (each well contained 1µg of

the MTBs). 100 μ l of CRPMI was added to 3 control wells. The plates were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air (henceforth mentioned as a 5% CO₂ incubator).

Cells were harvested after 48 hours of incubation in the last 16-18 hours of which they received tritiated thymidine ³H TdR (1 μ ci/well). After 18 hours of pulsing, the cultures were harvested on 3mm Wattman filter paper discs, air dried and soaked in 10% distilled water and finally washed with 20% methanol for 5 minutes. The discs were dried and transferred in to the scintillation vials containing 4 ml scintillation fluid (Appendix I). The incorporation of labelled thymidine was assayed in β scintillation counter (Racbeta 1211, LKB) and expressed as counts per minute (CPM). Degree of stimulation was expressed as stimulation index (SI), which is the ratio of CPM of the wells stimulated with antigen/mitogen and CPM of the wells containing unstimulated cells.

D. Generation of stimulated cell culture supernatants for estimation of cytokines :

PBMNCs separated from the blood were washed thrice with RPMI 1640 with intermittent centrifugation for 10 minutes at 1000 rpm to pellet down the cells. The cells were counted with haemocytometer, viability checked with trypan blue. Cells were resuspended in CRPMI so that the cell density would be 2 $\times$ 10⁶ cells /ml. 100 μ l/well of the well mixed cell suspension was added to the wells of a U bottom microplate so as to obtain 2 $\times$ 10⁵cells/well. Some of the wells were added with 100 μ l of 5 μ g/ml PHA (Sigma) in CRPMI and 100 μ l of 10 μ g/ml of MTBs in CRPMI. The plates were incubated at 37°C for different duration and supernatants were collected, pooled and centrifuged. The cell free supernatants were suitably aliquoted and stored at -20°C till assayed for cytokines.

Different cytokines were found to be secreted at their peak levels at different period on stimulation. In the present study, we found that in our experimental conditions,

IL-2 and IL-4 were found to be maximum in secretion 24 hours after stimulation with PHA and 48 hours after stimulation with MTBs. IL-10 was found to be secreted to the maximum levels in supernatants at 24 hours after stimulation with PHA and 72 hours after stimulation with MTBs. IFN- γ was found to be maximally secreted in 72 hours supernatant of PHA and MTBs stimulated cells. Seventy-two hours of incubation with MTBs and 24 hours of incubation with PHA were required for maximum levels of TNF- α to be secreted in the supernatants. Accordingly, PBMNCs from all the patients and controls were stimulated with PHA and MTBs for the optimal duration.

QUANTITATIVE DETERMINATION OF CYTOKINES

IL-2, IL-4, TNF- α , IFN- γ , IL-10 were assayed from the cell supernatants generated. IL-2 was assayed using IL-2 dependent CTLL-2 cell line and IL-4, TNF- α , IFN- γ , IL-10 were assayed using commercially available ELISA kits (ENDOGEN, USA).

A. CTLL bioassay

The IL-2 dependent cell line was used to assess the IL-2 activity in PHA/MTBs activated lymphocyte culture supernatant as per the method of Gills *et al* (1978) (333). The CTLL cell line was routinely maintained in IL-2 containing Concavalin A (Con A) stimulated rat spleenocyte culture supernatant.

Generation of Rat IL-2

A spleen of young (6-8 weeks) Wistar Rat (200gms) was excised under aseptic conditions and transferred to a petriplate containing RPMI 1640. The spleen was carefully freed of the attached muscle pieces and rinsed with RPMI 1640 thoroughly. The spleen minced on a wire mesh (0.4mm diameter) and the finely ground suspension in RPMI 1640 was transferred into 2 tubes. The suspension was centrifuged at 1500 rpm, for 20 minutes and the cell pellet was washed twice in RPMI 1640 with intermittent centrifugation at 1500 rpm for 20 minutes. The pellet was then suspended in CRPMI. Cells counted

in haemocytometer and checked for *viability* using trypan blue staining. Cells were divided so as to get 100×10^6 cells/20ml. To the 20ml cell suspension 100 μ g of Conavalin A (ConA) was added. The suspension was transferred to cell culture flasks (Nunc, Denmark) and incubated in 5% CO₂ incubator at 37°C for 48 hours. The stimulated contents of the flasks were transferred to centrifuge tubes and centrifuged at 1500 rpm for 20 minutes and the supernatant was carefully collected without disturbing the pellet. The supernatant was sterilised by passing it through 0.22 μ m filter (Millipore). Sterility was checked using sodium thioglycolate liquid medium and then the sterile Rat IL-2 was suitably aliquoted and stored at -20°C till used to support growth of CTLL cells.

Maintenance of CTLL

The cell line was a gift from Dr. S.V. Chiplunkar, Cellular Immunology, CRI, Parel, Mumbai. The CTLL cells were found to be highly susceptible for IL-2 deprivation and needed a split and reconstitution with fresh IL-2 containing medium every 48 hours. For routine maintenance, 4×10^5 cells from a 48 hour old culture were suspended in 5 ml CRPMI with 10% Rat IL-2. The cells were seeded in tissue culture flasks (25cm², 50ml volume, Nunc Denmark) and incubated in 5% CO₂ incubator at 37°C. The optimum concentration of rat IL-2 in the ConA stimulated Rat spleenocyte culture was checked for every preparation of Rat IL-2 which was generally found to be 10% v/v.

Assay for IL-2

The stored supernatants were first added in 50 μ l/well quantities in triplicates in 96 well, round bottom plates. CTLL cells were washed extensively thrice with cold GKN (Appendix I) solution to remove residual Rat IL-2 and suspended in CRPMI 1640. The *viable* cells were counted and 1×10^4 cells/150 μ l/well were added to wells already containing 50 μ l of supernatants to be tested (final concentration of the supernatants was 25% v/v). To the three wells to which supernatants were not added, 50 μ l of CRPMI 1640 was added to make up the volume to 200 μ l. These wells served as negative control. Another three wells contained 10% Rat IL-2 (v/v) (20 μ l of Rat IL-2 + 150 μ l of

cells + 30µl of CRPMI) and served as positive controls. The cells were incubated for 48 hours in a CO₂ incubator. During last 16 hours of incubation, they were pulsed with ³H TdR and ³H TdR incorporation was detected as described earlier. Results were expressed as Net CPM, which represents the proliferation in presence of activated lymphocyte culture supernatants in excess of the proliferation in the negative control cultures. Only those experiments in which Rat IL-2 added cultures (positive controls) showed optimal desired CPM (about 60,000-80,000) were considered for evaluation.

Generation of standard curve: Human recombinant IL-2 (Appendix I)(ENDOGEN) was added in different dilutions at concentration of 2U/ml to 10U/ml. Each concentration is added to wells in triplicate and the CPM for the CTLL was evaluated as above. The CPM for each concentration is plotted against the corresponding concentration to generate a standard curve. The amount of IL-2 in each supernatant was found out by interpolating from the CPM (Y-axis) to the corresponding U/ml of rIL-2 (X-axis). Thus IL-2 content of each supernatant is expressed in U/ml in the following report.

Quantitative Cytokine ELISA

IL-4, TNF-α, IFN-γ and IL-10 were assayed using commercially available ELISA kits (ENDOGEN, USA).

IFN-γ

The ENDOGEN Human IFN-γ ELISA was *in vitro* enzyme linked immunosorbent assays for the quantitative measurement of IFN-γ in culture supernatants, plasma, serum and urine.

Principle:

Standard curve:

Two vials of lyophilised standards were provided with each kit. The standard was reconstituted with CRPMI containing 10% FCS, same as that used for supporting growth of the cells) and diluted according to the instructions given in the kits. The concentrations 1000 pg/ml, 400 pg/ml, 160 pg/ml, 64 pg/ml, 25.6 pg/ml and 0 pg/ml were selected to be the standard curve points and

50µl from each dilution was added to the wells in duplicated for each concentration.

The kit had supplied wells, which were precoated with anti-human IFN- γ antibody. Fifty microlitres of biotinylated antibody was added to each well with 50µl of standards or samples in duplicate. The solid phase antibody and the biotinylated antibody recognise two different epitopes for the same cytokine giving more specificity to the test (as per information given in the kit).

After incubation and subsequent washing the plates were incubated with streptavidin-HRP and developed in dark with TMB in subsequent steps. The reaction was stopped and the absorbance of the plate was read at 450-550nm on a plate reader (Multiscan).

The standard curve was generated by plotting the average absorbency obtained for each of the standard concentration the vertical (Y) axis versus the corresponding IFN- γ concentration on the horizontal (X) axis. A graph was plotted using Microsoft Excel curve fitting package giving a linear fit.

Estimation of IFN- γ in test supernatants:

The amount of IFN- γ in each sample was determined by interpolating from the absorbance value (Y-axis) to the IFN- γ concentration (X-axis) using the standard curve

The optical density values obtained for duplicates should be within 10% of the mean. Duplicate values that differed from the mean by greater than 10% were considered suspect and were repeated.

If the IFN- γ concentration in any of the test supernatant was found to be exceeding the highest point on the standard curve, a ten fold dilution was prepared by adding 50µl of the test supernatant to 450µl of CRPMI and mixing thoroughly. The value obtained by interpolating OD of the diluted supernatant on the standard plot was multiplied by 10 (dilution factor) to get the concentration of IFN- γ in the test supernatant.

As per the instructions given in the kit, the kit was able to evaluate IFN- γ in the range of 0-1000pg/ml and the sensitivity of this assay for the lower limit of detection (LLD) was <2 pg/ml of human IFN- γ which was not zero with 95%

confidence with good reproducibility (intra assay cv <10% and interassay cv <10%). The ELISA was specific for the measurement of natural and recombinant human IFN- γ . It did not cross react with human IL-1 α , IL-1 β , IL-2, IL-3, IL-6, IL-7, IL-8, TNF- α or GM-CSF. The standards in this ELISA were calibrated to WHO reference Gg23-901-530. 1pg of ENDOGEN standard = 56 WHO pg.

IL-4

The principle and the procedure of the assay for IL-4 are the same as that for IFN- γ . IL-4 in the supernatants was estimated by interpolating the absorbance values of each supernatant from the standard graph generated with standard recombinant human IL-4 given along with the kit in lyophilised powdered form. The standard curve points were 400, 160, 64, 25.6, 10.24, and 0 pg/ml. According to the kit insert, the standards in this ELISA were calibrated to WHO reference 88/656.

One pg of Endogen standard = 35 WHO pg.

The ENDOGEN Human IL-4 ELISA was *in vitro* enzyme linked immunosorbent assays for the quantitative measurement of IL-4 in culture supernatants, plasma, serum and urine. It did not cross react with Human IL-2, IL-6, IL-10, IFN- γ , GM-CSF, TNF- α , TNF- β or mouse IL-4.

The kit was able to evaluate IL-4 in the range of 0-400 pg/ml and the sensitivity of this assay for the lower limit of detection (LLD) was <2 pg/ml of human IL-4 which was not zero with 95% confidence with good reproducibility (intra assay cv <10% and interassay cv <10%).

IL-10

The principle and the procedure of the assay for IL-10 are the same as that for IFN- γ . IL-10 in the supernatants was estimated by interpolating the absorbance values of each supernatant from the standard graph generated with standard recombinant human IL-10 given along with the kit in lyophilised powdered form. The standard curve points were 600, 240, 96, 38.4, 15.36, and 0 pg/ml.

According to the kit insert, the standards in this ELISA were calibrated to WHO reference lot 92/516.

One pg of Endogen standard = One WHO pg.

The ENDOGEN Human IL-10 ELISA was *in vitro* enzyme linked immunosorbent assays for the quantitative measurement of IL-10 in culture supernatants, plasma, serum and urine. It did not cross react with Human IL-1 α , IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IFN- γ .

The kit was able to evaluate IL-10 in the range of 0-600 pg/ml and the sensitivity of this assay for the lower limit of detection (LLD) was <3 pg/ml of human IL-10 which was not zero with 95% confidence with good reproducibility (intra assay cv <10% and interassay cv <10%).

TNF- α

The principle and the procedure of the assay for TNF- α are the same as that for IFN- γ . TNF- α in the supernatants was estimated by interpolating the absorbance values of each supernatant from the standard graph generated with standard recombinant human TNF- α given along with the kit in lyophilised powdered form. The standard curve points were 1000, 400, 160, 25.6, 0 pg/ml.

According to the kit insert, the standards in this ELISA were calibrated to WHO reference lot 87/650.

One pg of Endogen standard = One WHO pg.

The ENDOGEN Human TNF- α ELISA was *in vitro* enzyme linked immunosorbent assays for the quantitative measurement of TNF- α in culture supernatants, plasma, serum and urine. It did not cross react with Human IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, TNF- β , IFN- α , IFN- γ .

The kit was able to evaluate TNF- α in the range of 0-1000 pg/ml and the sensitivity of this assay for the lower limit of detection (LLD) was <5pg/ml of human TNF- α which was not zero with 95% confidence with good reproducibility (intra assay cv <10% and interassay cv <10%).

STATISTICAL ANALYSIS

For the lymphocyte proliferation tests and the cytokine profiles, comparisons among different groups were performed with the Analysis of Variance

(ANOVA). The comparisons between the two groups were done by modified LSD procedure at 0.01 level.

Correlation coefficients were calculated between IL-2 production, IFN- γ levels and the magnitude of lymphocyte proliferation among the patients and tested for its significance.

All the data obtained were analysed on computer using the SPSS/PC package[334].



RESULTS

Our study included a total of 10 healthy volunteers and 240 patients, who were divided into five groups according to the infections they harboured.

Controls: Controls consisted of 10 healthy volunteers who were either employee or students of the Haffkine Institute. The sex and age wise distribution of control group is given in the Table 1.1.

7/10 (70%) healthy volunteers were male, 3/10 (30%) were female. All were in the age group of 21-30 years.

Table 1.2 shows demographic characteristics of the control group. Nine (90%) of the volunteers were graduate and only one (10%) was matriculate. Two (28.57%) of the male volunteers were employed in the Haffkine Institute where as remaining 5 (71.43%) were postgraduate students of the Institute. 2/7 (28.57%) male candidates were married and 2/3 (66.66%) female volunteers were married. Only 1(10%) of the male volunteers was alcoholic whereas 3(30%) of the male volunteers smoked.

Group A: 16 HIV seropositive and Western blot confirmed cases visiting the OPDs of the JJ hospital were recruited in this group. Age and sex wise distribution of Group A candidates is given in the Table 1.3. Twelve (75%) were male, 8 (66.66%) in the age group 21-30 yrs, 3 (25%) in the age group of 31-40 yrs and 1 (8.33%) in the age group 41-50 yrs. Four (25%) were females. Two (50%) in the age group 21-30 yrs and 2 (50%) in 31-40 yrs.

The demographic characteristics of Group A are given in Table 1.4.

Of the 12 male patients, 3 (25%) were illiterate, 7 (58.33%) had received primary education and 2(16.66%) had done matriculation. 10 (83.33%) were employed, All of them (100%) confessed of drinking alcohol regularly, 7 (58.33%) smoked and 2(16.66%) chewed tobacco. One (8.33%) was drug addict.

All the four female patients had received primary education and 3 were commercial sex workers and the remaining one was a housewife. Two (50%) of them confessed of drinking alcohol and 1(25%) chewed tobacco.

The probable source of acquiring HIV infection was through heterosexual contact in majority of the cases. 9 (75%) male and 4 female (100%), thus a total of 13(81.25%) out of 16 patients were infected in this way. One male

patient (8.33%) gave history of blood transfusion and 1(8.33%) other male patients was a helper, working in the post-mortem section of hospitals, so would have acquired the infection due to lack of proper biosafety measures. (Table 1.5).

Group B

Fourteen patients having advanced immunodeficiency due to HIV infection and at least one AIDS defining syndrome were included in this group. The age and sex wise distribution of the patients in this group is given in the Table 1.6.

All 14 were male. Nine (64.29%) were in the age group 21-30, 5 (35.71%) in the age group 31-40yrs.

The demographic characteristics of the group B are given in the Table 1.7. Three (21.43%) had done matriculation, 2 (14.29%) were illiterate, 9 (64.29%) received primary education.

Nine of them were employed and others worked as unskilled labourers.

Out of the 14 patients, 11 (78.57%) consumed alcohol regularly and 9 (64.29%) smoked, 2(14.28%) chewed tobacco and 1 (7.14%) was a drug addict. Ten (71.43%) were married.

Out of 14, 11 (78.57%) cases gave history of promiscuous sexual behaviour, which would have made them prone to acquire the virus. One (7.14%) had received blood transfusion during an operation, which was probably the source of infection. One (7.14%) was addicted to intravenous drug. One (7.14%) was an attendant in the primary health care centre and would have acquired the infection as a result of accidental injury with infected instruments. The possible mode of acquiring infection among Group B patients is given in the Table 1.8.

The spectrum of opportunistic infections as presented in the studied patients was as follows:

8/14 (57.14%) were suffering from oral candidiasis, 2 (14.29%) were suffering from cryptococcal meningitis, 2 (14.29%) were patients of gastroenteritis and 2 (14.29%) were infected with *Herpes simplex* virus I. (Table 1.9)

Group C: Two hundred and ten clinically and radiologically confirmed pulmonary tuberculosis patients were screened for the presence of HIV I&II antibodies in the serum.

The age and sex wise distribution of the patients in Group C is given in the Table 1.10.

Out of the 210 patients studied 170 (80.93%) males and 40 (19.04%) were females. Among males, 68 (40%) were in the age group of 21-30 yrs followed by 52 (30.59%) in the age group 31-40 yrs, 44 (25.88%) in the age group 41-50. Only one (0.058%) was below the age of 20yrs and 5 (2.94%) above the age of 51 yrs were included in this study. Among the female patients almost similar age wise distribution was observed. Twenty (50%) were in the age group of 21-30 yrs, 13 (32.5%) in the 31-40 yrs.3 (7.5%) in the age group of 41-50yrs.

The demographic characteristics of the patients in this group are given in the Table 1.11.

Only 66/170 (38.82%) of the male patients were illiterate. 92 (54.11%) had primary education and only 12 (7.05%) were matriculate. 127/170 (74.70%) were employed and others worked on daily wages, most of them being unskilled labourers. The monthly income of all of them was less than Rs 4500/-. They showed high rate of addiction. 81(47.65%) were alcoholics, 31(18.24%) smoked and 15 (8.82%) chewed tobacco. Out of 170 male patients 137(80.59%) were married..

Of the 40 female patients recruited in this study, 31(77.5%) were married, 26 (65%) were illiterate, 10 (32.5%) had primary education, 3(7.5%) could reach high school and only 1(2.5%) was matriculate. Most of them, 36 (90%) were housewives but some worked as household workers. Four (10%) chewed tobacco while 3 (7.5%) confessed of drinking alcohol.

HIV seroprevalence in Group C:

Fifty (24.29%) out of 210 pulmonary tuberculosis patients (Group C) showed presence of HIV I&II antibodies in their sera on ELISA. This was confirmed by a repeat test and the 51 HIV seropositive pulmonary tuberculosis patients

were grouped as Group C1 and the remaining 159 (75.71%) HIV seronegative pulmonary tuberculosis patients were included in Group C2.

Group C1:

Sex and age wise seroprevalence among Group C1 is given in the Table 1.12.

Out of the 51 seropositive patients, 42 (82.35%) were male and 9 (17.64%) were females. Among the male patients the maximum seroprevalence was found among sexually most active age group of 21-30 yrs [23/42 (54.76%)] and 15 (35.71%) in the age group of 31-40 yrs. Only 1 (2.3%) was in age group of 11-20 yrs and above 51 yrs each. Two (4.76%) were in the age group of 41-50 yrs.

Among the female patients, 6 (66.66%) were in the age group of 21-30 yrs and 3 (33.33%) were in the age group 31-40 yrs. Thus, out of 51, 47 (92.15%) were in the age group 21-40 yrs, which is the sexually most active group.

The demographic characteristics of the patients in this group are given in the Table 1.13.

Out of the 42 male patients, 27 (62.29%) were married. Fifteen (35.71%) were illiterate, 22 (52.38%) had received primary education, and only 5 (11.90%) had done matriculation. The rate of addiction was found to be very high among the male patients of this group. Nineteen (45.24%) were alcoholics, 7 (16.66%) smoked, and 6 (14.29%) chewed tobacco.

Out of 9 female patients, 3 (33.33%) were married. Three (33.33%) were illiterate, 5 (55.55%) had received primary education, and only one (11.11%) had passed matriculation. All 9 were unemployed. 6(66.66%) were commercial sex workers and 3 (33.33%) were housewives apparently infected from their heterosexual partner. Two (22.22%) of the 9 confessed of addicted to alcohol and 1 (11.11%) chewed tobacco.

The risk behaviour of the patients, which would have probably got them infected, is illustrated in Table 1.14.

Thirty-six (79.19%) of the male patients had promiscuous sexual behaviour, 3 (7.14%) gave history of blood transfusion, 1(2.38%) was intravenous drug addict, and 2(4.76%) was thought to have acquired the infection due to needle stick injury. However, in case of 4 (9.52%) patients, the cause of the infection could not be traced.

Six (66.66%) of the female patients were commercial sex workers, thus must have acquired the infections from infected clients. Other 2 (22.22%) were housewives whose husbands were HIV seropositive positive and thus they acquired HIV from their husbands. One (11.11%) gave history of blood transfusion.

Group C2: Table 1.15 shows age wise distribution of HIV seronegative pulmonary tuberculosis patients.

Out of the 159 HIV seronegative pulmonary tuberculosis patients, 128 (80.50%) were male and 31 (19.5%) were female. 45 (35.16%) were in the age group 21-30 yrs, 37(28.90%) were in the age group 31-40 yrs, 42 (32.81%) were in the age group 41-50 yrs and only 4 (3.13%) aged above 51 yrs. Two (6.45%) were below 20 years of age, 14 (45.16%) were in the age group 21-30 yrs, 10 (32.25%) were in the age group 31-40yrs, 3 (9.68%) were in the age group 41-50 yrs and only 2 (6.45%) aged above 51 yrs.

The demographic characteristics of the group are given in Table 1.16.

Out of 170 male patients included in this group, 137 (80.59%) were married. Sixty-six (38.82%) were illiterate, 92 (54.11%) received primary education and 12 (7.05%) had done matriculation. One hundred and twenty seven (74.70%) were employed. Rate of addiction found among the male patients in this group is as follows: Eighty one (47.65%) were alcoholic, 31 (18.24%) had the habit of smoking, and 15 (8.82%) were addicted to tobacco.

Among the female patients, 31 (77.5%) were married, 26 (65%) were illiterate, 13 (32.5%) had attended primary school and only 1 (2.5%) had matriculation certificate. Four (10%) of them were employed and 36 (90%) were housewives although some of them worked as household workers. Only 3 (7.5%) confessed of drinking alcohol, and 4 (10%) chewed tobacco.

TABLE 1.1
Age and sex wise distribution of the Healthy Volunteers (Controls)
n=10.

	11-20 yrs	21-30 yrs	31-40 yrs	41-50 yrs	51 & above
MALE n=7 (70%)	-	5 (71.43%)	2 (28.57%)	-	-
FEMALE n=3 (30%)	-	3 (100%)			
TOTAL n=10	-	8 (80%)	2 (20%)	-	-

TABLE 1.2
Demographic characteristics of Controls

Characteristics	Male n=7	Female n=3	Total n=10
Married	2 (28.57%)	2 (66.66%)	4 (40%)
Unmarried	5 (71.43%)	1 (33.33%)	6 (60%)
Education			
Illiterate	-	-	-
Primary	1 (14.29%)	-	1 (10%)
Matriculate & above	6 (85.71%)	3 (100%)	9 (90%)
Employment			
Employed	2 (28.57%)	1 (33.33%)	3 (30%)
Unemployed	5 (71.43%)	2 (66.66%)	7 (70%)
Addiction			
Alcohol	1 (14.29%)	-	1 (10%)
Smoking	2 (28.57%)	-	2 (20%)
Tobacco	-	-	-

TABLE 1.3
Age and sex wise distribution of HIV seropositive
Asymptomatic patients (Group A).
n=16.

	11-20 yrs	21-30 yrs	31-40 yrs	41-50 yrs	51 & above
MALE n=12 (75%)	-	1 (8.33%)	9 (75%)	2 (16.66)	-
FEMALE n=4 (25%)	-	2 (50%)	1 (25%)	1 (25%)	-
TOTAL n=16	-	3 (18.75%)	10 (62.5%)	3 (18.75%)	-

TABLE 1.4
Demographic characteristics of Group A

Characteristics	Male n=12	Female n=4	Total n=16
Married	8 (66.66%)	1 (77.5%)	9 (56.25%)
Unmarried	4 (33.33%)	3 (22.5%)	7 (43.75%)
Education			
Illiterate	3 (25%)	-	3 (18.75%)
Primary	7 (58.33%)	1 (32.5%)	8 (50.00%)
Matriculate & above	2 (16.66%)	3 (2.5%)	5 (31.25%)
Employment			
Employed	10 (83.33%)	-	10 (62.5%)
Unemployed	2 (16.66%)	4 (100%)	6 (37.5%)
Addiction			
Alcohol	12 (100%)	2 (7.5%)	14 (87.5%)
Smoking	7 (58.33%)	-	7 (43.75%)
Tobacco	2 (16.66%)	1 (10%)	3 (18.75%)
Drugs	1 (8.33%)		1 (6.25%)

TABLE 1.5
Mode of transmission of HIV In Group A

	Heterosexual	Blood transfusion	Professional hazard	Intravenous drugs
Male n=12	9 (75%)	1 (8.33%)	1 (8.33%)	1 (8.33%)
Female n=4	4 (100%)	-	-	-

TABLE 1.6
Age and sex wise distribution AIDS patients
without pulmonary tuberculosis (Group B).
n=14

	11-20 yrs	21-30 yrs	31-40 yrs	41-50 yrs	51 & above
MALE n=14		9 (64.29%)	5 (35.17%)	-	-

TABLE 1.7
Demographic characteristics of Group B

Characteristics	Male n=14
Married	10 (71.43%)
Unmarried	4 (28.57%)
Education	
Illiterate	2 (14.29%)
Primary	9 (64.29%)
Matriculate & above	3 (21.43%)
Employment	
Employed	9 (64.29%)
Unemployed	5 (35.71%)
Addiction	
Alcohol	11 (78.57%)
Smoking	9 (64.29%)
Tobacco	2 (14.29%)
Drugs	-

TABLE 1.8
Opportunistic infections among group B patients.

Infections	Oral candidiasis	Cryptococcal meningitis	Gastroenteritis	<i>Herpes simplex I</i>
Male n=14	8 (57.14%)	2 (14.29%)	2 (14.29%)	2 (14.29%)

TABLE 1.9
Mode of transmission of HIV in Group B

	Heterosexual	Blood transfusion	Professional hazard	Intravenous drugs
Male n=14	11 (78.57%)	1 (7.14%)	1 (7.14%)	1 (7.14%)

TABLE 1.10
Age and sex wise distribution of the pulmonary tuberculosis patients
tested for HIV sero-prevalence Group C.
n=210.

	11-20 yrs	21-30 yrs	31-40 yrs	41-50 yrs	51 & above
MALE n=170 (80.95%)	1 (0.59%)	68 (40%)	52 (30.59%)	44 (25.88%)	5 (2.94%)
FEMALE n=40 (19.04%)	2 (5%)	20 (50%)	13 (32.5%)	3 (7.5%)	2 (5%)
TOTAL n=210	3 (1.42%)	88 (41.90%)	65 (30.95%)	47 (22.38%)	7 (3.33%)

TABLE 1.11
Demographic characteristics of Group C

Characteristics	Male n=170	Female n=40	Total n=210
Married	137 (80.59%)	31 (77.5%)	168 (80%)
Unmarried	33 (19.41%)	9 (22.5%)	42 (20%)
Education			
Illiterate	66 (38.82%)	26 (65%)	92 (43.81%)
Primary	92 (54.11%)	13 (32.5%)	105 (50.00%)
Matriculate & above	12 (7.05%)	1 (2.5%)	13 (6.19%)
Employment			
Employed	127 (74.70%)	4 (10%)	131 (62.38%)
Unemployed	43 (25.29%)	36 (90%)	79 (37.62%)
Addiction			
Alcohol	81 (47.65%)	3 (7.5%)	84 (40%)
Smoking	31 (18.24%)	-	31 (14.76%)
Tobacco	15 (8.82%)	4 (10%)	19 (9.04%)

TABLE 1.12
Age and sex wise distribution of the pulmonary tuberculosis patients
co-infected with HIV Group C1 (n=51).

	11-20 yrs	21-30 yrs	31-40 yrs	41-50 yrs	51 & above
MALE n=42 (82.35%)	1 (2.38)	23 (54.76)	15 (35.71%)	2 (4.76%)	1 (2.3%)
FEMALE n=9 (17.64%)	-	6 (6.66%)	3 (3.33%)	-	-
TOTAL n=51	1 (1.96%)	29 (56.86%)	18 (35.29%)	2 (23.92%)	1 (1.96%)

TABLE 1.13
Demographic characteristics of Group C1

Characteristics	Male n=42	Female n=9	Total n=51
Married	27 (62.29%)	3 (33.33%)	30 (58.82%)
Unmarried	15 (35.71%)	6 (66.66%)	21 (41.18%)
Education			
Illiterate	15 (35.71%)	3 (11.11%)	16 (31.37%)
Primary	22 (52.38%)	5 (55.55%)	27 (65.85%)
Matriculate & above	5 (11.90%)	1 (33.33%)	8 (15.67%)
Employment			
Employed	32 (76.19%)	-	32 (62.75%)
Unemployed	10 (23.81%)	9 (100%)	19 (37.25%)
Addiction			
Alcohol	19 (45.24%)	2 (22.22%)	21 (41.18%)
Smoking	7 (16.66%)	-	7 (16.66%)
Tobacco	6 (14.29%)	1 (11.11%)	7 (16.66%)

TABLE 1.14
Mode of Transmission in Group C1
n=51

	Heterosexual	Blood	Professional hazard	Drug	Unknown
MALE n=42 (82.32%)	36 (79.19%)	3 (7.14%)	2 (4.76%)	1 (2.40%)	4 (9.52%)
FEMALE n=9 (17.65%)	6 (66.66%)	1 (11.11%)	-	-	2 (22.22%)
TOTAL n=51	42 (82.35%)	4 (7.84%)	2 (3.92)	1 (1.96)	6 (11.76%)

TABLE 1.15
Age and sex wise distribution of the HIV seronegative pulmonary tuberculosis patients Group C2.
n=159.

	11-20 yrs	21-30 yrs	31-40 yrs	41-50 yrs	51 & above
MALE n=128 (80.50%)	-	45 (35.16%)	37 (28.90%)	42 (32.81%)	4 (3.13%)
FEMALE n=31 (19.5%)	2 (6.45%)	14 (45.16%)	10 (28.90%)	3 (9.68%)	2 (6.45%)
TOTAL n=159	2 (1.26%)	59 (37.11%)	47 (29.56%)	45 (28.30%)	6 (3.77%)

TABLE 1.16
Demographic characteristics of Group C2

Characteristics	Male n=128	Female n=31	Total n=159
Married	110 (85.94%)	28 (90.32%)	138 (86.8%)
Unmarried	18 (14.06%)	3 (9.68%)	21 (13.20%)
Education			
Illiterate	51 (38.71%)	23 (74.19%)	78 (49.06%)
Primary	70 (54.69%)	8 (25.81%)	78 (49.06%)
Matriculate & above	7 (5.47%)	-	7 (4.40%)
Employment			
Employed	95 (74.22%)	4 (12.90%)	101 (63.52%)
Unemployed	33 (25.78%)	27 (87.10%)	60 (37.73%)
Addiction			
Alcohol	62 (48.44%)	1 (3.23%)	63 (39.62%)
Smoking	24 (18.75%)	-	24 (15.09%)
Tobacco	9 (7.03%)	3 (9.68%)	12 (7.54%)

IN VITRO LYMPHOCYTE PROLIFERATION AND CYTOKINE PRODUCTION

The lectin PHA induces T lymphocytes to undergo proliferation, preceded by metabolic changes which include production of various lymphokines. PHA and other T cell mitogens like Con A are known to signal for activation events similar to antigens in an amplified manner [335]. The ultimate event after primary activation with antigen and IL-1 production by activated monocytes and interaction with secreted IL-2 is the proliferation of lymphocytes and clonal expansion [336]. Lymphocyte proliferation to PPD and recall antigens helps in detecting cellular immune deficiency. CMI responses to, mycobacterial antigens like PPD, *M tuberculosis* sonicate, and short term culture filtrate, can be evaluated using lymphocyte proliferation technique [337].

Proliferation responses of PHA and MTBs activated responses from healthy donors and patients and their relation with production of IL-2, IFN- γ and other cytokines (IL-4, IL-10, and TNF- α) is given in this chapter.

All the 10 healthy volunteers were included in the *in vitro* proliferation and cytokine estimation study and are grouped as Controls. Group A consisted of 16 HIV seropositive asymptomatic cases as mentioned earlier. Group B consisted of 14 AIDS syndrome cases without *M tuberculosis* infection. Group C1 consisted of 25 patients out of the 51 HIV seropositive pulmonary tuberculosis patients of Group C1 described earlier. Group C2 consisted of 25 HIV seronegative pulmonary tuberculosis patients out of the 159 of earlier described C2 group. In Group C1 and C2, there was no specific discrimination among the patients while selecting for *in vitro* test other than their own willingness to donate blood.

STIMULATION WITH PHA

PROLIFERATION OF PBMNCs

The capacity of PBMNCs to proliferate in response to *in vitro* stimulation by PHA was evaluated as the extent of DNA synthesis by incorporation of tritiated thymidine and expressed as mean stimulation index (mean SI) (as described in Materials and Methods).

A total of 10 healthy volunteers and 80 patients were included in this study.

The proliferation responses of PBMNCs are given in Table 2.1

Controls: All 10/10 (100%) volunteers gave highest proliferation of PBMNCs in response to PHA stimulation, the mean being 54.21 ± 2.10 .

Group A: All 16/16 (100%) HIV seropositive asymptomatic cases showed significantly lowered lymphocyte proliferation the mean SI being 30.30 ± 2.37 , at $p < 0.01$ (ANOVA).

Group B: All 14/14 AIDS syndrome cases without tuberculosis showed the lowest proliferation (Mean 5.72 ± 0.81) as compared controls and asymptomatic patients at $p < 0.01$ (ANOVA).

Group C1: PBMNCs of 4/25 (16%) patients did not show any proliferation in response to PHA. This group also showed similar proliferation as Group B the mean being 6.76 ± 1.13 which was significantly low as compared to controls and Group A at $p < 0.01$ (ANOVA).

Group C2: Active pulmonary tuberculosis patients without HIV showed considerably lowered lymphocyte proliferation (Mean 20.99 ± 2.04) as compared to controls. The difference is found to be significant at $p < 0.01$ (ANOVA).

IL-2 production

Activated lymphocyte culture require a growth factor (IL-2) for clonal expansion which is synthesised by activated cells themselves and acts in an aurocrine and paracrine manner on cells expressing IL-2R to induce proliferation [338,339]. In the present study, amounts of IL-2 in culture

supernatants of activated lymphocytes from 10 healthy donors and 80 patients were determined. As stated expansion of IL-2 dependent murine CTLL-2 cell line in response to IL-2 present in the PHA activated lymphocyte culture supernatants was quantitated. IL-2 production in response to PHA stimulation of PBMCs of the patients as well as the controls is given in Table 2.2

Controls: All 10/10 (100%) controls showed the highest IL-2 production in response to PHA, the mean being 10.44 ± 0.42 U/ml, as compared to the patients in all the four groups at $p < 0.01$ (ANOVA).

Group A: 16/16 (100%) patients showed detectable IL-2. The Mean was 7.4 ± 0.34 U/ml. This was significantly reduced as compared to controls at $p < 0.01$ (ANOVA).

Group B: 14/14 (100%) patients showed detectable IL-2, Mean 0.92 ± 0.16 U/ml although, it was significantly reduced as compared to controls as well as asymptomatic cases (Group A) at $p < 0.01$ (ANOVA).

Group C1: 25/25 (100%) showed detectable amounts of IL-2 which were similar to those of Group B. The mean IL-2 detected was 2.53 ± 0.28 U/ml. This was significantly lowered as compared to controls as well as Group A cases at $p < 0.01$ (ANOVA).

Group C2: Active pulmonary tuberculosis cases showed Mean 4.23 ± 0.47 U/ml IL-2 production which was significantly reduced as compared to controls at $p < 0.01$, but the IL-2 production was significantly higher than HIV and tuberculosis co-infected patients Group C1 at $p < 0.01$ (ANOVA).

IFN- γ PRODUCTION

The activated state of lymphocytes can be assessed by their ability to produce IFN- γ as well as IL-2.

IFN- γ production by PBMCs of patients and controls is given in Table 2.3

Controls: Healthy volunteers produced the highest amount of IFN- γ in supernatants of PBMCs activated with PHA. All 10/10 (100%) produced detectable IFN- γ giving a mean of 1285.40 ± 184.21 pg/ml.

Group A: Asymptomatic HIV patients showed similar levels of IFN- γ as compared to controls (1228.05 ± 221.84 pg/ml) at $p < 0.01$ (ANOVA).

Group B: All the 14 patients in Group B showed the least IFN- γ in supernatants of PHA stimulated PBMNCs. The mean value was 192.61 ± 56.59 pg/ml. This was significantly reduced as compared to the controls and Group A patients at $p < 0.01$.

Group C1: Group C1 patients showed similar levels of IFN- γ in secretion. The mean value was 381.49 ± 84.19 pg/ml which was significantly lowered than controls and Group A patients at $p < 0.01$ (ANOVA).

Group C2: Pulmonary tuberculosis patients not co-infected with HIV showed slightly decreased IFN- γ production 733.44 ± 99.79 pg/ml, but this was not significantly different than the healthy volunteers (Controls) at $p < 0.01$ (ANOVA).

IL-4

IL-4 production by PBMNCs of patients and controls is given in Table 2.4

Controls: Only 5/10 (50%) of controls showed IL-4 production in the culture supernatants of their PBMNCs. The mean was 7.81 ± 3.21 pg/ml.

Group A: 2/16 (12.5%) did not show detectable IL-4 in the supernatants. The mean was 8.60 ± 2.89 pg/ml.

Group B: Only 4/14 (28.57%) showed detectable IL-4 in the supernatants. The mean IL-4 production was 3.43 ± 1.04 pg/ml.

Group C1: 7/25 (28%) HIV positive pulmonary tuberculosis patients did not show detectable IL-4 in the PBMNCs culture supernatants. From the remaining 18/25 (72%) patients the mean IL-4 produced was 7.46 ± 4.60 pg/ml.

Group C2: 2/25 (8%) pulmonary tuberculosis patients did not show detectable IL-4 in supernatants of PHA activated lymphocytes. The mean IL-4 produced was 6.14 ± 2.22 pg/ml.

There was no significant difference among all the groups and controls at $p < 0.01$ (ANOVA).

IL-10

IL-10 production by PBMCs of patients and controls is given in Table 2.5

Controls: Healthy volunteers gave mean IL-10 value of 555.51 ± 49.57 pg/ml, which was not significantly different from patients in any of the four groups at $p < 0.01$ (ANOVA).

Group A: The mean IL-10 produced was 540.52 ± 37.88 pg/ml.

Group B: The mean IL-10 produced was 383.94 ± 67.30 pg/ml.

Group C1: The mean IL-10 produced was 337.71 ± 44.20 pg/ml.

Group C2: The mean IL-10 produced was 554.52 ± 26.04 pg/ml.

There was no significant difference among all the groups and controls at $p < 0.01$ (ANOVA).

TNF- α

TNF- α production by PBMCs of patients and controls is given in Table 2.6

Controls: All the 10 controls included in this study showed a mean TNF- α production of 67.10 ± 15.66 pg/ml.

Group A: Sixteen asymptomatic patients in this group were studied for the production of TNF- α in response to MTBs. The mean TNF- α produced was 309.30 ± 77.95 pg/ml. This was not significantly different from the amount produced by controls.

Group B: Fourteen AIDS syndrome cases without tuberculosis produced 438.98 ± 125.58 pg/ml of TNF- α which was similar to the controls and Group A.

Group C1: The HIV and tuberculosis co-infected patients produced highest amount of TNF- α in response to MTBs stimulation, Mean 721.37 ± 101.07 pg/ml, which was significantly higher than all the other HIV infected groups and control at $p < 0.01$ (ANOVA).

Group C2: HIV seronegative pulmonary tuberculosis patients produced significantly high, Mean 622.08 ± 81.65 pg/ml, which was higher than controls at $P < 0.01$ (ANOVA).

STIMULATION WITH MTBs

PROLIFERATION OF PBMNCs:

Proliferation by PBMNCs of patients and controls is given in Table 2.7

Controls: 10/10 healthy volunteers showed proliferation with stimulation index 14.22 ± 1.54 . It was significantly higher than any other group at $p < 0.01$ (ANOVA).

Group A: 16/16 showed considerably reduced proliferation in response to MTBs and gave a stimulation index of 6.36 ± 0.48 . This was significantly lowered as compared to controls at $p < 0.01$ (ANOVA).

Group B: 9/14 (64.29%) showed proliferation in response to MTBs and gave a stimulation index of 2.55 ± 0.29 which was significantly lowered as group A and controls at $p < 0.01$ (ANOVA).

Group C1: 15/25 (60%) showed proliferation in response to MTBs and gave a stimulation index of 2.79 ± 0.29 which was significantly lowered as group A and controls at $p < 0.01$.

Group C2: 1/25 (4%) did not show proliferation of PBMNCs in response to MTBs stimulation. The mean stimulation index was 5.61 ± 0.77 which was significantly lowered as compared to controls but significantly higher than Group C1 at $p < 0.01$ (ANOVA).

IL-2 production

IL-2 production by PBMNCs of patients and controls is given in Table 2.8

Controls: 10/10 healthy volunteers produced detectable amount of IL-2 in the MTBs activated lymphocyte culture supernatants. The mean amount of IL-2 produced by the 10 subjects was 7.80 U/ml as was significantly higher than the patients in any of the groups included in the study at $p < 0.01$ (ANOVA).

Group A: 16/16 HIV asymptomatic patients produced detectable amount of IL-2, the mean being 3.53 ± 0.44 U/ml which was significantly reduced as compared to controls at $P < 0.01$ (ANOVA).

Group B: The mean IL-2 produced by the 14 AIDS syndrome cases was 0.67 ± 0.13 U/ml, which was the least among all the patients, as well as controls. The amount of IL-2 produced was significantly reduced as compared to controls and asymptomatic HIV infected cases (Group A) at $p < 0.01$ (ANOVA).

Group C1: HIV-infected pulmonary tuberculosis patients produced a mean of 0.73 ± 0.13 U/ml of IL-2 which was comparable to that produced by Group B AIDS syndrome cases without tuberculosis. Production of IL-2 was considerably reduced as compared to controls, Group A as well as pulmonary tuberculosis patients without HIV (Group C2) this reduction was significant at $p < 0.01$ (ANOVA).

Group C2: Pulmonary tuberculosis patients showed significant decline in IL-2 production in response to MTBs (Mean 2.01 ± 0.31 U/ml) as compared to the controls at $p < 0.01$ (ANOVA).

IFN- γ production:

IFN- γ production by PBMCs of patients and controls is given in Table 2.9

Controls produced the highest amount of IFN- γ . Mean of IFN- γ produced in response to MTBs was 1763.75 ± 1530.03 pg/ml which was significantly higher than patients in any other group included in the present study at $p < 0.01$ (ANOVA).

Group A: Asymptomatic patients show considerable decline in the production of IFN- γ in response to MTBs antigen Mean 980.63 ± 165.82 pg/ml as compared to the controls at $p < 0.01$, but this was higher than the AIDS syndrome cases without tuberculosis Group B Mean 222.76 ± 50.34 pg/ml or with tuberculosis Group C1 Mean 301.58 ± 47.68 pg/ml at $p < 0.01$.

Group B: The AIDS syndrome cases produced the least amount of IFN- γ Mean 222.76 ± 50.34 pg/ml in response to MTBs that was significantly lower than the Controls as well as the asymptomatic HIV patients (Group A) at $p < 0.01$ (ANOVA).

Group C1: 8/25 patients co-infected with HIV and tuberculosis did not produce any detectable amounts of IFN- γ in response to stimulation with

MTB. The remaining 17 HIV seropositive pulmonary tuberculosis patients produced significantly lower IFN- γ (Mean 301.58 ± 47.68 pg/ml) as compared to Controls at $p < 0.01$. However, it was significantly higher than the amount produced by pulmonary tuberculosis patients co-infected with HIV (Group C2).

Group C2: 2/25 HIV seronegative active pulmonary tuberculosis patients did not produce detectable IFN- γ in the MTBs activated PBMNCs supernatants. The remaining 23 patients produced a mean of 653.85 ± 59.42 pg/ml which was significantly higher than the HIV seropositive pulmonary tuberculosis patients (Group C1) at $p < 0.01$, but was significantly reduced than the controls at $p < 0.01$ (ANOVA).

IL-4 production

IL-4 production by PBMNCs of patients and controls is given in Table 2.10

Controls: Only 2/10 (20%) of the controls produced detectable amount of IL-4 in response to MTBs stimulation, the mean value was 0.033 ± 0.013 pg/ml.

Group A: 6/16 HIV asymptomatic cases produced detectable IL-4 and the mean amount produced was 3.39 ± 1.86 pg/ml.

Group B: 4/14 AIDS syndrome cases without tuberculosis produced detectable IL-4 in the MTBs activated PBMNCs culture supernatants. The mean of the amounts produced was 0.69 ± 0.16 pg/ml.

Group C1: 7/25 HIV seropositive pulmonary tuberculosis patients produced a mean of 1.26 ± 0.31 pg/ml of IL-4 in response to stimulation with MTBs.

Group C2: 7/25 pulmonary tuberculosis patients without HIV infection produced a mean of 2.75 ± 1.51 pg/ml of IL-4 in response to stimulation with MTBs.

There was no statistical significance found among any of the groups of patients and the controls at $P < 0.01$ (ANOVA).

IL-10 production

IL-10 production by PBMCs of patients and controls is given in Table 2.11

Controls: Healthy volunteers produced a mean of 535.84 ± 41.99 pg/ml of IL-10 in response to stimulation with MTBs.

Group A: HIV asymptomatic cases produced slightly raised, a mean of 582.67 ± 66.74 pg/ml of IL-10, though it was not found to be statistically significant at $p < 0.01$.

Group B: HIV syndrome cases without tuberculosis produced a mean of 500.94 ± 66.74 pg/ml IL-10 in response to MTBs stimulation of PBMCs *in vitro*.

Group C1: HIV seropositive pulmonary tuberculosis patients produced slightly increased 646.12 ± 44.17 pg/ml of IL-10. The increase was not significant as compared to any other group at $p < 0.01$.

Group C2: HIV seronegative pulmonary tuberculosis patients produced a mean 490.29 ± 46.25 pg/ml of IL-10 in response to MTBs stimulation.

There was no significant difference found in the levels of IL-10 produced by any of the patient groups or controls at $p < 0.01$ (ANOVA).

TNF- α production

TNF- α production by PBMCs of patients and controls in response to MTBs stimulation is given in Table 2.12

Controls: Controls produced the lowest amount of TNF- α in response to MTBs stimulation. The mean was 136.64 ± 17.36 pg/ml.

Group A: Asymptomatic HIV infected individuals (Mean TNF- α 138.62 ± 43.39 pg/ml) showed no difference in TNF- α production as compared to healthy controls.

Group B: AIDS syndrome patients without tuberculosis patients (Mean 56.65 ± 9.88 pg/ml) showed slightly lower TNF- α production than controls and Group A but the difference was not significant at $p < 0.01$ (ANOVA).

Group C1: Pulmonary tuberculosis patients co-infected with HIV showed highest production of TNF- α in response to MTBs (Mean 1236.46 ± 103.61

pg/ml) as compared to any other group in the present study at $p < 0.01$ (ANOVA).

Group C2: HIV seronegative pulmonary tuberculosis patients showed significantly higher TNF- α production (Mean 542.94 ± 81.60 pg/ml) as compared to healthy controls at $p < 0.01$, but it was significantly less as compared to the HIV seropositive pulmonary tuberculosis patients (Group C1) at $p < 0.01$ (ANOVA).



Fig 1: Light micrograph of human PBMNCs cultured without any stimulation (control) in complete RPMI 1640 medium in tissue culture plate, for 48 hours.

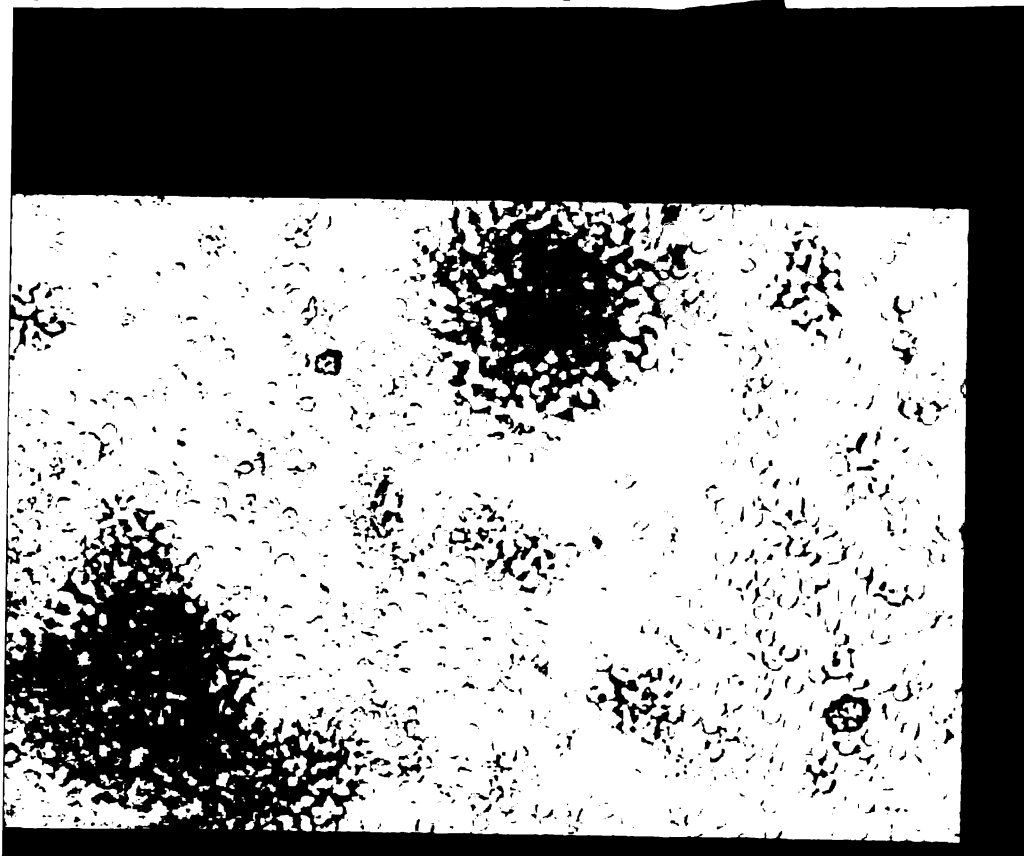


Fig 2: Light micrograph of human PBMNCs cultured with PHA (5µg/ml) stimulation in complete RPMI 1640 medium in tissue culture plate, for 48 hours.

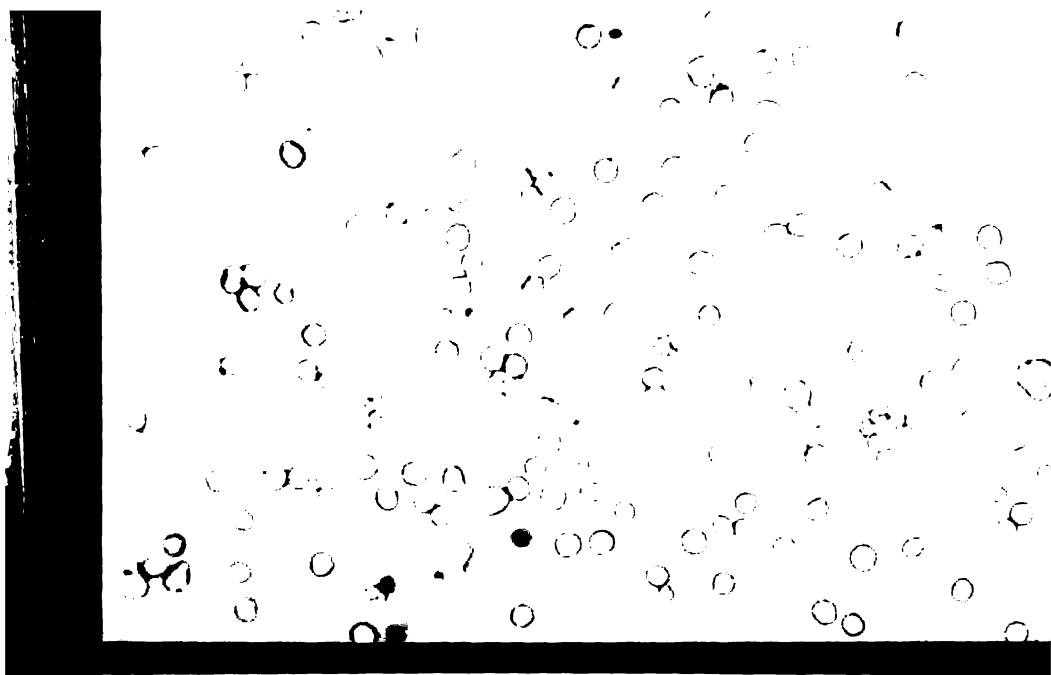


Fig 3: Light micrograph of murine CTLL-2 cell line growing in CRPMI with 10% Ra for maintains in the tissue culture flasks (50ml volume, Nunc), incubated for 48 hou

TABLE 2.1
PBMNCs PROLIFERATION IN RESPONSE TO PHA STIMULATION

GROUPS	STIMULATED MEAN CPM $\pm$ SE	UNSTIMULATED MEAN CPM $\pm$ SE	MEAN SI $\pm$ SE
CONTROLS	55501.00 $\pm$ 2424.48	1027.60 $\pm$ 35.67	54.21 $\pm$ 2.10
GROUP A	33580.52 $\pm$ 1568.29	1178.69 $\pm$ 75.00	30.30 $\pm$ 2.37
GROUP B	3924.93 $\pm$ 647.27	684.93 $\pm$ 39.22	5.72 $\pm$ 0.81
GROUP C1	5436.40 $\pm$ 1062.81	762.40 $\pm$ 35.37	6.76 $\pm$ 1.13
GROUP C2	19320.16 $\pm$ 1386.61	985.52 $\pm$ 43.61	20.99 $\pm$ 2.04

COMPARISON OF GROUPS	SIGNIFICANCE
CONTROLS > GROUP A	P < 0.01
CONTROLS > GROUP B	P < 0.01
CONTROLS > GROUP C1	P < 0.01
CONTROLS > GROUP C2	P < 0.01
GROUP A > GROUP B	P < 0.01
GROUP A > GROUP C1	P < 0.01
GROUP A > GROUP C2	P < 0.01
GROUP C2 > GROUP C1	P < 0.01

TABLE 2.2
IL-2 PRODUCTION IN RESPONSE TO PHA

	CONTROL	GROUP A	GROUP B	GROUP C1	GROUP C2
IL-2 (units/ml)	10.44 $\pm$ 0.42	7.4 $\pm$ 0.34	0.92 $\pm$ 0.16	2.53 $\pm$ 0.28	4.23 $\pm$ 0.47

COMPARISON OF GROUPS	SIGNIFICANCE
CONTROLS > GROUP A	P < 0.01
CONTROLS > GROUP B	P < 0.01
CONTROLS > GROUP C1	P < 0.01
CONTROLS > GROUP C2	P < 0.01
GROUP A > GROUP B	P < 0.01
GROUP A > GROUP C1	P < 0.01
GROUP C2 > GROUP C1	P < 0.01

CONTROLS : Healthy volunteers.

GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

TABLE 2.3
IFN- γ PRODUCTION IN RESPONSE TO PHA

	CONTROLS	GROUP A	GROUP B	GROUP C1	GROUP C2
IFN- γ (pg/ml)	1285.40 $\pm$ 184.21	1228.05 $\pm$ 221.84	192.61 $\pm$ 56.53	381.49 $\pm$ 84.19	733.44 $\pm$ 99.79

COMPARISON OF GROUPS	SIGNIFICANCE
CONTROLS > GROUP A	-
CONTROLS > GROUP B	P < 0.01
CONTROLS > GROUP C1	P < 0.01
CONTROLS > GROUP C2	-
GROUP A > GROUP B	P < 0.01
GROUP A > GROUP C1	P < 0.01

TABLE 2.4
IL-4 PRODUCTION IN RESPONSE TO PHA

	CONTROLS	GROUP A	GROUP B	GROUP C1	GROUP C2
IL-4 (pg/ml)	7.46 $\pm$ 4.60	6.14 $\pm$ 2.22	3.43 $\pm$ 1.04	8.60 $\pm$ 2.89	7.81 $\pm$ 3.21

NO TWO GROUPS ARE SIGNIFICANTLY DIFFERENT AT 0.01 LEVEL.

CONTROLS : Healthy volunteers.
 GROUP A : HIV seropositive asymptomatic cases.
 GROUP B : AIDS patients having opportunistic infections other than tuberculosis
 GROUP C1 : HIV seropositive pulmonary tuberculosis patients.
 GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

TABLE 2.5
IL-10 PRODUCTION IN RESPONSE TO PHA

	CONTROLS	GROUP A	GROUP B	GROUP C1	GROUP C2
IL-10 (pg/ml)	555.51 ± 49.57	540.52 ± 37.88	383.94 ± 67.30	337.71 ± 44.20	554.52 ± 26.04

NO TWO GROUPS ARE SIGNIFICANTLY DIFFERENT AT 0.01 LEVEL.

TABLE 2.6
TNF- α PRODUCTION IN RESPONSE TO PHA

	CONTROLS	GROUP A	GROUP B	GROUP C1	GROUP C2
TNF- α (pg/ml)	67.10 ± 15.66	309.30 ± 77.95	438.98 ± 125.58	721.37 ± 103.07	622.08 ± 81.65

COMPARISON OF GROUPS	SIGNIFICANCE
GROUP C1 > CONTROLS	P < 0.01
GROUP C2 > CONTROLS	P < 0.01

CONTROLS : Healthy volunteers.

GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

TABLE 2.7
PBMNCs PROLIFERATION IN RESPONSE TO MTB_s STIMULATION

GROUPS	STIMULATED MEAN CPM $\pm$ SE	UNSTIMULATED MEAN CPM $\pm$ SE	MEAN SI $\pm$ SE
CONTROLS	14239.70 $\pm$ 965.89	1050.80 $\pm$ 76.69	14.22 $\pm$ 1.54
GROUP A	7315.19 $\pm$ 524.76	1177.19 $\pm$ 48.84	6.36 $\pm$ 0.48
GROUP B	2311.07 $\pm$ 380.40	681.93 $\pm$ 74.11	2.55 $\pm$ 8.29
GROUP C1	2950.00 $\pm$ 425.90	933.92 $\pm$ 70.26	2.79 $\pm$ 0.29
GROUP C2	6281.00 $\pm$ 811.51	1206.80 $\pm$ 75.82	5.61 $\pm$ 0.77

COMPARISON OF GROUPS	SIGNIFICANCE
CONTROLS > GROUP I	P < 0.01
CONTROLS > GROUP II	P < 0.01
CONTROLS > GROUP III	P < 0.01
CONTROLS > GROUP IV	P < 0.01
GROUP IV > GROUP I	P < 0.01
GROUP IV > GROUP III	P < 0.01
GROUP II > GROUP I	P < 0.01

CONTROLS : Healthy volunteers.

GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

TABLE 2.8
IL-2 PRODUCTION IN RESPONSE TO MTBs

	CONTROLS	GROUP A	GROUP B	GROUP C1	GROUP C2
IL-2 (unit/ml)	7.80 ± 0.55	3.53 ± 0.44	5.72 ± 0.81	6.76 ± 1.13	20.99 ± 2.04

COMPARISON OF GROUPS	SIGNIFICANCE
CONTROLS > GROUP A	P < 0.01
CONTROLS > GROUP B	P < 0.01
CONTROLS > GROUP C1	P < 0.01
CONTROLS > GROUP C2	P < 0.01
GROUP A > GROUP B	P < 0.01
GROUP A > GROUP C1	P < 0.01
GROUP C2 > GROUP C1	P < 0.01

CONTROLS : Healthy volunteers.

GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

TABLE 2.9
IFN- γ PRODUCTION IN RESPONSE TO MTBs

	CONTROLS	GROUP A	GROUP B	GROUP C1	GROUP C2
IFN- γ (pg/ml)	1763.75 $\pm$ 1530.03	980.63 $\pm$ 165.82	222.76 $\pm$ 50.34	301.58 $\pm$ 47.68	653.85 $\pm$ 59.42

COMPARISON OF GROUPS	SIGNIFICANCE
CONTROLS > GROUP A	P < 0.01
CONTROLS > GROUP B	P < 0.01
CONTROLS > GROUP C1	P < 0.01
CONTROLS > GROUP C2	P < 0.01
GROUP A > GROUP B	P < 0.01
GROUP A > GROUP C1	P < 0.01
GROUP C2 > GROUP C1	P < 0.01

TABLE 2.10
IL-4 RESPONSE TO MTBs

	CONTROLS	GROUP A	GROUP B	GROUP C1	GROUP C2
IL-4 (pg/ml)	0.033 $\pm$ 0.013	3.39 $\pm$ 1.86	0.69 $\pm$ 0.16	1.26 $\pm$ 0.31	2.75 $\pm$ 1.51

NO TWO GROUPS ARE SIGNIFICANTLY DIFFERENT AT 0.01 LEVEL.

CONTROLS : Healthy volunteers.

GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

TABLE 2.11
IL-10 PRODUCTION IN RESPONSE TO MTBs

	CONTROLS	GROUP A	GROUP B	GROUP C1	GROUP C2
IL-10 (pg/ml)	535.84 ± 41.99	582.67 ± 66.74	500.94 ± 66.74	646.12 ± 44.17	490.29 ± 46.25

NO TWO GROUPS ARE SIGNIFICANTLY DIFFERENT AT 0.01 LEVEL.

TABLE 2.13
TNF- α PRODUCTION IN RESPONSE TO MTBs

	CONTROLS	GROUP A	GROUP B	GROUP C1	GROUP C2
TNF- α (pg/ml)	136.64 ± 17.38	138.62 ± 43.39	56.65 ± 9.88	1236.46 ± 103.61	542.94 ± 81.60

COMPARISON OF GROUPS	SIGNIFICANCE
GROUP C1 > CONTROLS	P < 0.01
GROUP C1 > GROUP C2	P < 0.01
GROUP C1 > GROUP B	P < 0.01
GROUP C1 > GROUP A	P < 0.01
GROUP C2 > GROUP A	P < 0.01
GROUP C2 > GROUP B	P < 0.01
GROUP C2 > CONTROLS	P < 0.01

CONTROLS : Healthy volunteers.

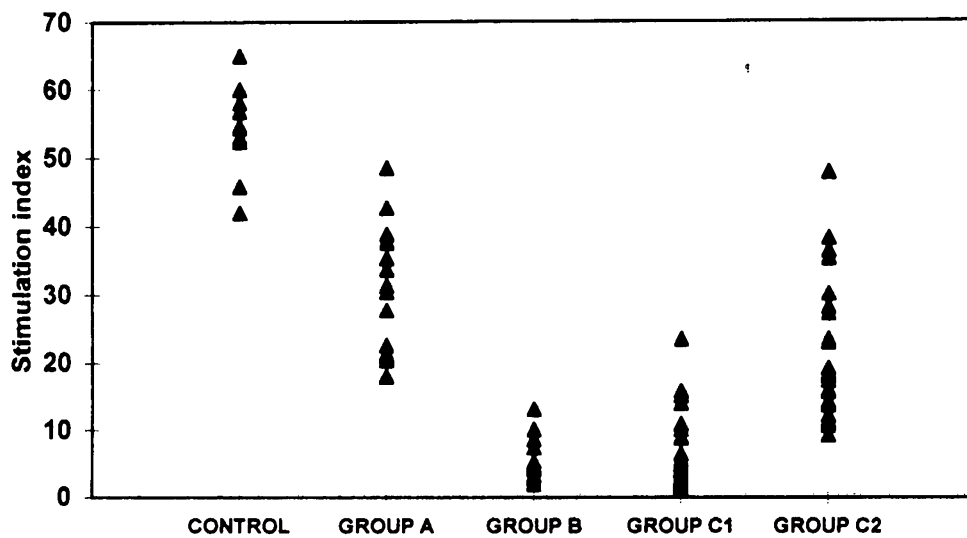
GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

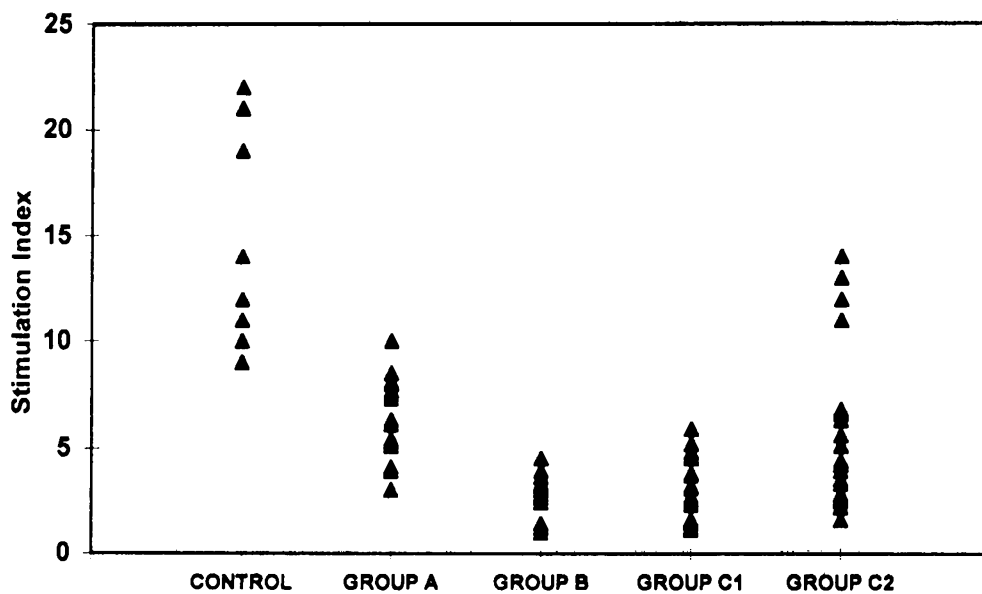
GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Proliferation of PBMCs in response to PHA stimulation



Proliferation of PBMCs in response to MTBs stimulation



CONTROLS : Healthy volunteers.

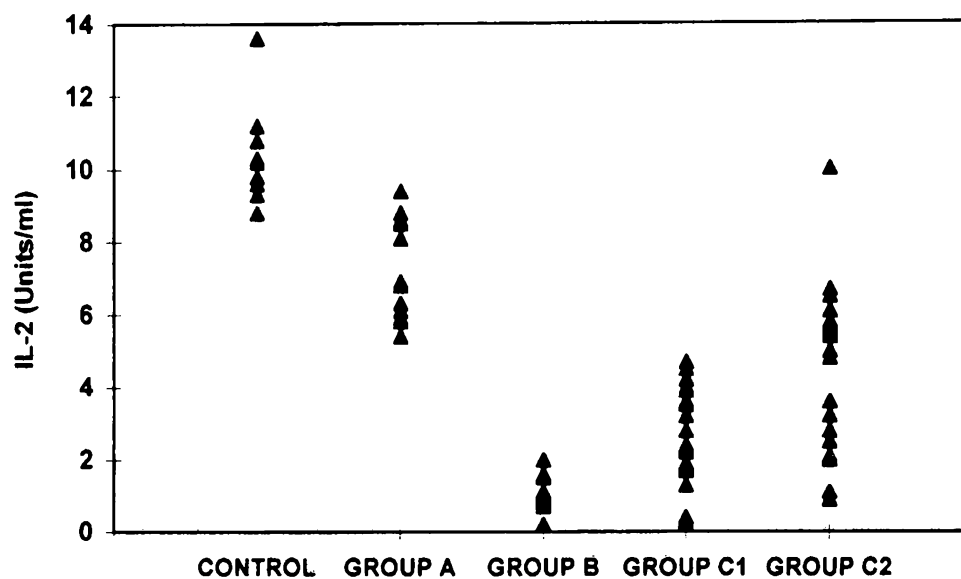
GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

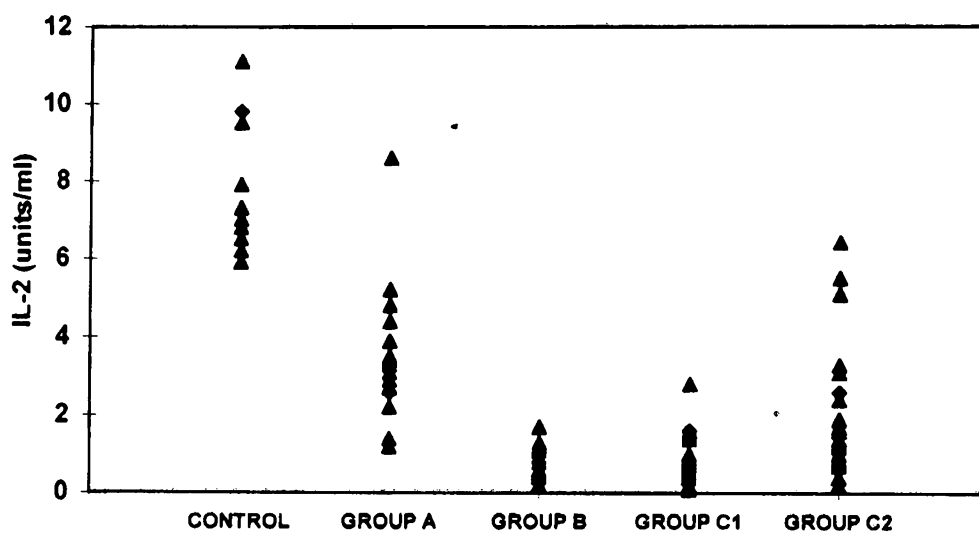
GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

IL-2 prodction by PBMCs in response to PHA stimulation



IL-2 production by PBMCs in response to MTBs stimulation



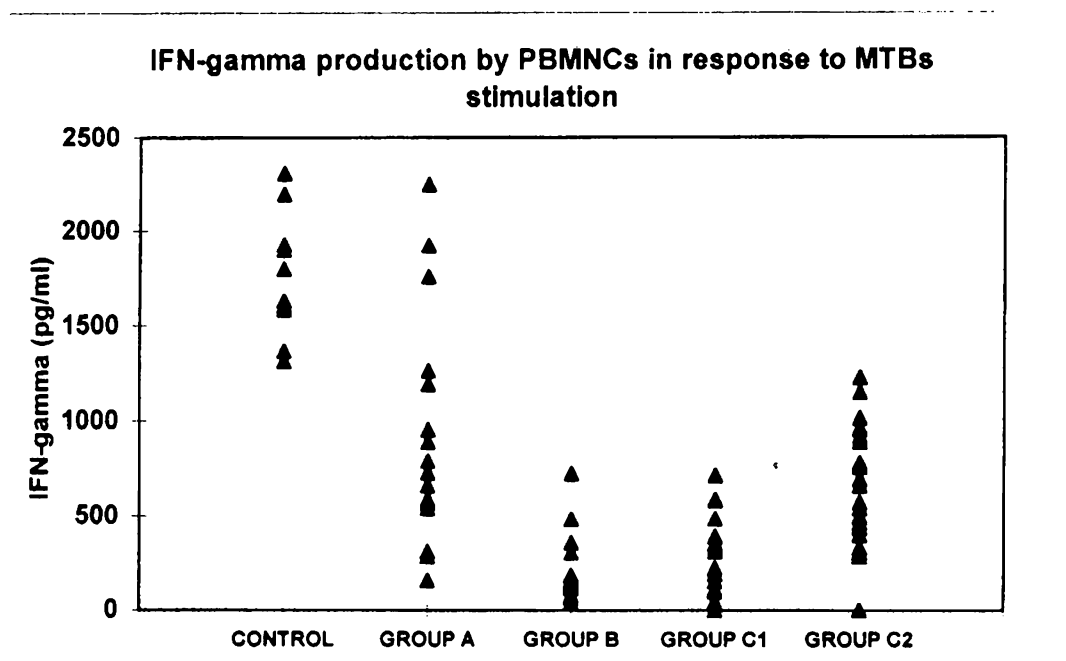
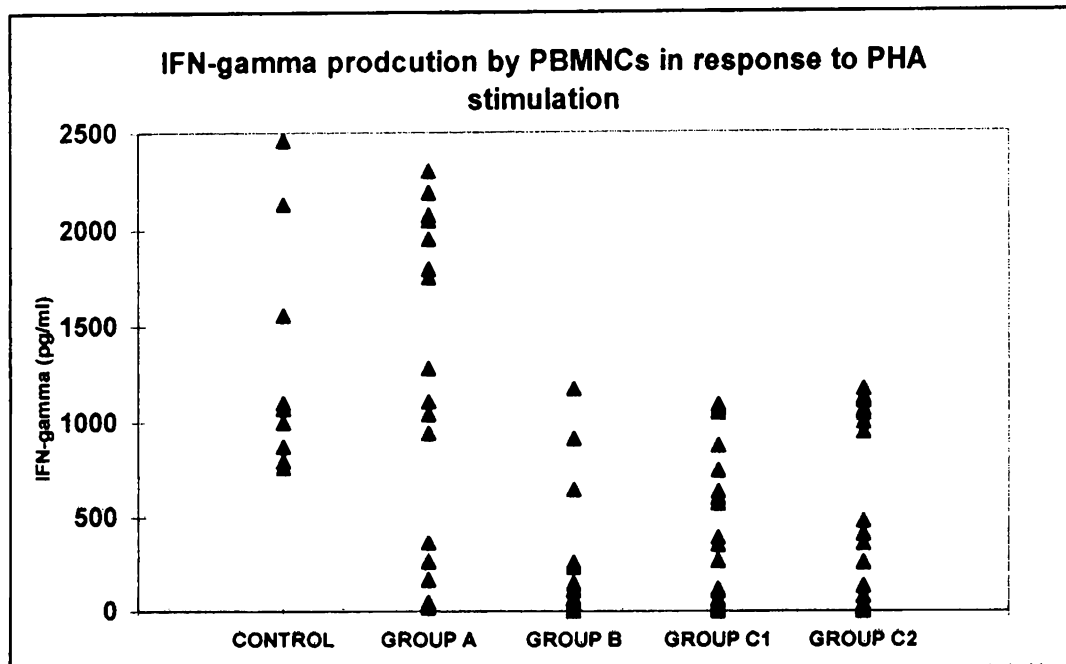
ONTROLS : Healthy volunteers.

ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.



CONTROLS : Healthy volunteers.

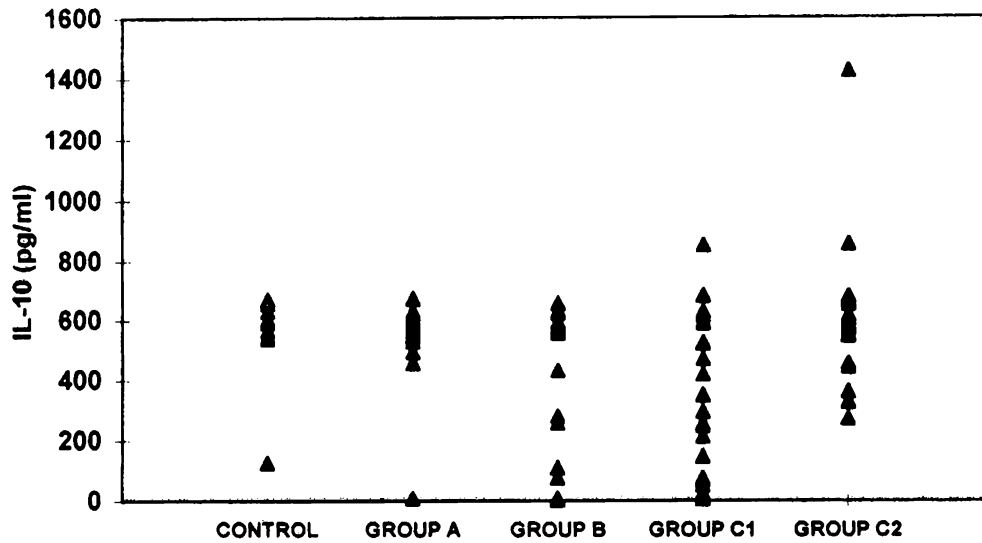
GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

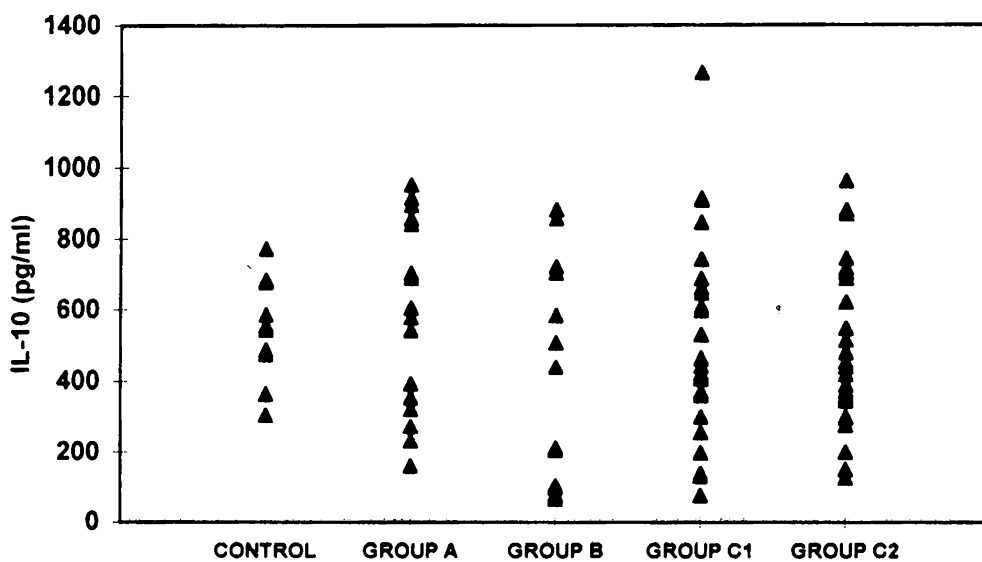
GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

IL-10 production by PBMCs in response to PHA stimulation



IL-10 production by PBMCs in response MTBs stimulation



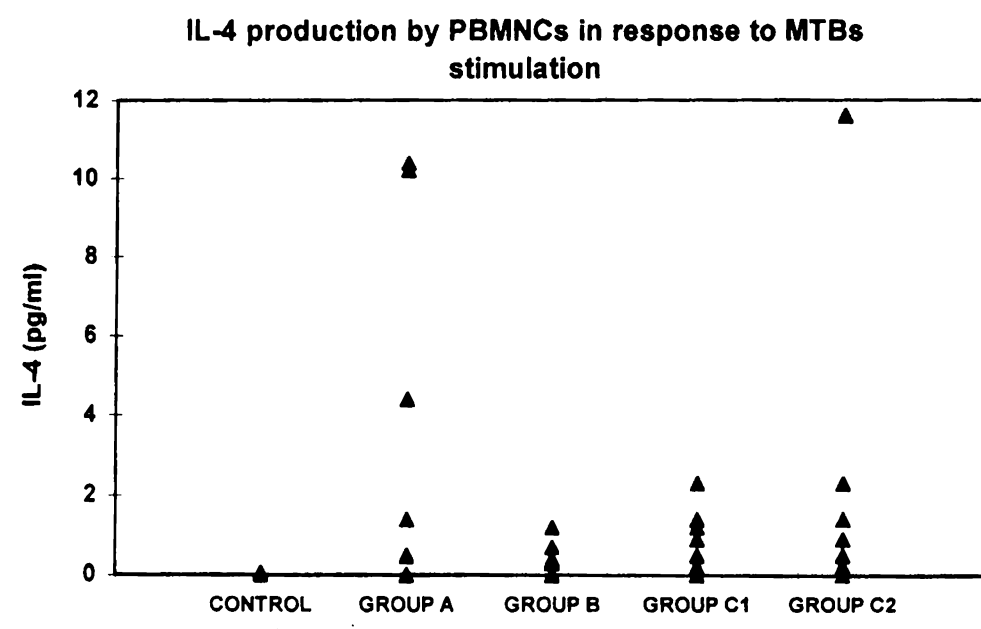
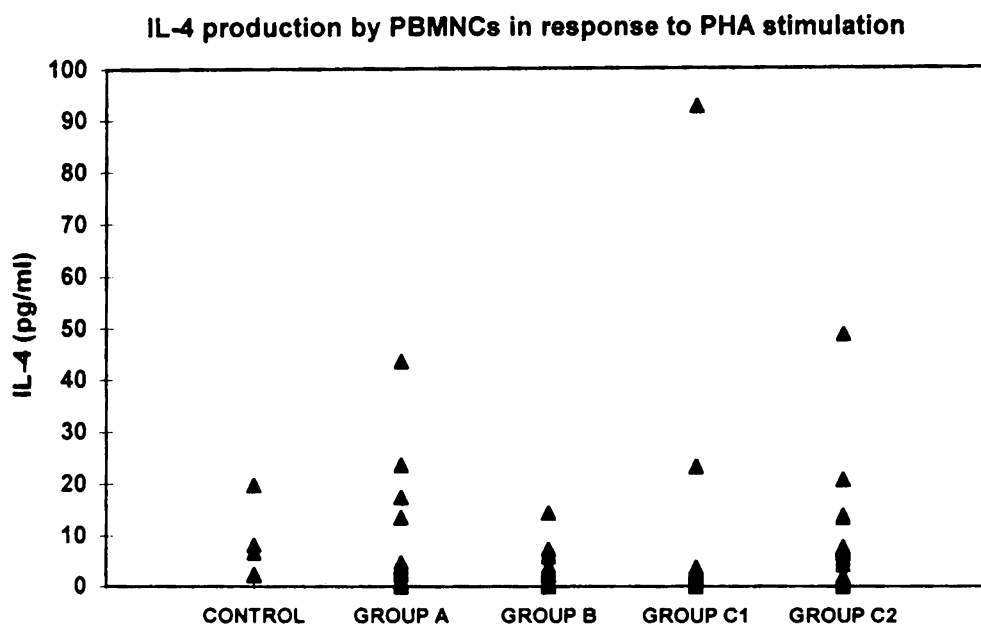
CONTROLS : Healthy volunteers.

GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.



ONTROLS : Healthy volunteers.

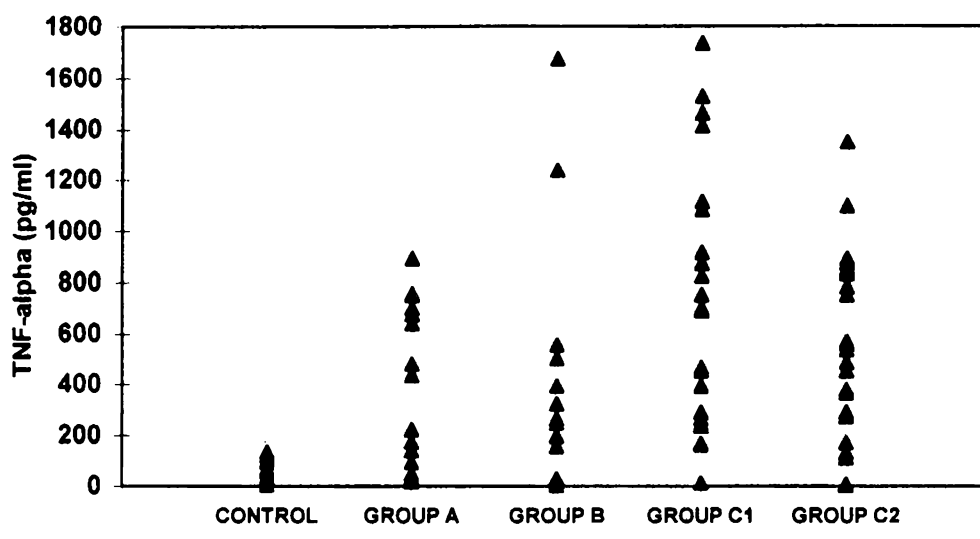
ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

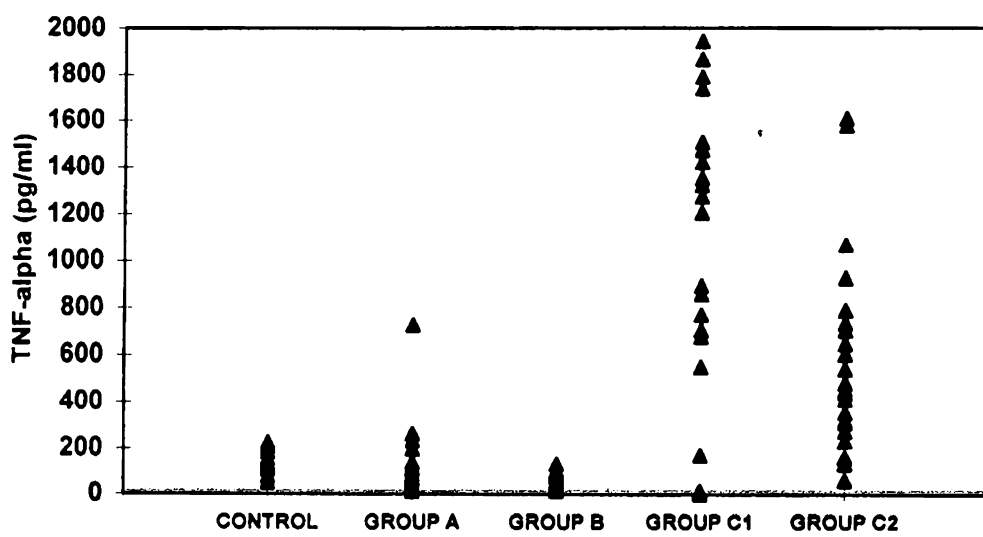
ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.

TNF-alpha production by PBMCs in response to PHA stimulation



TNF-alpha production by PBMCs in response to MTBs stimulation



ONTROLS : Healthy volunteers.

ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.



DISCUSSION

Today, as it has been for centuries, tuberculosis remains the leading cause of death in the world from infectious disease. Around 1 out of 3 people on earth is infected with the *M tuberculosis*. Tuberculosis is the cause of one fifth of all deaths in developing countries. Tuberculosis is a re-emergent problem in industrialised countries. The current global re-emergence of tuberculosis can be attributable the compromise of immune mechanisms in HIV infected individuals that leads either to reactivation of old tuberculosis infections or to increased susceptibility to new infection. In USA, the long lasting annual decline in tuberculosis cases ended abruptly in 1985, at the peak of HIV spread. Particularly ominous is the emergence of multi-drug resistant tuberculosis. Millions of tuberculosis carriers who would otherwise have escaped active tuberculosis are now developing the disease because their immune system is under attack from HIV. In Africa, with wide spread of HIV in late 1970's, exists a disastrous dual epidemic. In Asia, where HIV epidemic arrived almost a decade later and is expanding dramatically, 14% of all tuberculosis cases are expected to be attributable to HIV by the end of year 2000. Thus alliance between AIDS and tuberculosis is tuberculosis has emerged as a devastating global threat to health [340].

Prevalence of tuberculosis is highest in the conditions of poverty and overcrowding. In some of the poorest and most overcrowded cities in developing world, up to 80% of adults are infected with tuberculous bacilli [340]. Cities are also epicentres of spread of the HIV epidemic. The interaction between the tuberculosis epidemic and HIV epidemic is lethal. Tuberculosis adds to burden of HIV infected people and shortens their life expectancy, while the HIV epidemic spurs the spread of tuberculosis. Increase in active tuberculosis cases means more transmission of tuberculosis bacillus, more tuberculosis carriers, more tuberculosis in population. This becomes very important in countries like India, where 50% of adult population carries the tuberculosis germ and even more in cities like Mumbai, where population is extremely dense and people are surviving in very compromising living conditions.

Two hundred and ten clinically and radiologically confirmed pulmonary tuberculosis patients admitted to the Group of TB hospitals, Sewree, Mumbai were screened for presence of HIV infection. In this group C, maximum number of patients (41.90%) were in the age group 21-30 years followed by 30.95% patients in the age group 31-40 years. In this group male patients (80.95%) were found to outnumber female patients (19.04%) approximately in the ratio 4:1 [Table 1.10].

Tuberculosis has been reported at higher rate in adult males as compared to females [341]. Also, in a study by Styblok EDA, 40-50% of tuberculosis cases have been shown to be occurring between ages 15-49 years and rates were higher in males [342]. In social settings like that in India, where female members are not always given adequate amount of health care, and are most of the time deprived of the medical attention even in case of fatal diseases like tuberculosis, the lower rate of tuberculosis in females just be due to underreporting.

The socio-economic and cultural factors cannot be overlooked while analysing data from such a cohort. In female patients 65% were illiterate, 32.5% had primary education. Most of them (90%) were housewives. This lack of education and lack of financial independence may be one of the causes of less number of women seeking medical help. Also, 38.92% of male patients were illiterate, while 54.11% of male patients had merely primary education. Although 74.70% were employed their average monthly income never exceeded Rs. 4500/- [Table 1.11]. This cohort can be thus considered to be the representative of majority of the Indians, except the fact that these city dwellers were living in crowded households with congregate living facility. Increased risk of tuberculosis is found in such living conditions [343]. Also poverty, unemployment, illiteracy, malnourishment, are always found to be associated with increased risk of tuberculosis [344]. Poverty, unemployment and lack of education leading to high rates of addiction increase risk of tuberculosis. In our study, 47.65% of male patients were alcoholics, 18.24% smoked and 8.82% chewed tobacco. High prevalence of tuberculosis was shown among alcoholics and drug addicts [345]. Cigarette smoking was

shown to be a risk factor for pulmonary tuberculosis in young people [346]. [Table 1.11]

Our study reports an alarming 24.29% rate of HIV infection among pulmonary tuberculosis patients. In 1992, 20-67% of HIV seroprevalence among tuberculosis patients in Sub-Saharan African countries was reported by De Cock KM *et al* [347]. Similarly, 73% in Zambia [348], 32% in Tanzania [349], 48% in Zimbabwe [350] represent the caseloads as a result of dual epidemic which are overwhelming health care systems that are stretched to breaking point. In India we are experiencing a similar situation with reported rise from 2.32% in 1988 to 8.90% in 1992-1993 [351]. Bhave *et al* have reported a rise from 10.5% to 14.5% of HIV seroprevalence among tuberculosis patients in Mumbai from 1993-1994 [352]. In 1996, Rao S *et al* reported 13.89% HIV seroprevalence among tuberculosis patients [353]. In Pune, Paranjpe RS *et al* reported a rise in HIV seroprevalence from 3.2% in 1991 to 20.1% in 1996 [354].

Very low HIV seroprevalence rates are reported among other parts of the country e.g. in Lucknow, HIV seroprevalence in tuberculosis patients was 1.41% in 1997 [355], Talib *et al* reported 4.71% HIV seroprevalence in Marathwada, Maharashtra [356] in 1998. These two cities are not so densely populated as compared to metropolitan cities like Mumbai. Also Mumbai is national and international commercial centre where large number of people from various parts of the world as well as India come as labours, tourists, traders. Such a large influx of people and money, had caused prostitution to prosper and thus the promiscuous heterosexual contact becomes the main cause of spread of HIV.

All the HIV infected patients included in our study were very young, majority of them in the third or fourth decade of their lives. In the patients co-infected with HIV and tuberculosis (Group C1), 54.76% of the males were in age group 21-30 years and 35.71% in the age group of 31-40 years. Also in females, 66.66% were in the age group 21-30 years and 33.33% were in the age group 31-40 years which the sexually most active age group [Table 1.12].

Epidemiological studies conducted world wide also show similar trends. AIDS is reported to be the second leading cause of death in the United States among people aged 25-44 years [357]. In Zaire, the largest number of HIV seropositives were in the age group of 16-29 years [358]. In a study conducted in Madras, in 1993, 88.3% of HIV seropositives belonged to the age group if 21-40 years [359]. In Upper Assam, 48.4% of HIV infected cases belonged to the age group 21-30 years, followed by 31.3% in the age group of 31-40 years [360]. In Punjab, peak age incidence of HIV seropositivity was found in the third decade of life [361].

The reason for high seroprevalence among this group could be the fact that subjects belonging to this group are sexually most active and being younger have higher rate of multiple sex partners. Also young women are more susceptible to genital infections [362].

Sexual promiscuity among HIV infected patients or their sexual partners was found to be the single most important risk factor for acquiring infection among pulmonary tuberculosis patients who tested HIV seropositive in Group C1 (42%) as well as Group A (81.25%) and Group B (78.57%). World wide, more than 75% of all adult HIV infections have resulted from heterosexual intercourse [363].

In our study, transmission through blood [Group C1 (7.84%), Group A (6.25%), Group B (7014%)] were also found [Tables 1.14, 1.5, 1.9].

All the other groups taken for comparison viz. Group A, Group B, Controls and strictly in the same age group and socio-economic strata as that of Group C1. This can be seen from their sex and age wise distribution and demographic characters described in the results [Table 1.2,1.4,1.7].

AIDS syndrome cases free of tuberculosis infection in our study, Group B, showed various other opportunistic infections; oral candidiasis occurred in maximum (57.14%) number of patients, followed by cryptococcal meningitis (14.29%), gastroenteritis (14.29%), *Herpes simplex I* (14.29%). [Table 1.8]. Oral and pharyngeal candidiasis is the most frequently found infection among HIV seropositive patients [364]. In United States and Europe, 95% of heterosexual seropositive men are also infected with *Herpes simplex 1* or 2 or

both [365]. Cryptococcosis in AIDS patients in United States range from 6 to 12% [366]. Incidence rate in our study is differing from general trend. This may be due to small sample size included in our study.

Lymphocyte proliferation and cytokine production:

In the present study, all the controls were healthy volunteers who were in the same age group and belonging to both the sex similar to the patients included in the study.

PBMNCs of all the controls showed highest proliferation and IL-2 and IFN- γ production in response to PHA as well as in response to MTBs, where as there was no difference in the production of IL-4 as well as IL-10 both in response to PHA and MTBs. However, only 2 of the controls produced detectable IL-4 in response to MTBs. This may be because PHA is a T cell mitogen capable of producing full range of cytokine response. Mycobacterial antigens are known to induce a predominant TH1 response [367].

It is possible that normal individuals are exposed to environmental mycobacterial antigens that are cross reactive with antigens used in this study as stimuli. There are also different cells such as CD4⁺CD8⁻ and CD8⁺ $\alpha\beta$ CD1 restricted T cells from normal donors, that have been shown to produce IFN- γ in response to mycolic acids and lipoglycans [368]. Furthermore human $\gamma\delta$ T cells from tuberculin positive donors were found to produce IFN- α and IL-2 in response to tuberculosis antigen [275,369]

Asymptomatic HIV infected patients (Group A):

Asymptomatic HIV infected patients showed significant decrease in proliferation as well as IL-2, IFN- γ production in response to PHA as well as MTBs as compared to controls.

Pinching AJ found abnormalities in CMI including T lymphopenia, T helper lymphopenia, decrease Th/Ts ratio, monocyte functional defects and anergy [370]. Marijke TL have shown low T cell reactivity to CD3 monoclonal antibody among HIV seropositive patients and also have concluded that T cell function *in vitro* is of value for staging HIV infection [371]. Similar results

were obtained by Lucey *et al.* [372]. T lymphocyte proliferation has been shown to be decreased in response to ant-CD3 monoclonal antibody [299].

T helper functions in terms of IL-2 and IFN- γ production has been shown to be decreased years before CD4⁺ cell number was critically reduced in asymptomatic human immunodeficiency virus infected individuals [184]. Antigen specific T cell response was lost during asymptomatic phase of HIV infection [180,373]. Also, some of the asymptomatic patients failed to respond to even PHA along with HLA alloantigen, influenza virus, tetanus toxoid in terms of proliferation and IL-2 production in a study carried out by Clerici M [181].

In our study, proliferation and IL-2 , IFN- γ production in response to PHA stimulation was found to be decreased as compared to controls. The responses to MTBs stimulation are further reduced. The reasons for such reduced cell mediated responses could be :

1. CD4 cells could be infected without being killed and such infected cells would be non functional [374]. However, this possibility appears to be unlikely because frequency of infected leukocytes is less than $1/10^3$ [375].
2. Autoantibodies binding to human Class II MHC molecules might block interactions between CD4 and APC required for antigen specific responses. These antibodies are found in sera of AIDS patients [181] as well as sera of asymptomatic patients [109].
3. Since HIV infects CD4⁺ monocytes, macrophages defective antigen presenting function is proposed to account for early defect in T lymphocyte function [120].
4. Proliferative unresponsiveness to specific antigens can be accounted for by the observed disturbed ratio of naive and memory T cells since HIV preferentially deletes memory T cells [181].

IL-4 and IL-10 production in response to PHA as well as MTBs stimulation was not found to be any different from that observed in controls. Clerici M *et al* had been able to show increase in IL-4 and IL-10 production in response to mitogens [181], but many other studies have failed to repeat this observation. The production of both IL-4 and IL-10 in response to PHA by PBMNCs of HIV

infected patients was not increased, but rather was decreased in comparison with IL-4 and IL-10 production by randomly matched HIV seronegative healthy donors [188,376,377]. More importantly, PBMCs from HIV infected patients with reduced numbers of circulating CD4⁺T cells produced lower amounts of both IL-4 and IL-10 in comparison with HIV infected patients showing quite normal or only slightly decreased values of circulating CD4⁺T cells [188].

TNF- α production in response to PHA or MTBs stimulation was similar to that found in the controls. Some studies have shown increase in TNF- α production in PBMCs of HIV seropositive patients both spontaneously and in response to cell activation [378]. Our observation is in agreement with the reports by Wallis RS *et al* [328] and Miller F [379] that found similar TNF- α production in HIV patients without opportunistic infections as compared to healthy controls.

AIDS cases (Group B) patients without tuberculosis:

All the 14 AIDS patients having opportunistic infection other than tuberculosis, showed further decrease in proliferation, IL-2 and IFN- γ production in response to PHA as compared to the asymptomatic patients as well as controls ($p < 0.01$). Proliferation, IL-2 and IFN- γ production in response to MTBs was further reduced and was the lowest in this group in comparison with all the group studied ($p < 0.01$). These observations were predictable as the patients were having atleast one opportunistic infection other than tuberculosis. The above results may be reflection of quantitative absence of responsive CD4⁺T lymphocytes in addition to qualitative dysregulation.

The probable cause of loss of T helper activity may be due to inhibitory signals mediated by HIV viral or regulatory gene products [96] selective infection and destruction of memory T cells, syncytia formation between infected and uninfected cells [380], immune suppression by HIV products [181]. However, we did not find concomitant increase in IL-4 or IL-10 even in response to PHA. The IL-4 and IL-10 production in response to PHA or MTBs stimulation was similar to that in controls. Some workers have found increase in IL-4 production in response to PHA [181] while others have not observed

such an increase in these TH2 type of response [188,376,377]. The possible reasons could be that IL-4 is secreted is barely detectable in *in vitro* culture. MTBs stimulates production of TH1 type of cytokines and not TH2 type of cytokines [367,381]. The average amount of IL-4 produced by AIDS patients is higher than the controls. The reason that it could not reach significant values as compared to controls could be

1. Inability of our experimental system to detect it.
2. The sample size being small, the difference did not reach statistically significant values.

Present published data is contradictory regarding the level of IL-10 production PBMNCs from patients with HIV infection and contribution of IL-10 to HIV immunopathogenesis is still unclear [186,382,383,384,385]. Chemini *et al* [384] did not find any differences between IL-10 levels in HIV infected and controls. Cultures stimulated by PHA in AIDS patients in our study produced slightly decreased although not significantly different amounts of IL-10 as compared to controls. Several cell types produced IL-10 including lymphocytes and monocytes. Thus reduced number of CD4⁺ lymphocytes might be important for the lower IL-10 production. There are discrepant reports concerning IL-10 production in PBMNCs culture after PHA stimulation during HIV infection [182,186,384,385,386,387,388] and conflicting results may partly due to different proportions of CD4⁺ lymphocytes in PBMNCs cultures in different studies.

After the highly provocative model of TH1-TH2 shift by Clerici and Shearer [182] an avalanche of information has accumulated on the role of cytokines during HIV infection and conflicting results have been generated [188,389,390]. Such differences are observed may be due to different cell systems used, different patient population studies and the detection assays and also the complexity of the cytokine network and heterogeneity of the AIDS population.

In vitro stimulation of PBMNCs in short term cultures in our study showed a marked decrease in TH1 responses (proliferation, IL-2 and IFN- γ production], but did not show simultaneous increase in the TH2 (IL-4,IL-10) type of

responses which were either maintained in most of the asymptomatic as well as AIDS syndrome cases or slightly reduced.

TNF- α production was found to be slightly decreased as compared to controls in response to MTBs stimulation. This may be the direct result of decrease in cell producing TNF- α as a result of CD4⁺ cell destruction as well as monocyte depletion due to HIV replication. Wallis RS *et al* also showed reduced TNF- α production in AIDS patients without tuberculosis [328,379].

HIV seronegative pulmonary tuberculosis patients (Group C2):

The 25 patients included in this group showed significantly reduced lymphocyte proliferation as well as IL-2 and IFN- γ production as compared to healthy volunteers. IL-4 and IL-10 production in response to PHA as well as MTBs were similar to that in controls.

The results of present study confirm the previous reports showing that patients with active tuberculosis have altered immune responsiveness to mycobacterial antigen. IL-2 and IFN- γ expression has shown to be depressed when PBMCs were stimulated with heat killed *M. tuberculosis* [329], live H₃₇Rv [325], live H₃₇Ra [326], or PPD [391]. In all the above reports depressed expression of IL-2, IFN- γ was not found to be associated with increased IL-4, IL-10 (TH2). In another report IFN- γ production was shown to be maintained without concomitant increase in IL-4 but showed decreased proliferation to PPD [392]. This decrease in the protective type of response as indicated by proliferation, IL-2 and IFN- γ production, does not seem to be the result of crossregulation by suppressive (IL-4, IL-10) immune response. These responses may be depressed by mechanisms independent of TH2 cells, such as dysregulation of co-stimulatory molecules, anergized TH1 cells and enhanced production of immunosuppressive monokines such as TFG- β [289].

IFN- γ is thought to have protective role in anti-microbial defences. Presence of IL-2 is required for optimal IFN- γ production *in vitro* [376]. Active tuberculosis is associated with transient CD4⁺T lymphocyte depression in

patients even without HIV infection [393,394]. Patients having low number of CD4⁺T cells have decreased number of antigen specific cells capable of responding to stimulation with antigen. This decreased proliferative response on stimulation with mitogen also. In earlier studies, measuring IFN- γ production from patients of tuberculosis, it was shown that IFN- γ was deficient in some patients [395,396]. Defect in IL-12 was proposed to account for lack of IFN- γ in case of multi-drug resistant tuberculosis patients with low CD4⁺T cell counts [397]. There is a correlation between IFN- γ production by PBMNCs with different clinical manifestations of the disease [398]. Because the immune response to tuberculosis is compartmentalized [399] IFN- γ , IL-2 production in lungs or lymphnodes in patients with tuberculosis may not be similar as that shown by PBMNCs. Stimulation of the antigen specific CD4⁺T cells in tissue spaces may alter their distribution and properties in peripheral blood [400]. The sequestration of antigen specific T cells in tissue can result in further decrease in IFN- γ and IL-2 production as well as proliferation of lymphocytes in response to MTBs.

After *in vitro* stimulation with live virulent *M tuberculosis*, cells from tuberculosis patients expressed levels of IL-2 and IFN- γ mRNA lower than those of sensitized controls [325].

TNF- α production was very high as compared to controls. High serum levels of TNF- α as well as high production by PBMNCs has been shown in tuberculosis patients. This is due to its production by monocytes and T cells in response to stimulation with *Mycobacterium tuberculosis* which is an essential step in pathogenesis. However excess production of TNF- α is always found to correlate with the cachexia, weight loss, fever and morbidity associated with active pulmonary tuberculosis.

HIV and tuberculosis coinfectd group (Group C1):

All the 25 patients in this group showed similar proliferation as well as IL-2, IFN- γ , IL-4, IL-10 production as compared to AIDS syndrome cases without

tuberculosis (Group B). Proliferation, IL-2 and IFN- γ production in response to PHA and MTBs was significantly reduced as compared to asymptomatic patients (Group A) ($p < 0.01$) and controls ($p < 0.01$) and tuberculosis patients without HIV infection (Group C2) ($p < 0.01$). Although there is no apparent difference between the two groups, if we see these results in the light of the fact that tuberculosis is generally considered to be the first infection to occur [when CD4⁺ T cell count is still relatively high (350 cells/mm³)] worldwide [340] and more so in country like India where almost every second adult is infected with tuberculosis, it can be seen that tuberculosis has caused major functional damage to the immune system before CD4⁺ count deplete. This might add to faster progression of the disease. Although we do not have CD4⁺ counts of our patients to substantiate this speculation previous studies have shown that survival is shortened in HIV infected persons following the diagnosis of tuberculosis, when compared to CD4 matched controls without tuberculosis [401]. Similarly among all patients with tuberculosis mortality is increased fourfold in those who are HIV infected [305]. Previous study in the department of Virology, Haffkine Institute found almost 40% death rate among HIV-TB co-infected patients within 6-18 months of detection of HIV serostatus [Dr. R.A. Deshmukh, Personal Communication].

Tuberculosis must be facilitating HIV induced immunological deterioration as

1. Active tuberculosis is associated with transient CD4⁺T lymphocyte depression even in patients without HIV infection [396,397,398].
2. Decrease in IL-2 and IFN- γ production is seen in pulmonary tuberculosis patients without HIV infection also. Thus functional anergy of protective responses caused because of tuberculosis may further damage the immune system.
3. Mycobacteria and mycobacterial products stimulate production of TNF- α by macrophages and T cells [235,276,328,]. TNF- α in return promotes HIV expression by latently infected cells [102,103,404]. This probably occurs *in vivo* in the cellular interaction within granulomas and in circulation.

It has been shown that HIV replication in mononuclear phagocyte cell lines exposed *in vitro* to *M. tuberculosis* may be increased due to generation of TNF- α [310]. Correlation between *M. tuberculosis* induced HIV production and secretion of TNF- α has also been observed [309,310]. Enhanced susceptibility of peripheral blood monocytes from patients with tuberculosis and TNF- α could be associated with this effect has been shown by Toossi *et al* [319]. Ability of TNF- α to promote HIV replication has been proved in several monocytic and lymphocytic cell lines [152,155,405] as well as monocytes and lymphocytes recovered from peripheral blood [312,406,107]. The upregulation acts through the activation of NF- κ B [312,408].

The average TNF- α production by PBMCs of the co-infected patients was found to be 10 times more than controls and 2 times more than HIV negative tuberculosis patients. These results are similar to those found in other studies by Garrit *et al* have shown that lymphocytes in the pleural fluid of tuberculosis patients are more susceptible to HIV infection without any prior *in vitro* activation and could support a consistent replication [409]. Thus tuberculosis generated a microenvironment by chronic activation of immune responses with increased TNF- α , decreased IFN- γ , IL-2 at the site of mycobacterial infection and thus support enhanced replication of HIV at the site of mycobacterial infection [409]. This has been confirmed by *in vivo* observation that HIV plasma viral load increased 5-160 fold during acute phase of tuberculosis and that *in vitro* tuberculosis mediated viral production likely occurs through antigen specific activation and infection of responding cells [316].

The cytokines induced during the immune response to mycobacterial antigen result in activation of latently infected cells and induction of virus expression. Also they cause increase in replication of virus from infection of newly activated cells. These factors could participate in an increased viral burden subsequent to the development of tuberculosis and might partially explain the HIV disease progression after tuberculosis.

The present investigation support to the speculation of association between immune dysregulation and increased mortality between HIV and tuberculosis

co-infected patients. Highly increased production of TNF- α is responsible for high fever, weight loss and acceleration of HIV disease in co-infected patients.

The hypothesis is supported by observation of Pape *et al* that preventive therapy of tuberculosis appears to delay the onset of other HIV related disease as well as preventing tuberculosis [410].

Correlation between proliferation and cytokine production.

Since the proliferative responses and cytokine production (IL-2, IFN- γ , IL-4, IL-10, TNF- α) have been studied simultaneously using mitogen and MTBs activated PBMNCs from patients as well as controls we analysed these parameters for correlation between them.

A good correlation existed between lymphocyte proliferation and IL-2 production as well as lymphocyte proliferation and IFN- γ production in all the patients as well as controls.

It can be seen from the graphs [pg 107-121] that although the individual parameters of IL-2, IFN- γ and proliferation are significantly impaired in tuberculosis and HIV infected patients, those patients who show lymphocyte proliferation are also capable of IL-2 and IFN- γ production, thus emphasising functional efficiency of these lymphocytes.

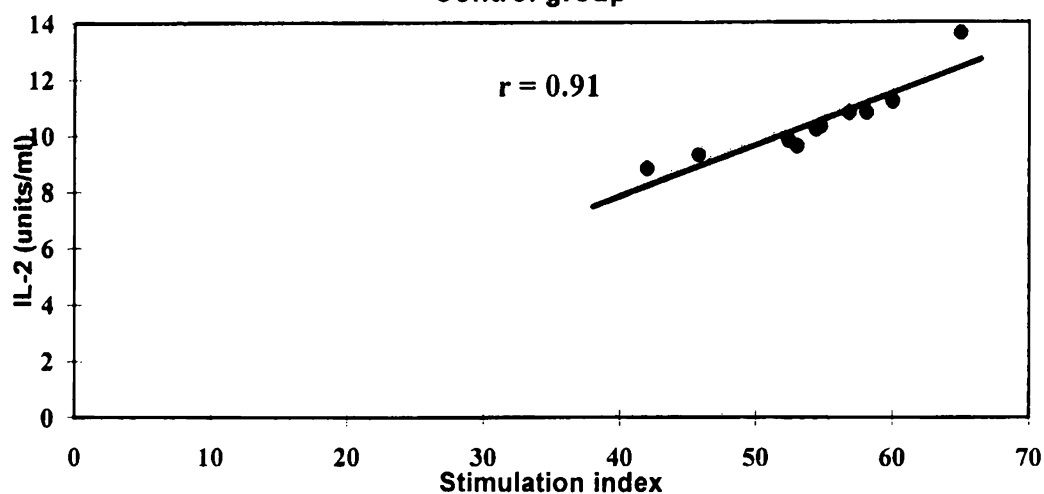
Also correlation between the levels of IL-2 and IFN- γ secretion was assessed by determination of correlation coefficient. There existed a statistically significant correlation between IL-2 and IFN- γ production in HIV and tuberculosis patients as well as controls. This might be due to the fact that IFN- γ is dependent on IL-2 secretion for its optimal production.

However, there was no correlation found between IL-4, IL-10 and TNF- α production and proliferation, IL-2 or IFN- γ secretion. Thus the decrease in TH1 type of response cannot be attributed to increase in the suppressive TH2 (IL-4 and IL-10) responses.

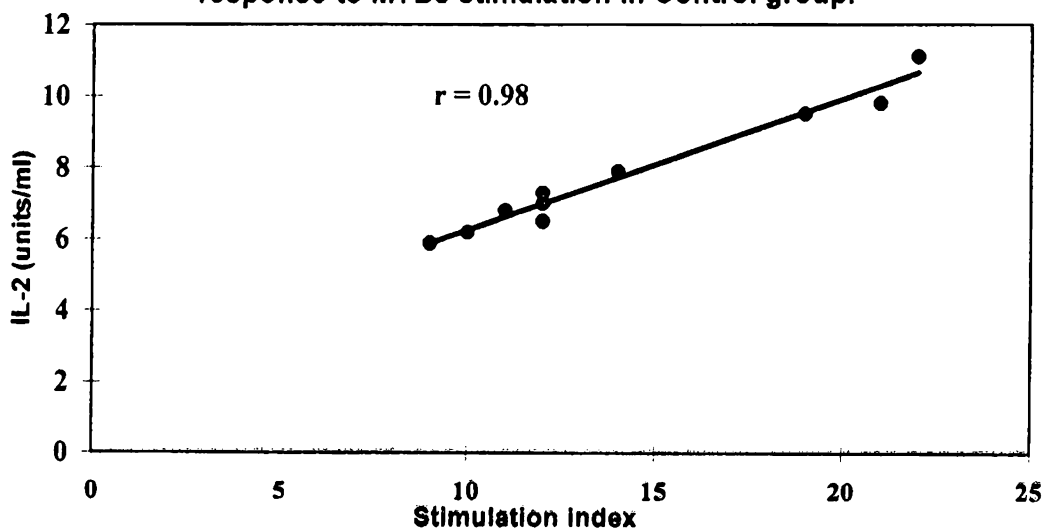
Therefore, it appears on the whole that HIV and tuberculosis patients showed impairment in T helper type 1 functions viz. proliferation, IL-2 and IFN- γ

production which is not due to simultaneous increase in TH2 type (IL-4 and IL-10) responses but numerical absence or functional silence (anergy) of CD4⁺T lymphocytes in patients infected with HIV or tuberculosis or both together.

**Correlation between Proliferation and IL-2 production by
PBMNCs in response to PHA stimulation in
Control group**



**Correlation between proliferation and IL-2 production in
response to MTBs stimulation in Control group.**



ONTROLS : Healthy volunteers.

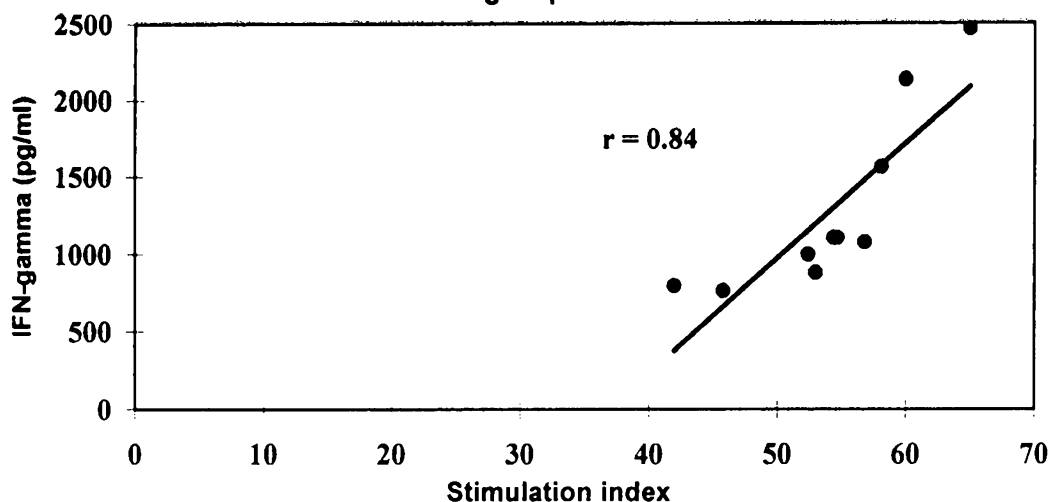
ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

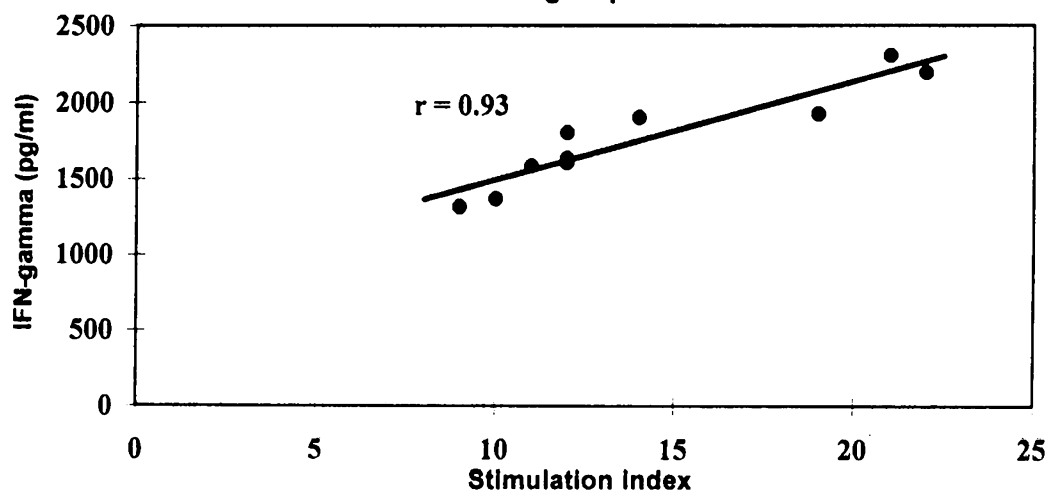
ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.

**Correlation between proliferation and IFN-gamma secretion
by PBMCs in response to PHA stimulation in Control
group.**



**Correlation between proliferation and IFN-gamma
production by PBMCs in response to MTBs stimulation in
Control group**



ONTROLS : Healthy volunteers.

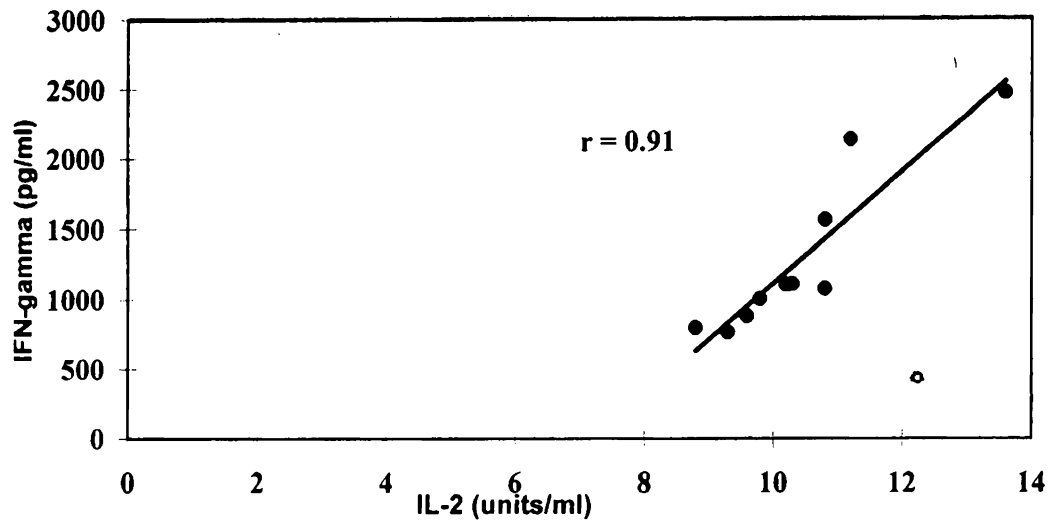
ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

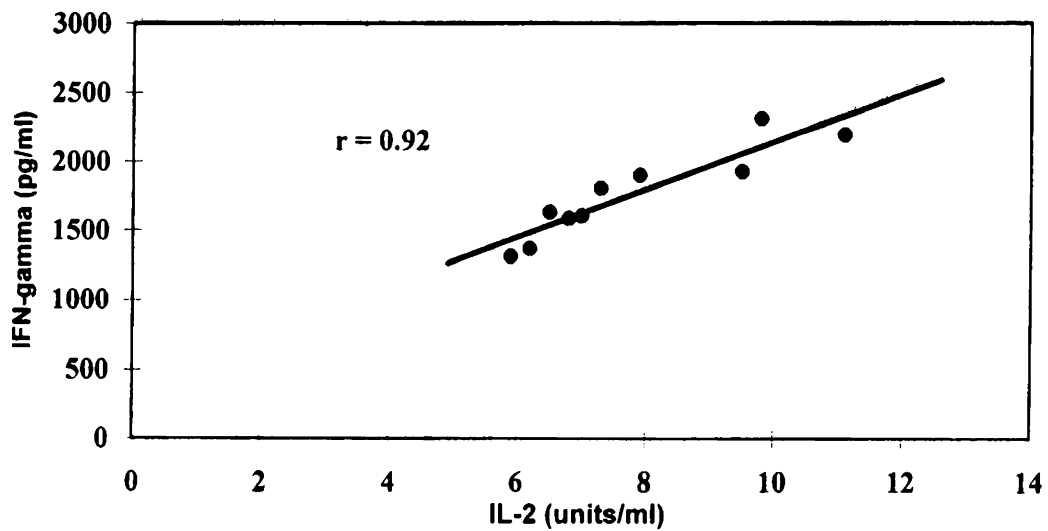
ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between IL-2 and IFN gamma secretion by PBMNCs in response to PHA stimulation in controls.



Correlation between IL-2 and IFN-gamma secretion in response to MTBs stimulation in control group



ONTROLS : Healthy volunteers.

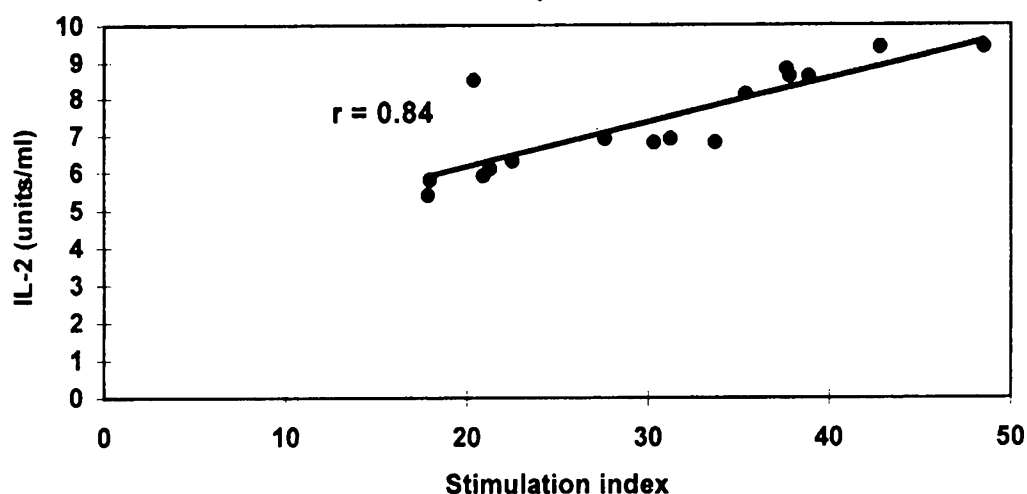
ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

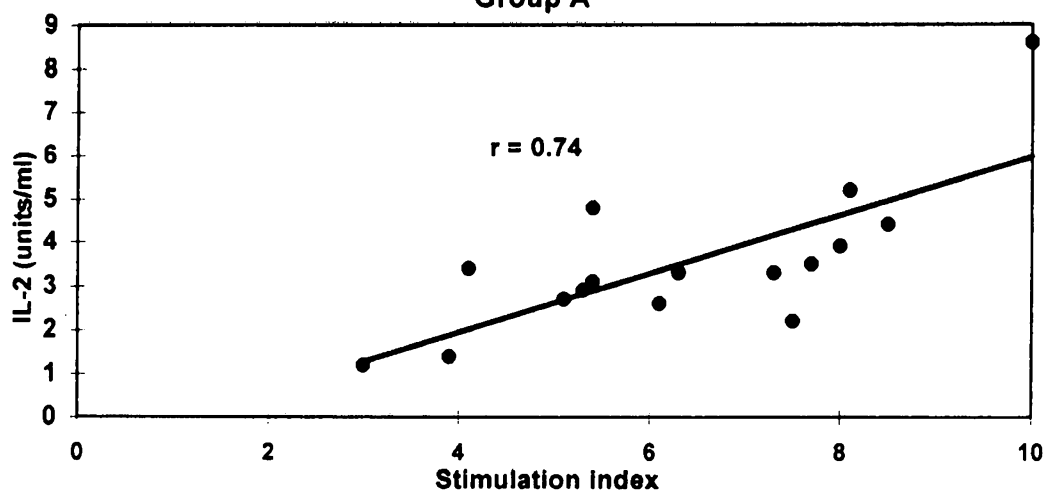
ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.

**Correlation between proliferation and IL-2 secretion by
PBMNCs in response to PHA stimulation in
Group A.**



**Correlation between proliferation and IL-2 production by
PBMNCs in response to MTBs stimulation in
Group A**



CONTROLS : Healthy volunteers.

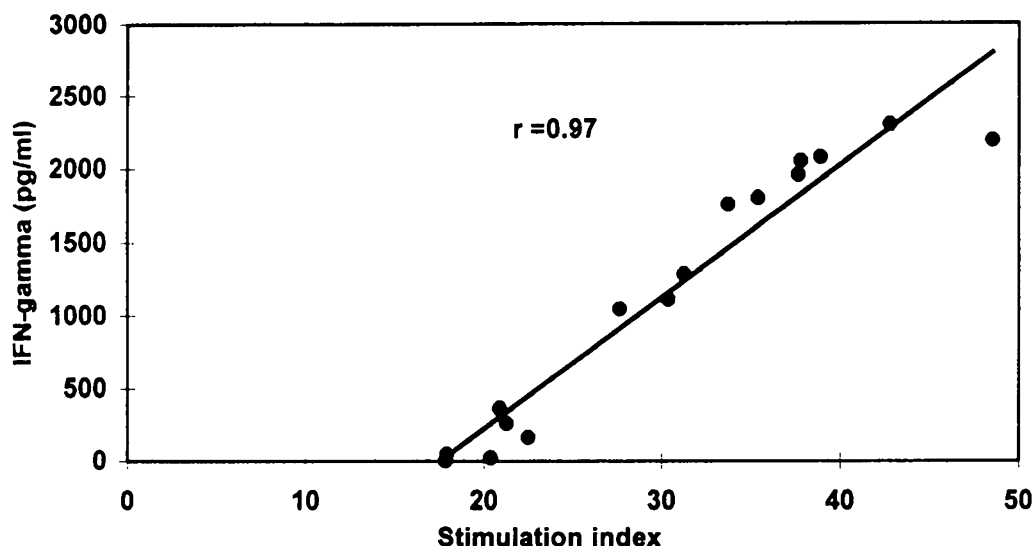
GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

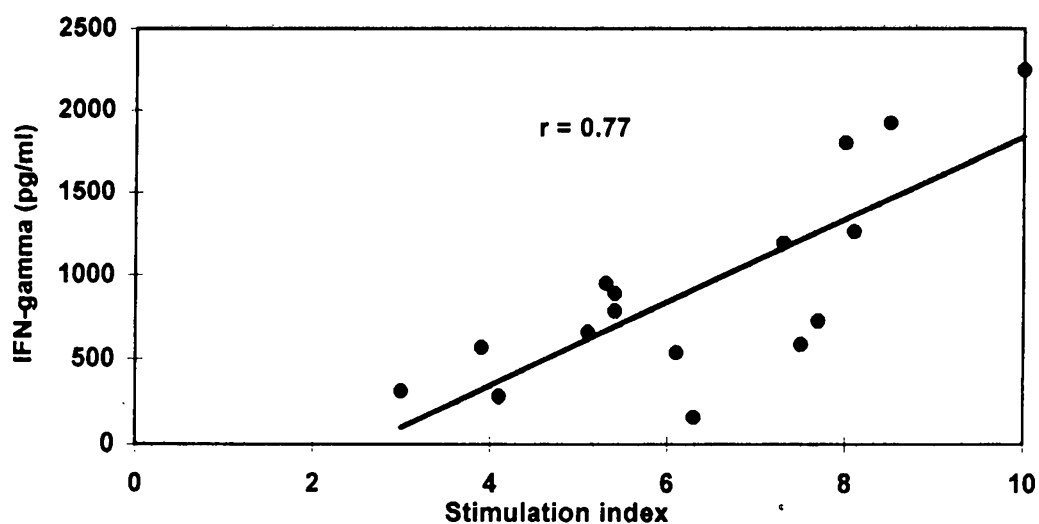
GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between proliferation and IFN-gamma production by PBMNCs in response to PHA stimulation in group A



Correlation between proliferation and IFN-gamma production by PBMNCs in response to MTBs stimulation in Group A.



ONTROLS : Healthy volunteers.

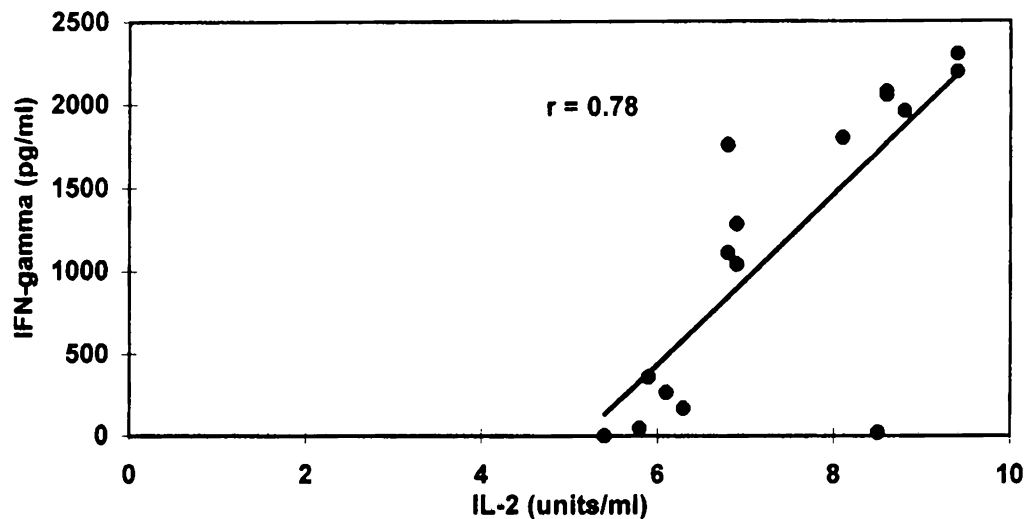
GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

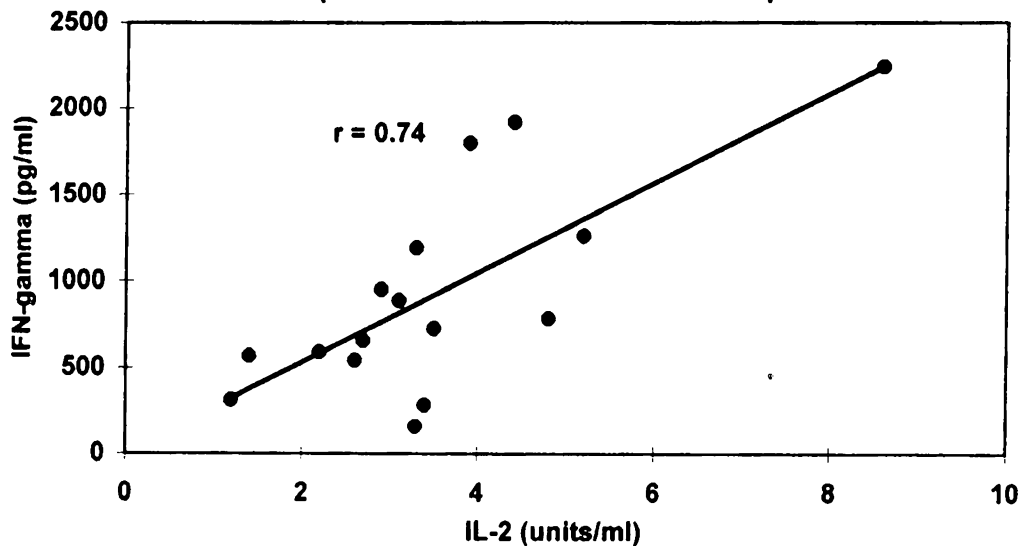
GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between IL-2 and IFN-gamma production in response to PHA stimulation in Group A



Correlation between IL-2 and IFN-gamma secretion by PBMNCs in response to MTBs stimulation in Group A



ONTROLS : Healthy volunteers.

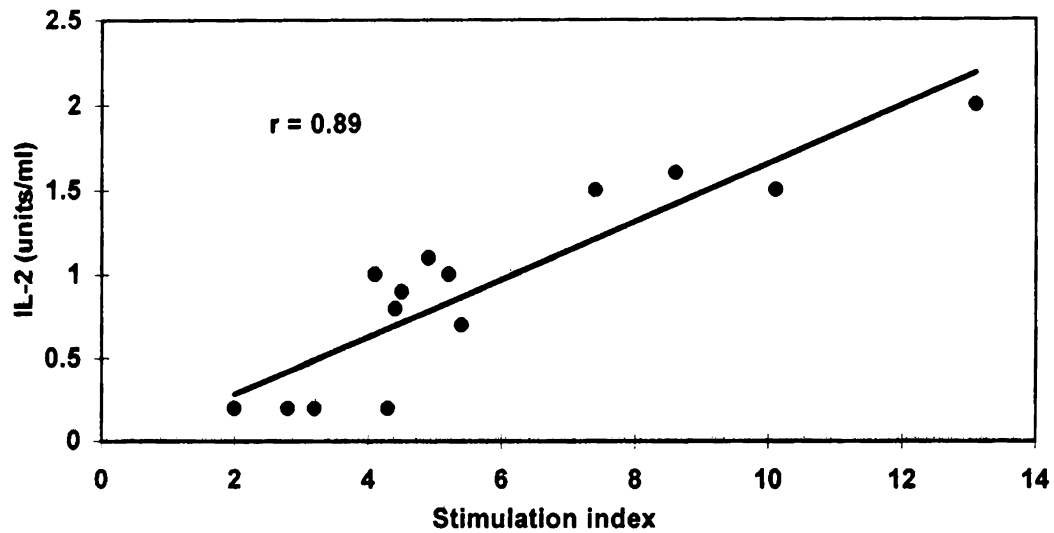
ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

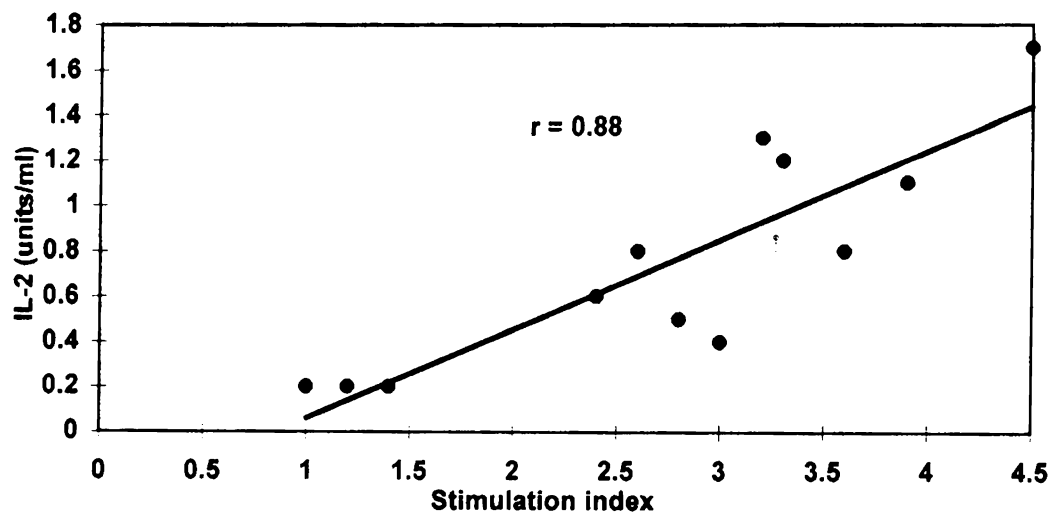
ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between proliferation and IL-2 production by PBMNCs in response to PHA stimulation in group B



Correlation between proliferation and IL-2 production by PBMNCs in response to MTBs stimulation in Group B



CONTROLS : Healthy volunteers.

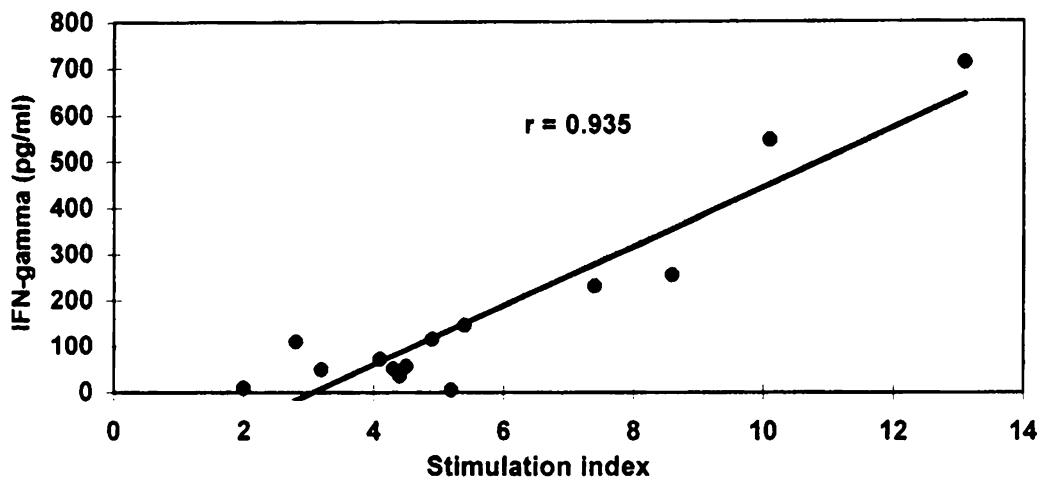
GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

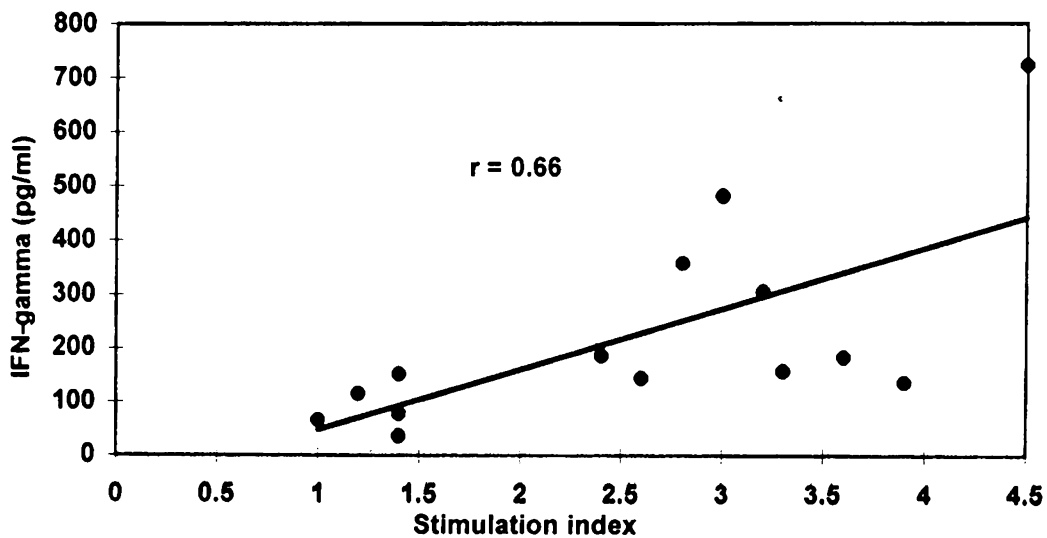
GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between proliferation and IFN-gamma production by PBMCs in response to PHA stimulation in group B.

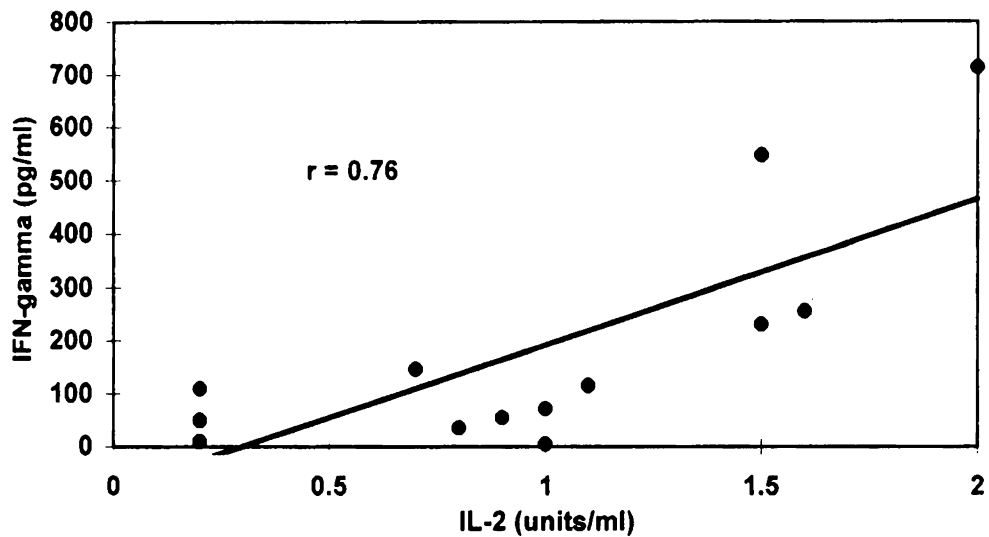


Correlation between proliferation and IFN-gamma production in response to MTBs stimulation in group B

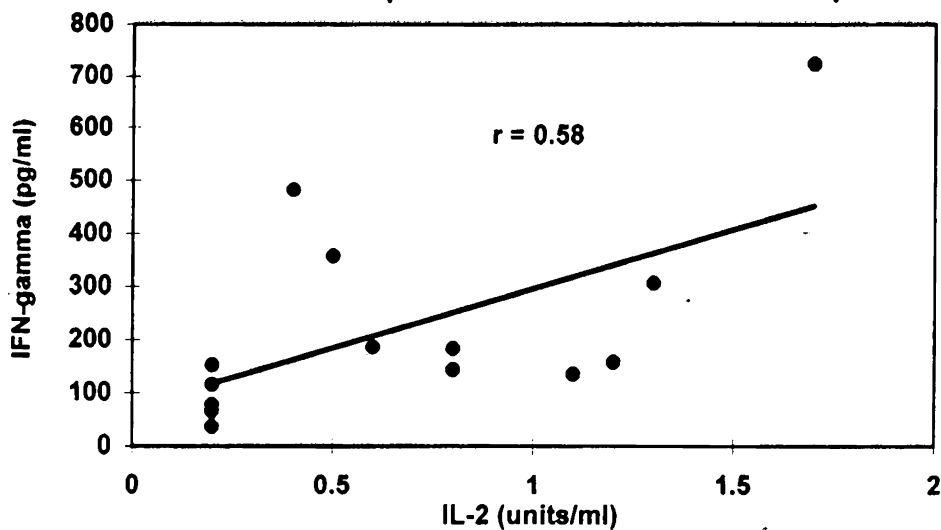


- ONTROLS : Healthy volunteers.
 ROUP A : HIV seropositive asymptomatic cases.
 ROUP B : AIDS patients having opportunistic infections other than tuberculosis
 ROUP C1 : HIV seropositive pulmonary tuberculosis patients.
 ROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between IL-2 and IFN-gamma secretion by PBMNCs in response to PHA in group B.



Correlation between IL-2 and IFN-gamma secretion by PBMNCs in response MTBs stimulation in Group B



CONTROLS : Healthy volunteers.

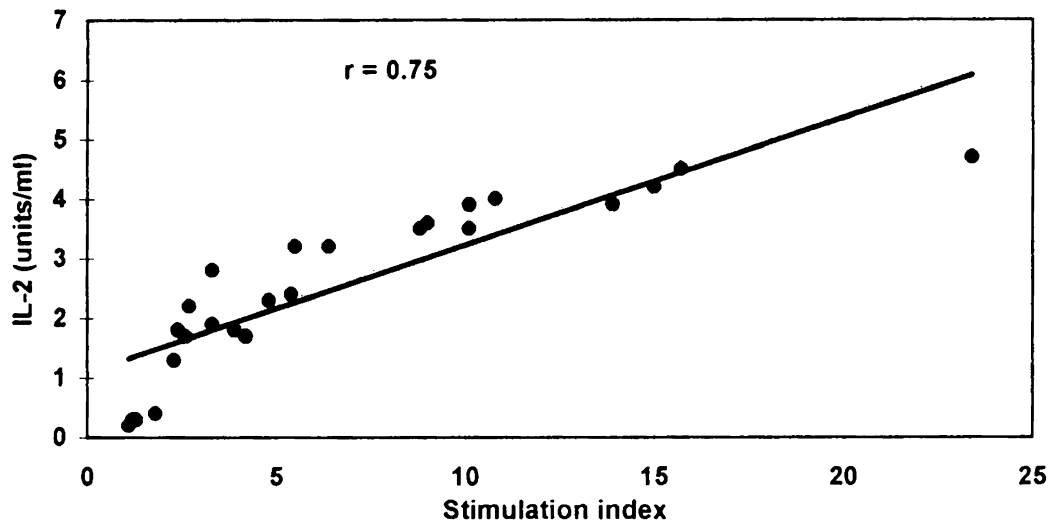
GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

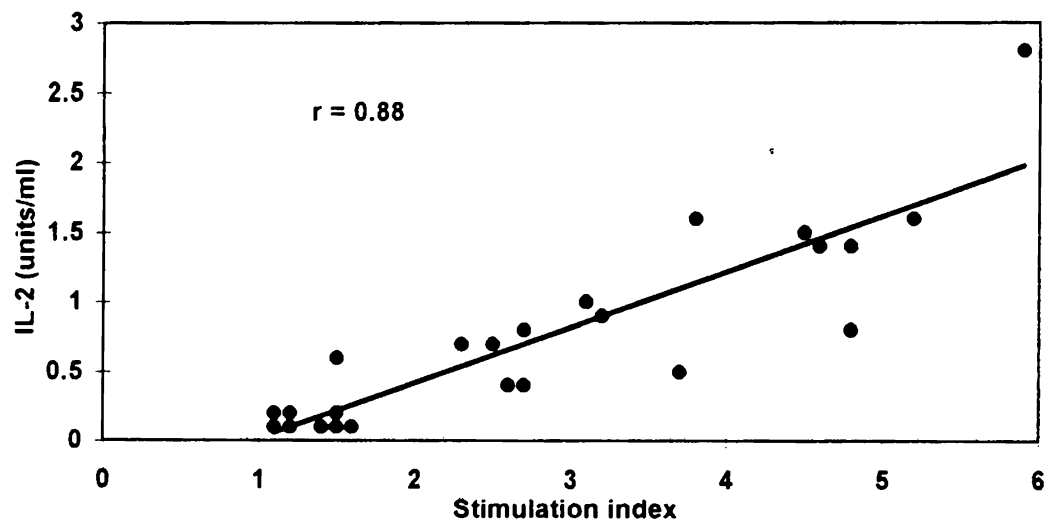
GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between proliferation and IL-2 production by PBMNCs in response to PHA stimulation in Group C1.



Correlation between proliferation and IL-2 production by PBMNCs in response to MTBs stimulation in group C1



ONTROLS : Healthy volunteers.

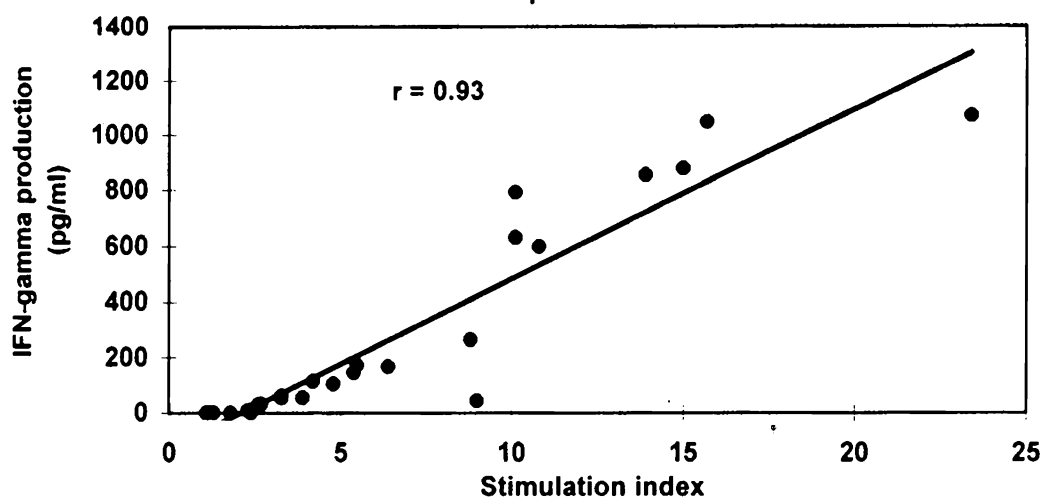
ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

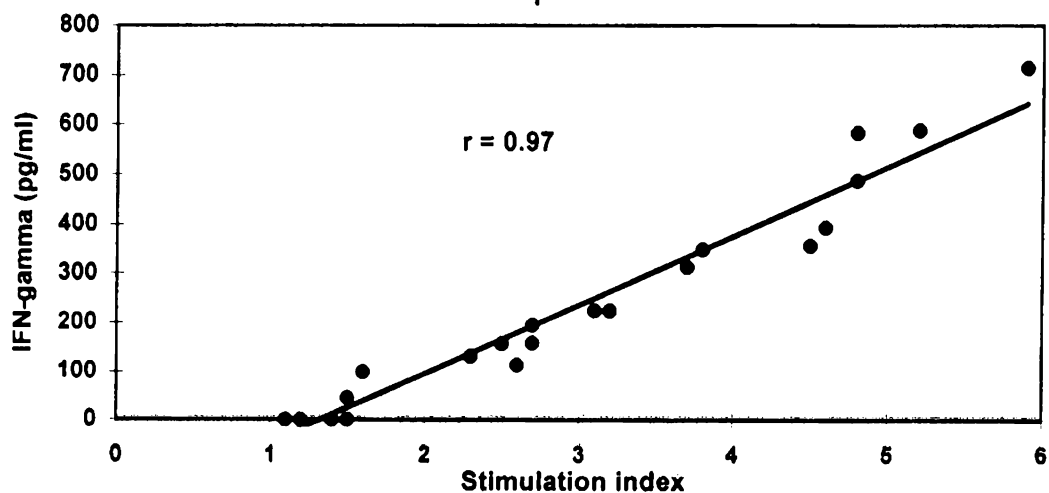
ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.

**Correlation between proliferation and IFN-gamma
production by PBMCs in response to PHA stimulation in
Group C1**



**Correlation between proliferation and IFN-gamma
production by PBMCs in response to MTBs stimulation in
Group C1**



CONTROLS : Healthy volunteers.

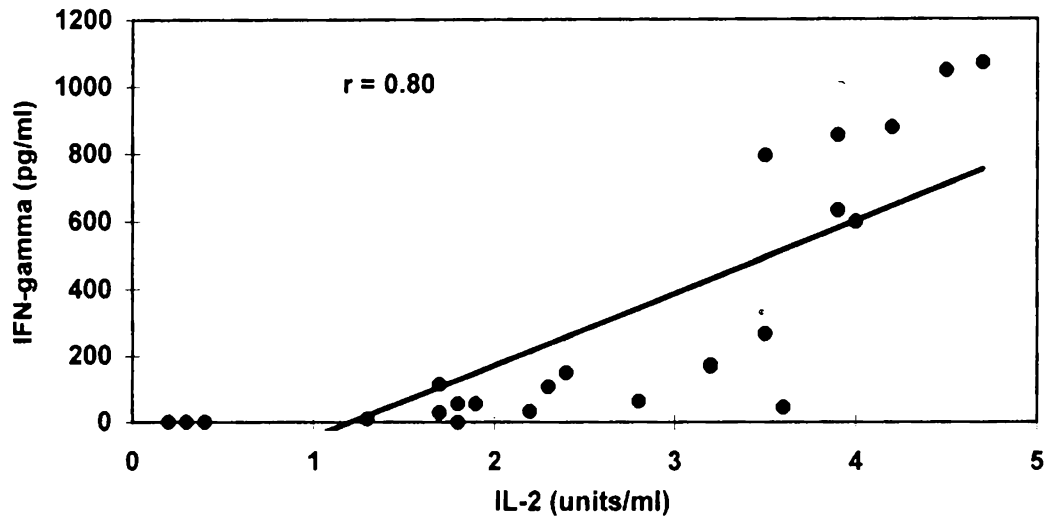
GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

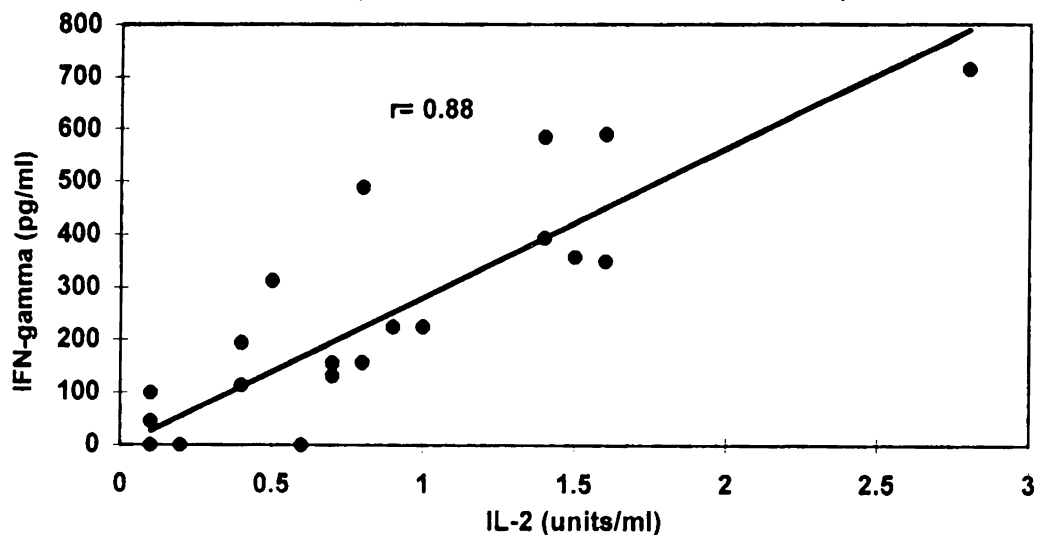
GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between IL-2 and IFN-gamma secretion by PBMNCs in response to PHA stimulation in Group C1.



Correlation between IL-2 and IFN gamma production by PBMNCs in response to MTBs stimulation in Group C1.



ONTROLS : Healthy volunteers.

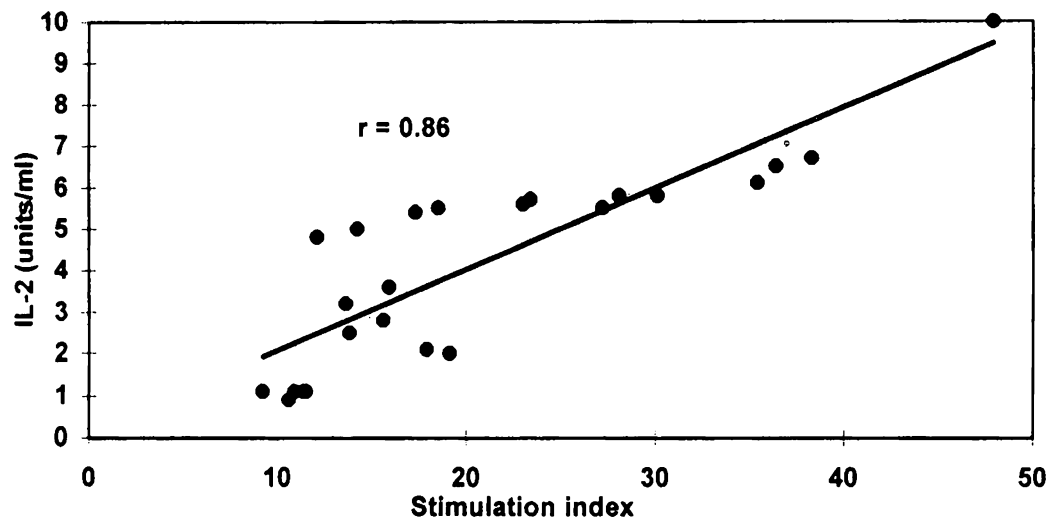
ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

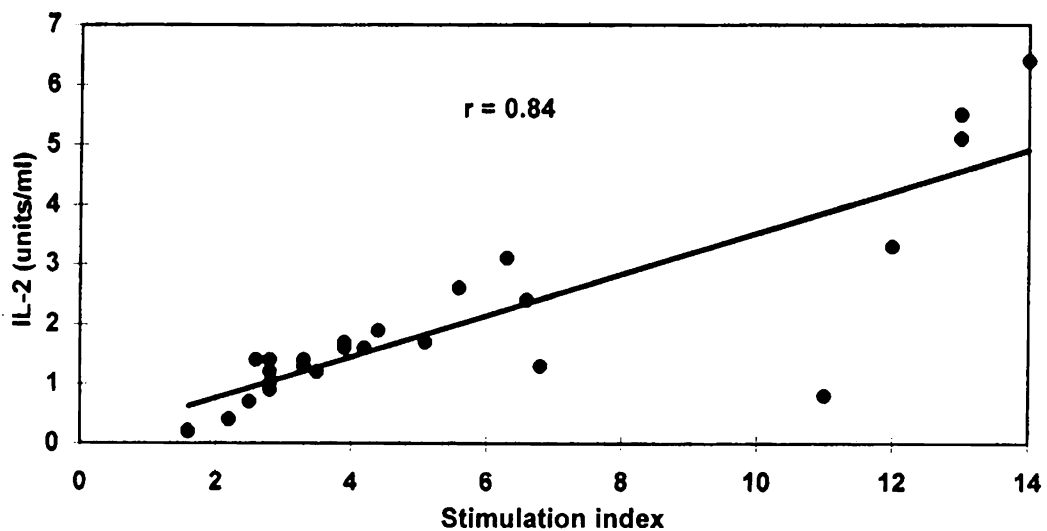
ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between proliferation and IL-2 production by PBMNCs in response to PHA stimulation in Group C2.



Correlation between proliferation and IL-2 production in response to MTBs stimulation in Group C2



CONTROLS : Healthy volunteers.

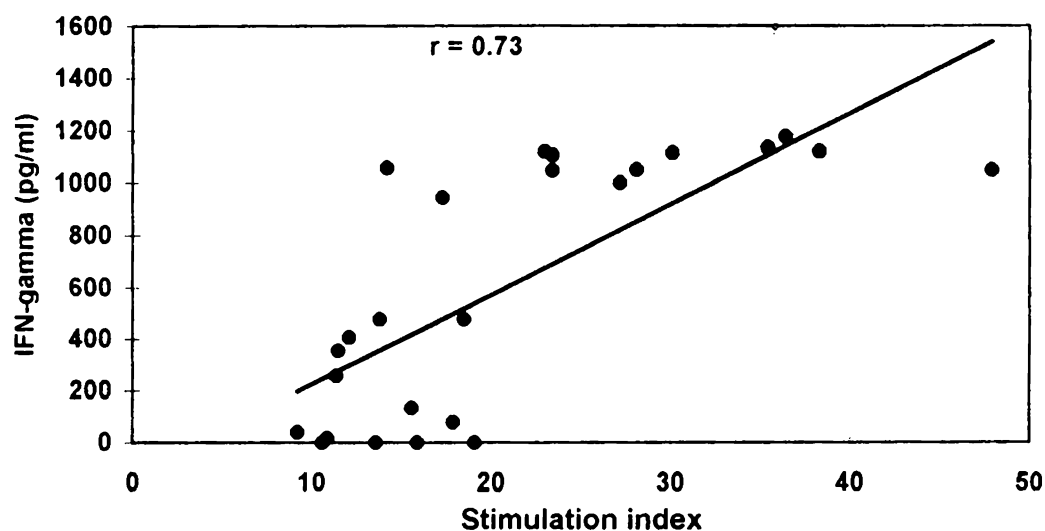
GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

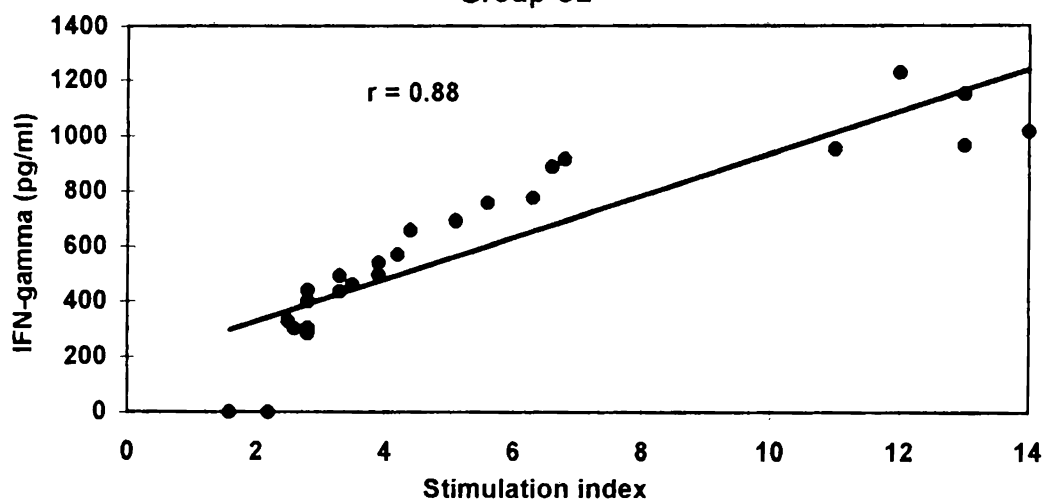
GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between proliferation and IFN-gamma production in response to PHA stimulation in Group C2.



Correlation between proliferation and IFN-gamma production by PBMNCs in response to MTBs stimulation in Group C2



ONTROLS : Healthy volunteers.

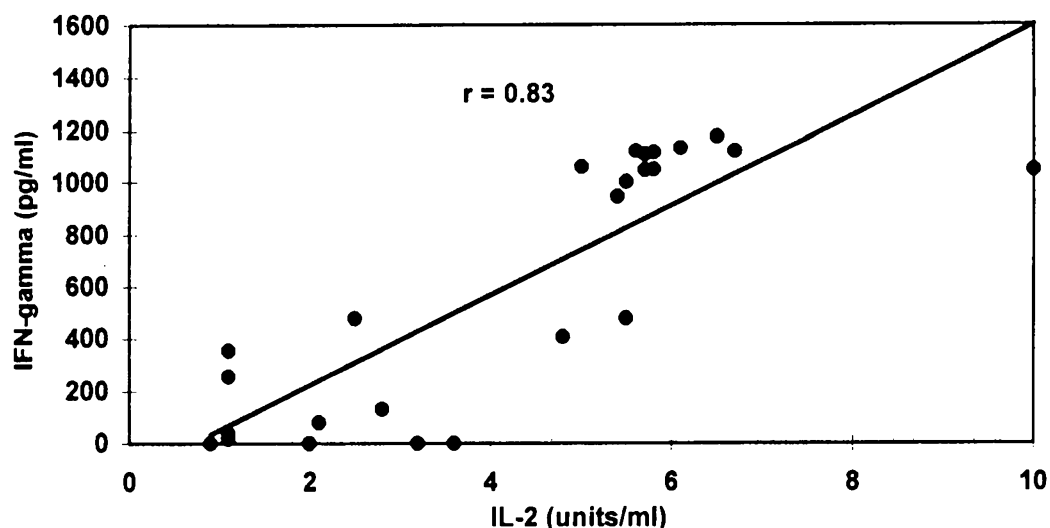
ROUP A : HIV seropositive asymptomatic cases.

ROUP B : AIDS patients having opportunistic infections other than tuberculosis

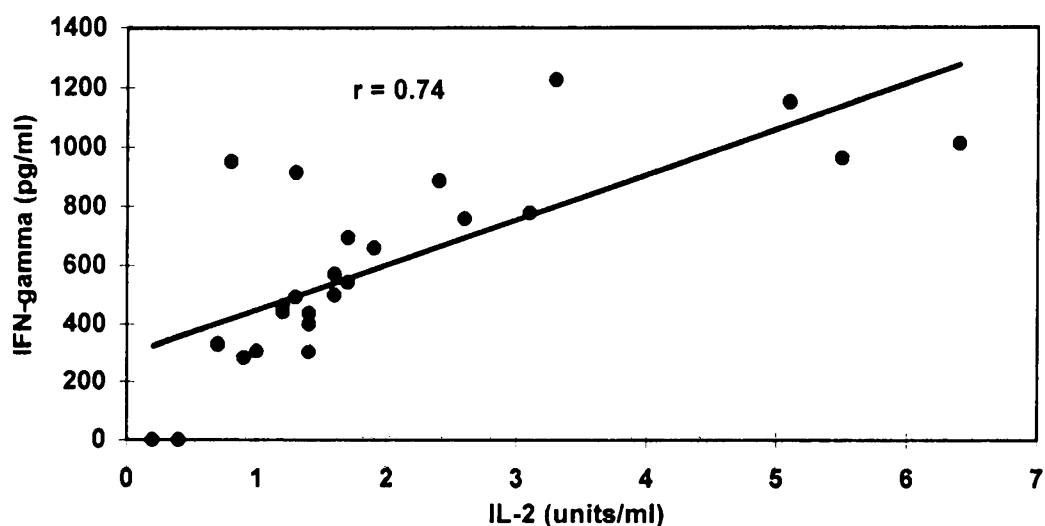
ROUP C1 : HIV seropositive pulmonary tuberculosis patients.

ROUP C2 : HIV seronegative pulmonary tuberculosis patients.

Correlation between IL-2 and IFN-gamma production by PBMNCs in response to PHA stimulation in group C2.



Correlation between IL-2 and IFN gamma production by PBMNCs in response to MTBs stimulation in Group C2.



CONTROLS : Healthy volunteers.

GROUP A : HIV seropositive asymptomatic cases.

GROUP B : AIDS patients having opportunistic infections other than tuberculosis

GROUP C1 : HIV seropositive pulmonary tuberculosis patients.

GROUP C2 : HIV seronegative pulmonary tuberculosis patients.

SUMMARY & CONCLUSION

In the present study 210 clinically and radiologically confirmed pulmonary tuberculosis patients, 16 HIV seropositive asymptomatic cases, 14 AIDS patients without evidence of tuberculosis and 10 healthy volunteers were included.

Out of 210 pulmonary tuberculosis patients, majority (41.90%) were in the age group 21-30 years for both the sex. They also showed unemployment, lack of education, addiction to alcohol (40%) and smoking (14.76%). All these factors must have contributed to the high risk for acquiring tuberculosis.

Maximum HIV seroprevalence was observed in the age group of 21-40 years which is the sexually most active age group. Heterosexual contact was found to be the cause for acquiring infection in majority of the cases.

The effect of HIV, M tuberculosis and the dual infection on cellular arm of immune functions was estimated using *in vitro* PBMNCs proliferation and cytokine production (IL-2, IFN- γ , IL-4, IL-10, TNF- α) in response to PHA and MTBs stimulation. Decline in the protective responses was shown in asymptomatic patients on stimulation with PHA as well as MTBs which was significantly reduced as compared to healthy volunteers [controls] ($p < 0.01$). This decrease becomes serious as the disease progresses further to AIDS syndrome stage with opportunistic infections. AIDS syndrome cases without tuberculosis showed the least proliferation, IL-2 and IFN- γ production in response to PHA as well as MTBs which was significantly reduced as compared to controls [$p < 0.01$] and asymptomatic HIV infected cases [$p < 0.01$]. HIV and tuberculosis coinfecting patients also showed the similar results.

Pulmonary tuberculosis patients who were HIV seronegative, showed decrease in CMI potential of the PBMNCs in response to PHA as well as MTBs stimulation as assessed by the observed decrease in proliferation, IL-2 and IFN- γ production which was significantly low as compared to controls at $p < 0.01$.

There was no significant difference found in IL-4 and IL-10 production in all the five groups. Although number of IL-4 producers are more in the HIV

infected group, the difference in the IL-4 production did not reach significant values as compared to controls.

Thus, the study reports a definite decline in TH1 responses, on stimulation with both mitogen and antigen stimulation of PBMNCs in short term in vitro culture of patients with either HIV or tuberculosis or both infections. This was not found to be accompanied by increase in Th2 responses as indicated by similar production of IL-4 and IL-10 in all the groups.

TNF α is a proinflammatory cytokine which constitutes an essential factor in immune response against tuberculosis. HIV and tuberculosis co-infected patients showed 3 fold increase in TNF α production as compared to healthy controls and 2 fold TNF α production as compared to patients infected with tuberculosis alone. This increase in TNF α leads to increase in HIV expression and T cell loss due to direct cell killing by HIV due to enhanced viral expression and other mechanisms like enhanced apoptosis which occur due to decrease in protective response (IL-2) and increase or no change in TNF α and IL-4, IL-10 respectively which increase apoptosis.

Active tuberculosis, as reflected in results, leads to suppression of protective responses even in absence of HIV. Thus if occurs early in the course of HIV infection, weakens the immune system faster and adds to the damage caused by HIV. Also it being characterised by prolonged antigenic stimulation and increase in TNF α , promotes HIV replication. As HIV also destroys the immune system, protective response cannot be mount against tuberculosis, This results in further spread of tuberculosis and mutual synergy between the two pathogens leads to faster progression of disease.

From the present study we observed that cytokines play central role in the synergistic action of the two infections. Thus early detection and treatment of tuberculosis is necessary especially in Indian population where rate of co-infection was found to be very high.

APPENDIX I

1. GKN

NaCl	8 gms
KCL	0.40 gms
Na ₂ HPO ₄ .2H ₂ O	1.77 gms
NaH ₂ PO ₄ .2H ₂ O	0.69 gms
Glucose	2.0 gms
Phenol red	0.01 gms
Make volume to 1000ml with distilled water and filter sterilise.	

2. LOWENSTEIN-JENSEN (LJ) MEDIUM [411]

Mineral salt solution

Potassium dihydrogen phosphate - KH ₂ PO ₄	2.4 g
Magnesium sulfate	0.24 g
Magnesium citrate	0.6 g
Asparagine	3.6 g
Glycerol	12 ml
Distilled water	600 ml

Dissolve the ingredients except glycerol by heating and then add glycerol. Autoclave at 121⁰C for 21 minutes.

Malachite Green solution

Malachite green	2.0 g
Distilled water	100 ml
Dissolve by heating and autoclave at 121 ⁰ C for 15 minutes.	

Homogenized whole egg solution

Clean, about 20-22 hen eggs, 3-4 days old, by scrubbing with hand brush in 5% soap solution and allow to stand for 30 minutes. Rinse thoroughly in running water. Soak these eggs in 70% alcohol for 15 minutes. Break these eggs into sterile flask containing glass beads under aseptic condition and homogenized by shaking vigorously. Filter through 4 layers of sterile gauze to obtain 100ml of sterile solution.

Complete medium

Mineral salt solution	600 ml
Malachite green solution	20 ml
Homogenized egg solution	1000 ml

Prepare under aseptic condition. Mix well and dispense aseptically 8 ml of medium into 20 × 150 mm sterile screw capped culture tubers. Slant the tubes and inspissate at 85⁰ C for 50 minutes. Store the tubers at 4⁰ C until used.

3. Phosphate buffered saline (0.01 M pH 7.2)

NaCl	8.5 g
K ₂ HPO ₄ (anhydrous)	1.21 g
KH ₂ PO ₄ (anhydrous)	0.34 g
Distilled water	1000 ml

4. RPMI -1640 medium

Preparation : powdered medium obtained from Sigma (USA) was dissolved in 900ml of deionised water. Two grams of Sodium bicarbonate was added and stirred till it was completely dissolved. pH was adjusted to 7.0 using 1N HCl and 1N NaOH. Additional water was added to bring the volume to 1 litre. The medium was then filter sterilised using a membrane with a porosity of 0.22 microns. The filtered medium was aseptically dispensed into sterile bottles and stored at 4° C till use.

5. Complete RPMI-1640 medium (CRPMI-1640)

RPMI-1640	89ml
Foetal calf serum	10 ml
L-glutamine	1 ml

6. Scintillation cocktail

1-4 bis (5-phenyl oxazolyl) benzene (POPOP)	0.3016 g
2-5 diphenyl oxazole (PPO)	3.968 g
Toluene	1000 ml

PPO and POPOP were dissolved in 800ml toluene in volumetric flask and mixed thoroughly. After complete dissolution the volume was made to 1.0 litre with toluene.

7. Sodium thyoglycolate

Sodium thyoglycolate medium was obtained from HI Media in a solid powdered form.

29gms was suspended in 1000 ml distilled water. It was boiled to dissolve the medium completely. Sterilised by autoclaving at 15 lb. pressure, 121°C for 15 minutes and stored in refrigerator till used.

8. Youman's medium: (modification of Proskaur and Beck) [412]

Asparagine	0.5 g
Potassium dihydrogen phosphate KH_2PO_4	0.05 g
Glycerol	2.0 ml
Distilled water	100 ml

Dissolve the ingredients in the water in the above order, ensuring that one is dissolved before the next is added. Adjust the pH to 7.0 with 4% sodium hydroxide and add magnesium citrate 0.15g. Autoclave at 121°C for 20 minutes.

9. Recombinant Human Interleukin-2

It was purchased from ENDOGEN, USA as a lyophilised powder 10µg/ml. The powder was reconstitute in 100mM acetic acid and 10% BSA to suitable dilutions and was stored in working aliquots at -20°C. As given in the instructions accompanying vial
One unit activity = 0.1 ng/ml

APPENDIX II

EATIMATION OF PROTEIN BY LOWRY'S METHOD [413].

Reagents:

Reagent 1.

Sodium potassium tartarate- 0.2 gms.

Distilled water - 10 ml

Reagent 2

Cupric sulphate -0.1gms

Distilled water -10 ml Standard protein solution

Reagent 3.

Sodium carbonate - 2.0 gms.

Sodium hydroxide (0.1N)- 0.4gms.

Distilled Water-100ml

Reagent A

1 ml of reagent 1+ 1 ml of Reagent2 was mixed and added to 100ml of Reagent3.

Reagent B

Folin ciocalteau's Reagent(sisco) was diluted 1:1 with distilled water just before use .

Standard protein solution.

Bovine serum albumin (BSA,Sigma) in the concentration of 200ug/ml was used as the standard protein solution.

A set of clean glass tubers were taken to which 0.1, 0.2, 0.3, 0.4, 0.5 ml of standard BSA was added in duplicate. The volume of each tube was made upto 5ml with distilled water. Blanks of 0.5ml distilled water were also set up. The test solution was also taken in 0.5ml quantity with appropriate dilution for the protein estimation. 2.5ml reagent A was added to each tuber. The contents of each tuber were mixed and tuber were allowed to stand at room temperature for 15 minutes. 0.25 mm of reagent B was then added to each tuber with vigorous shaking and tubes were allowed to stand at room temperature for 30 minutes. Optical density (OD) readings were taken at 660nm adjusted against

the distilled water blank. A graph of OD against protein concentration was plotted from which the protein concentration of test sample was calculated.

BIBLIOGRAPHY

1. The Cytokine Handbook. Thomson A (ed), Academic Press; London, 1994.
2. Murray HW, Rubin BY, Rothermel CD.
Killing of intracellular *Leishmania donovani* by lymphokine stimulated human mononuclear phagocytes. Evidence that interferon gamma is the activating lymphokine.
J Clin Invest 1983; 72: 1506-1510.
3. Finkelman FD, Pearce EJ, Urban JJ, Sher A.
Regulation and biological function of helminth induced cytokine responses.
Immunol Today 1991; 12: A62-A66.
4. Sher A, Gassinelli RT, Oswald IP, *et al*.
Role of T cell derived cytokines in the down regulation of immune responses in parasitic and retroviral infection.
Immunol Rev 1992; 127: 183-204.
5. Mossman TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL.
Two types of murine helper cell clone I Definition according to profiles of Lymphokines activities and secreted proteins.
J Immunol 1986; 136: 2348-2357.
6. Janeway CA, Carding S Jr. Jones B, .
CD4⁺T cells: Specificity and function.
Immunol Rev 1988; 101: 39-80.
7. Romagnani S.
Human TH1 and TH2: doubt no more.
Immunol Today 1991; 12: 256-257.
8. Fiorentino DF, Bond MW Mosmann TR.
Two types of mouse T helper cell IV. TH2 clones secrete a factor that inhibits cytokine production by Th1 clones.
J Exp Med 1989; 170: 2081-1095.
9. Sornasse T, Larenas PV, Davis KA, de Vries JE, Yssel H.
Differentiation and stability of T helper 1 and 2 cells derived from naïve human neonatal CD4 T cells, analysed at the single cell level.
J Exp Med 1996; 184: 473-483.
10. Mossman TR, Coffman RL.
Th1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties.
Ann Rev Immunol 1989; 7: 145-173.

11. Bottomly K.
A functional dichotomy in CD4⁺T lymphocytes.
Immunol Today 1988;9: 268-274.
12. Snapper CM, Paul WF.
Interferon- γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production.
Science 1987; 236: 944-947.
13. Stevens TL, Bossie A, Sanders VM, .
Regulation of antibody isotype secretion by subsets of antigen specific helper T cells.
Nature 1988; 334: 255-258.
14. Coffman RL, Lebnan DA, Rothman P.
Mechanisms and regulation of immunoglobulin isotype switching.
Adv Immunol 1995; 60: 151-266.
15. Galli S.
New concepts about the mast cell.
N Engl J Med 1993; 328: 257-265.
16. Wardlaw AJ, Moqbel R, Kay AB.
Eosinophils: Biology and role in disease.
Adv Immunol 1995; 60: 151-266.
17. Finkelman FD, Holmes J, Katona IM, .
Lymphoic control of immunoglobulin isotype selection.
Ann Rev Immunol 1990; 8: 303-333.
18. Croft M, Swain SL.
Recently activated naive CD4⁺T cells can help resting B cells, and can produce sufficient autocrine IL-4 to drive differentiation to secretion of T helper 2 type cytokines.
J Immunol 1995; 154: 4269-4281.
19. Bloom BR, Modlin RL, Salgame P.
Stigma variations. Observations on suppressor T cells and leprosy.
Ann Rev Immunol 1992; 10: 453-488.
20. Seder RA, Paul WE.
Acquisition of lymphokine producing phenotype by CD4⁺T cells.
Ann Rev Immunol 1994;12: 635-373.
21. Rocken M, Muller KM, Suarat JH.
Lectin mediated induction of IL-4 producing CD4⁺T cells.
J Immunol 1991; 146: 577-584.

22. Seder R, Paul WE, Davis MM, Fazekas de st Groth B.
the presence of interleukin 4 during *in vitro* priming determines
the lymphokine producing potential of CD4 T cells from T cell receptor
transgenic mice.
J Exp med 1992; 176: 1091-1098.
23. Hsieh CS, Heimberger AB, Gold JS, O'Garra A, Murphy KM.
Differential regulation of T helper phenotype development by interleukin
4 and 10 in an alpha-beta T cell receptor transgenic system.
Proc Natl Acad Sci 1992; 89: 6065-6069.
24. Swain SL.
Regulation of the development of distinct subsets of CD4⁺T cells.
Res Immuno 1991; 142: 14-18.
25. Kamogawa Y, Minasi LI, Carding SR, Bottomly K, Flavell RA.
The relationship of IL-4 and IFN- γ producing T cells studied by lineage
ablation of IL-4 producing cells.
Cell 1993; 75: 985-995.
26. Openshaw P, Murphy EE, Hosken NA, .
Heterogeneity of intracellular cytokine. Synthesis at the single cell level
in polarized T helper 1 and T helper 2 populations.
J Exp Med 1995; 182: 1357-1367.
27. Chen Y, Kachroo VK, Inobe J, Hafler DA, Weiner HL.
Regulatory T cell clones induced by oral tolerance. Suppression
of autoimmune encephalomyelitis.
Science 1994; 265: 1237-1240.
28. O' Garra A, Murphy KM.
Role of cytokines in the development of TH1 and TH2 cells.
Chem Immunol 1996; 63: 1-13.
29. Kelso A.
TH1 and TH2 subsets: paradigm lost ?
Immunol Today 1995; 16 374-379.
30. Bucy RP, Karr L, Huang G-Q, .
Single cell analysis of cytokine gene co-expression during CD4⁺T
cell phenotype development.
Proc Natl Acad Sci 1995; 92: 7565-7569.
31. Abbas AK, Murphy KM, Sher A.
Functional diversity of helper T lymphocytes.
Nature 1996; 388: 787-793.

32. Heufler C, Koch F, Stanzl U.
Interleukin 12 is produced by dendritic cells and mediates TH1 development as well as IFN- γ production by Th1 cells.
Eur J Immunol 1996; 26: 659-668.
33. Hsieh CS, Macatonia SE, Tripp CS, .
Development of Th1 CD4 T cells through IL-12 produced by Leisteria induced macrophages.
Science 1996; 260: 547-549.
34. Macatonia SE, Hosken NA, Litton M .
Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4⁺T cells.
J Immunol 1995; 154: 5071-5079.
35. Szabo S, Jacobson NG, Dighe AS, Gubler U, Murphy KM.
Developmental commitment to the TH2 lineage by extinction of IL-12 signalling.
Immunity 1995; 2: 665-675.
36. Kaplan MH, Sun YL, Hoey T, Grusby MJ.
Impaired IL-12 responses and enhanced development of Th2 cells in Stat-4 deficient mice.
Nature 1996; 382: 174-177.
37. Jacobson NG.
Interleukin 12 signaling in T helper type (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription Stat 3 and Stat 4.
J Exp Med 1995; 181: 1755-1762.
38. Magram J, Connaughton SE, Warrier RR, .
IL-12 deficient mice are defect in IFN- γ production and type 1-cytokine responses.
J Immunol 1996; 4: 471-481.
39. Manetti R, Paronchi P, Giudizi M-G, .
Natural killer cell stimulatory factor (interleukin-12) induces T helper type 1 (TH1) specific immune responses and inhibits the development of IL-4 producing Th cells.
J Exp Med 1993; 177: 1199-1204.
40. Manetti R, Gerosa F, Giudizi M-G, .
Interleukin 12 induces stable priming for interferon γ (IFN- γ) production in established Th2 cell clones.
J Exp Med 1994; 179: 1273-1283.

41. Flesch IFA, Hess JH, Huang S, .
Early interleukin 12 production by macrophages in response to mycobacterial infection depends on interferon γ and tumor necrosis factor α .
J Exp Med 1995; 181: 1615-1621.
42. Wang Z-E, Zheng S, Corry DB, Dalton DK, Seder RA, Reiner S, Locksley RM.
Interferon γ independent effects of interleukin 12 administered during acute or established infection due to *Leishmania major*.
Proc Natl Acad Sci 1994; 91: 12932-12936.
43. Dighe AS, Campbell D, Hsieh C-S, .
Tissue specific targeting of cytokine unresponsiveness in transgenic mice.
Immunity 1995; 3: 657-666.
44. Trinchieri G.
Interleukin 12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity.
Ann Rev Immunol 1995; 13: 251-276.
45. Guler M, Garham JD, Hsieh CS, *et al*.
Genetic susceptibility to leishmania: IL- 12 responsiveness in Th1 cell development.
Science 1996; 271: 984-987.
46. Wenner C, Guler ML, Macatonia SE, O'Garra A, Murphy KM.
Roles of IFN- γ and IFN- α in IL-12 induced TH1 development.
J Immunol 1996; 156: 1442-1447.
47. Swain SL, Huston G, Tonlonogy S, Weinberg AD.
Transforming growth factor beta and IL-4 cause helper T cell precursors to develop into distinct effector helper cells that differ I lymphokine secretion pattern and cell surface phenotypes.
J Immunol 1991; 147: 2991-3000.
48. Sad S, Mosmann TR.
A single IL-2 secreting precursor CD4 ⁺T cell can develop into either a TH1 or TH2 secreting phenotype.
J Immunol 1994; 153: 3514-3522.
49. Santambrogio L, Houchwald GM, Saxena B .
Studies on mechanisms by which transforming growth factor β (TGF- β) protects against allergic encephalomyelitis.
J Immunol 1993; 151: 1116-1127.

50. Hoehn P, Goedert S, Germann T, .
Opposing effects of TGF- β on the TH2 cell development of naive CD4⁺T cell isolated from different mouse strains.
J Immunol 1995; 155: 3788-3793.
51. Maggi E, Parronchi P, Manetti R, .
Reciprocal regulatory role of IFN- γ and IL-4 on the *in vitro* development of human TH1 and Th2 cells.
J Immunol 1992; 148: 2142-2147.
52. Okamura H, Tsutsu H, Komatsu T, .
Cloning of a new cytokine that induces IFN- γ production by T cells.
Nature 1995; 25: 2625-2660.
53. Swain SL, Weinberg AD, English M, Huston G.
IL-4 directs the development of TH2 like helper effectors.
J Immunol 1990; 145: 3796-3806.
54. Seder RA, Paul WE, Davis MM, Fazekas De t Groth B.
The presence of interleukin 4 during *in vivo* priming determines the lymphokine producing potential of CD4⁺T cells from T cell transgenic mice.
J Exp Med 1992; 176:1091-1098.
55. Gross A, Ben Sasson SZ, Paul WE.
Anti-IL-4 diminishes priming for antigen specific IL-4 production by T cells.
J Immunol 1993; 150: 2112-2120.
56. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluthmann H, Kohler G.
Disruption of the murine IL-4 gene blocks Th2 cytokine responses.
Nature 1993; 362: 245-248.
57. D'Andrea A, Aste-Amezago M, Valiante NM, Ma X. Kubin M, Trinchieri G.
Interleukin 10 (IL-10) inhibits human lymphocyte interferon γ production by suppressing natural killer cell stimulatory factor IL-12 synthesis in accessory cells.
J Exp Med 1993; 178: 1041-1048.
58. Williams IR, Unanue ER.
Characterisation of accessory cell co-stimulation of Th1 cytokine synthesis.
J Immunol 1991; 147: 3752-3760.
59. Zubigo AM, Munoz E, Huber BT.
Production of IL-1 α by activated Th type 2 cells.
J Immunol 1991; 146: 3849-3856.

60. Macatonia SE, Hsieh C-S, Murphy KM, Ó'Garra A.
Dendritic cells and macrophages are required for TH1 development of CD4⁺T cells from $\alpha\beta$ TCR transgenic mice. IL-12 substitution for macrophage to stimulate IFN- γ production is IFN- γ dependent.
Intern Immunol 1993; 5: 1119-1128.
61. Manetti R, Barak V, Piccinni M-P.
Interleukin-1 favours the development of type 2 T helper (Th2) human T cell clones.
Res Immunol 1994; 145: 93-100.
62. Tanaka T, Hu-Li J, Seder RA,, de St. Groth BF, Paul WE.
Interleukin 4 suppresses interleukin 2 and interferon gamma production by naive T cells stimulated by accessory cell-dependent receptor engagement.
Proc Natl Acad Sci 1993; 90: 5914-5918.
63. Seder RA,.
The role of IL-12 in the regulation of TH1 and TH2 differentiation.
Res Immunol 1995; 146: 473-476.
64. Chensue SW, Ruth JH, Warmington K, Lincoln P, Kunkel SL.
regulation of macrophage IL-12 production during type 1 and type 2 cytokine mediated granuloma formation.
J Immunol 1995; 155: 3546-3551.
65. Del Prete GF, De Carli M, Almerigogna F, Giudizi M-G, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production.
J Immunol 1993; 150: 1-8.
66. Fiorentino DF, Zlotnik A, Vieira P, .
IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells.
J Immunol 1991; 146: 3444-3451.
67. Hino A, Nariuchi H.
Negative feedback mechanism suppresses interleukin-12 production by antigen presenting cells interacting with T helper 2 cells.
Eur J Immunol 1996; 26: 623-628.
68. Trincieri G, Scott P.
Interleukin-12: a pro-inflammatory cytokine with immunoregulatory function.
Res Immunol 1995; 146:423-431.

69. Fitch FW, McKisic MD, Lancki DW, Gajewski TF.
Differential regulation of murine T lymphocyte subsets.
Ann rev Immunol 1993; 11: 29-48.
70. Zurawaski G, de Vries JE.
Interleukin 13, an interleukin 4 like cytokine that acts on monocytes and B cells, but not on T cells.
Immunol Today 1994; 15:19-26.
71. de Vries JE.
Molecular and biological characteristics of interleukin 13.
Chem Immunol 1996; 63: 204-218.
72. Gajewski TF, Joyce J, Fitch FW.
Anti proliferative effect of IFN- γ in immune regulation. III. Differential selection of Th1 and Th2 murine helper T lymphocyte clones.
J Immunol 1989; 143: 15-22.
73. Gajewski TF, Fitch FW.
Anti-proliferative effect of IFN-gamma in immune regulation. IV. Murine CTL clones produce IL-3 and GM-CSF, the activity of which is masked by the inhibitory action of secreted IFN-gamma.
J Immunol 1990; 144: 548-556.
74. Abed NS, Chace JH, Cowdery JS.
T cell dependent B cell activation increases IFN-gamma receptor expression and renders B cells sensitive to IFN-gamma mediated inhibition.
J Immunol 1994; 253: 3369-3377.
75. Urban JF Jr, Madden KB, Cheever AW, Tritta PP, Katona IM, Finkelman FD.
IFN inhibits inflammatory responses and protective immunity in mice infected with the nematode parasite, *Nippostrongylus brasiliensis*.
J Immunol 1993; 151: 7086-7094.
76. Wenner C, Guler ML, Macatonia SE, O'Garra A, Murphy KM.
Roles of IFN- γ and IFN- α in IL-12 induced TH1 development.
J Immunol 1996; 156: 1442-1447.
77. Manetti R, Annunziato F, Tomasevic L, .
Polyinosinic acid: polycytidylic acid promotes T helper type-1 specific immune responses by stimulating macrophage production of IFN- α and IL-12.
Eur J Immunol 1995; 378: 88-91.

78. Parronchi P, Mohapatra S., Sampognaro S, .
Modulation by IFN- α of cytokine profile, T cell receptor repertoire and peptide reactivity of human allergen specific T cells.
Eur J Immunol 1996; 26: 697-703.
79. Seder RA, Gazzinelli R, Sher A, Paul WE.
Interleukin 12 acts directly of CD4⁺T cells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming.
Proc Natl Acad Sci 1993; 90:10188-10192.
80. Manetti R, Parronchi P, Giudizi M-G, .
Natural killer cell stimulatory factor (interleukin-12) induces T helper type (TH1) specific immune responses and inhibits the development of IL-4 producing Th cells.
J Exp Med 1993; 177: 1199-1204.
81. DeKruyff RH, Fang Y, Wolf SF, Umetsu DT.
IL-12 inhibits IL-4 synthesis in keyhole limpet hemocyanin primed CD4⁺T cells through an effect on antigen presenting cells.
J Immunol 1995; 154: 2578-2587.
82. Morris RC, Madden KB, Adamovic Z .
Effects of IL-12 *in vivo* cytokine gene expression and Ig isotype selection.
J Immunol 1994; 152: 1047-1056.
83. Bliss J, Van Cleave V, Murray K, .
IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response.
J Immunol 1996; 156: 887-894.
84. Meyaard L, Hovenkamp E, Otto SA, Miedema F.
IL-12 induced IL-10 production by human T cells as a negative feedback for IL-12 induced immune responses.
J Immunol 1996; 156: 2776-2782.
85. Mertens TE, Belsey E, Stoneburner RL, .
Global estimates of HIV infections and AIDS: further heterogeneity in spread and impact.
AIDS 1995;9(Suppl 1): S 251
86. UNAIDS : report on the global HIV/AIDS epidemic, June 1998.
87. Quinn T.
Global burden of HIV epidemic.
Lancet 1996;348:99-106.

88. Saag MS. Natural history of HIV-1 infection. In: Samuel Broder, Merigan TC Jr., Bolognes D, editors, *Text Book of AIDS Medicine*; Baltimore: Willian and Wilkins 1994; pg 45-54.
89. Stein DS, Korvick JA, Vermund SH.
CD4⁺ lymphocyte cell enumeration for prediction of clinical course of human immunodeficiency virus disease : a review.
J Infect Dis 1992;165:352-363.
90. Lange JMA, Parry JV, De Wolf F, Mortimer PP, Goudsmit J.
Diagnostic value of specific IgM antibodies in primary HIV infection.
AIDS 1988;2:31-35.
91. Clark SJ, Sagg MS, Decker WG,, .
High titres of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection.
N Engl J Med 1991; 324: 954-960.
92. Osmond D, Chaisson RE, Moss AR, Bacchetti P, Krampf W.
Lymphadenopathy in asymptomatic patients seropositive for HIV.
N Engl J Med 1987;317:246.
93. Grunfeld C, Feingold KR.
Seminars in medicine of the Beth Israel Hospital, Boston : metabolic disturbances and wasting in the acquired immunodeficiency syndrome.
N Engl J Med 1992;327:329-337.
94. Macdonell KB, Chimiel JS, Poggensee L, Wu S, Phair JP.
Predicting progression to AIDS : combined usefulness of CD4 lymphocyte counts and p24 antigenemia.
Am J Med 1990;89:706-712.
95. Moss AR, Bacchetti P.
Natural history of HIV infections.
AIDS 1989;3:55-61.
96. Lane HC, Fauci AS.
Immunologic abnormalities in the acquired immunodeficiency syndrome.
Ann Rev Immunol 1985; 3: 477-500.
97. Tindall B, Cooper DA.
Primary HIV infection: host responses and interaction strategies.
AIDS 1991; 5: 1-14.
98. Somasundaran M, Robinson HL,.
Unexpectedly high levels of HIV -1 RNA and protein synthesis is a cytotoxic function.
Science 1988; 242: 1554-1557.

99. Koga Y, Lindstrom E, Fenyo EM, Wigzell H, Mac TW.
High levels of heterodisperse RNA accumulate in T cells infected with human immunodeficiency virus and in normal thymocytes.
Proc Natl Acad Sci 1988; 85: 4521-4525.
100. Levnark R, Zagury D, Desportes I, Bernad J, Zagury IF, Gallo RC.
Cytopathic effect of human immunodeficiency virus in T4 cells is linked to the last stage of virus infection.
Proc Natl Acad Sci 1988; 85: 3510-3514.
101. Lynn WS, Tweedale A, Cloyd MW,.
Human immunodeficiency virus (HIV-1) cytotoxicity: perturbation of cell membrane of depression of phospholipid synthesis.
Virology 1988; 163: 43-51.
102. Hoxie JA, Alpers JD, Racowski JL, .
Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV.
Science 1986; 1123-1127.
103. Fisher AG, Ratner L, Mitsuya H .
Infectious mutants of HTLV-III with changes in 3' region and markedly reduced cytopathic effects.
Science 1986; 233: 655-659.
104. Koga Y, Sasaki M, Yoshida H, Wigzell H, Kimura G, Nomoto K.
Cytopathic effect determined by the amount of CD4 molecules in human cell lines expressing envelope glycoprotein of HIV.
J Immunol 1990; 144: 94-102.
105. Lifson JD, Reyes GR, McGrath MS, Stein BS, Engleman EG.
AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen.
Science 1986; 232: 1123-1227.
106. Lifson JD, Feinberg MB, Reyes GR, .
Induction of CD4 dependent cell fusion by the HTLV III/LAV envelop glycoprotein.
Nature 1986; 323: 725-728.
107. Crowe SM, Millis J, Elbeik J, .
Human Immunodeficiency virus infected monocyte derived macrophages express surface gp120 and fuse with CD4 lymphoid cells.
Clin Immunol Immunopathol 1992; 65: 143-151.

108. Brinchmann JR, Albert J, Vartdal F.
Few infected CD4 T cell but a high proportion of replication-competent provirus copies in asymptomatic human immunodeficiency type 1 infection.
J Virol 1991; 65: 2019-2023.
109. Golding H, Robey FA, Gates FTIII, *et al.*
Identification of homology regions in human immunodeficiency virus gp41 and human MHC class II β domain I monoclonal antibodies against the gp41 derived peptide in patient's sera react with naïve HLA class II antigens, suggesting a role of autoimmunity in pathogenesis of acquired immune deficiency syndrome.
J Exp Med 1988; 167: 914-923.
110. Grassi F, Meneveri R, Gullberg M, *et al.*
Human immunodeficiency virus type gp120 mimics a hidden monomorphic epitope borne by class II major histocompatibility complex heavy chains.
J Exp Med 1991; 194: 53-62.
111. Dorsett B, Cronin W, Chuma V, Ioachim HL.
Antilymphocyte antibodies in patients with the acquired immune deficiency syndrome.
Am J Med 1985; 78: 621-626.
112. Stricker RB, McHugh TM, Moody DJ, .
An AIDS related cytotoxic autoantibody reacts with a specific antigen on stimulated CD4⁺T cells.
Nature 1987; 327: 710-713.
113. Daniel V, Schimpf K, Oplez G.
Lymphocyte autoantibodies and alloantibodies in HIV positive haemophilia patients.
Clin Exp Immunol 1989; 75: 178-181.
114. Klaaseen RJ, Muddler JW, Vlekke AB, *et al.*
Autoantibodies against peripheral blood cells appear early in HIV infection and their prevalence increases with disease progression.
Clin Exp Immunol 1990; 81: 111-117.
115. Klaaseen RJ, Goldschmeding R, Dolman KM.
Antineutrophil cytoplasmic autoantibodies in patients with asymptomatic HIV infection.
Clin Exp Immunol 1992; 87:24-30.

116. Kowalski M, Ardman B, Basiripour L .
Antibodies to CD4 in individuals infected with human immunodeficiency virus type 1.
Proc Natl Acad Sci 1989; 86: 3346-3350.
117. Weimer R, Daniel V, Zimmermann R, Schimpf K, Oplez G.
Autoantibodies against CD4 cells are associated with CD4 helper defects in Human Immunodeficiency virus infected patients.
Blood 1991; 77:133-140.
118. Ardman B, Sikorski MA, Settles M, Stauton DE.
Human immunodeficiency virus type 1 infected individuals make autoantibodies that bind to CD43 on normal thymic lymphocytes.
J Exp Med 1990; 172: 1151-1158.
119. Bost KL, Hahn BH, Saag MS, Shaw GM, Weigent DA, Blalock JE.
Individuals with HIV possess antibodies against IL-2.
Immunology 1988; 65: 611-615.
120. Golding H, Shearer GM, Hillman K .
Common epitope in human immunodeficiency virus (HIV) 1 gp41 and HLA class II elicits immunosuppressive autoantibodies capable of contributing to immune dysfunction in HIV 1 infected individuals.
J Clin Invest 1989; 83: 1430-1455.
121. Zarlin JM, Ledbetter JA, Sias J .
HIV infected humans, but not chimpanzees, have circulating cytotoxic T lymphocytes that lyse uninfected CD4 T cells.
J Immunol 1990; 144: 2992-2998.
122. Weinhold KJ, Lyerly HK, Stanley SD, Austin AA, Matthews TJ, Bolognesi DP.
HIV1gp120 mediated immune suppression and lymphocyte destruction in the absence of viral infection.
J Immunol 1989; 142: 3091-3097.
123. Lansavecchia A, Roosnek E, Gregory T, Berman P, Abrignani S.
T cells can present antigens such as HIV gp120 targeted to their own surface molecules.
Nature 1988; 334: 530-532.
124. Siliciano RF, Lawton T, Knell c, .
Analysis of host virus interaction in AIDS with antigp120 T cell clones: effect of HIV sequence variation and a mechanism for CD4 cell depletion.
Cell 1988; 54: 561-575.

125. Katz JD, Nishanian P, Mitsuyasu R, Bonavida B.
Antibody dependent cellular cytotoxicity (ADCC) mediated destruction of human immunodeficiency virus (HIV) coated CD4⁺T lymphocytes by acquired immunodeficiency syndrome (AIDS) effector cells.
J Clin Immunol 1988; 8: 453-458.
126. Appler J, Kotzin B, Herron L, .
V-beta specific stimulation of human T cells by staphylococcal toxins.
Science 1989;244: 811-813.
127. Ameisoe JC Capron A,.
Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis.
Immunol Today 1991; 12:102-105.
128. Groux H, Tropeir G, Monte D, Mouton Y, Capron A, Ameisen JC.
Activation induced death by apoptosis in CD4⁺T cells from human immunodeficiency virus infected individuals.
J Exp Med 1992; 175: 331-340.
129. Amadori A, De Silvestro G, Zamarchi R .
CD4 epitope making by gp120/anti-gp120 antibody complexes. A potential mechanism for CD4⁺ cell function down regulation in AIDS patients.
J Immunol 1992; 148: 2709-2716.
130. Banda NK, Bernier J, Kurahara DK, .
Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation induced apoptosis.
J Exp Med 1992; 176: 1099-1106.
131. Terai C, Kornbluth RS, Pauza CP, Richman DD, Carson DA.
Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1.
J Clin Invest 1991; 87: 1710-1715.
132. Laurent-Crowford AG, Krust B, Muller S, .
The cytopathic effect of HIV is associated with apoptosis.
Virology 1991; 185: 829-839.
133. Muller S, Rixhalet P, Lawrent-Crowford A, .
Autoantibodies typical of non-organ specific autoimmune disease in HIV seropositive patients.
AIDS 1992; 6: 933-942.

134. Spivak JL, Bender BS, Quinn TC.
Haematologic abnormalities in the acquired immunodeficiency syndrome.
Am J Med 1984; 77: 224-228.
135. Schneider DR, picker LJ,.
Myelodysplasia in the acquired immune deficiency syndrome.
Am J Clin Pathol 1985; 84: 144-152.
136. Donahue RE, Johnson MM, Zn LI,Clark SC, Groopman JE.
Suppression of *in vitro* haematopoiesis following human immunodeficiency syndrome.
Nature 1987; 326: 200-203.
137. Folks TM, Kessker SW, Orenstein JM, Justement JS, Jaffe Es, Fauci AS.
Infection and replication of HIV-1 in purified progenitor cells of normal human bone marrow.
Science 1988; 242: 919-922.
138. Potts BJ, Hoggan MFD, Lamperth Ls, Spivak J.
Replication of HIV-1 and HIVII in human bone marrow cultures.
Virology 1992; 188: 840-849.
139. Baldwin GC, Fleischmann J, Chung Y, Koyanagi Y, Chen IS, Globe DW.
Human immunodeficiency virus causes mononuclear phagocyte dysfunction.
Proc Natl Acad Sci 1990; 87: 3933-3937.
140. Gendelman HE, Friedman RM, Joe S, .
A selective defect of interferon alpha production in human immunodeficiency virus infected monocytes.
J Exp Med 1990; 172: 1433-1442.
141. Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS.
Abnormalities of B cell activation and immuoregulation in patients with the acquired immunodeficiency syndrome.
N Engl J Med 1983; 309: 453-458.
142. Reddy MM, Goetz RR, Gorman JM, Grieco MH, Chess L, Lederman S.
Human immunodeficiency virus type-1 infection of homosexual men is accompanied by a decrease in circulating B cells.
J Acq Immune Def Syn 1991; 4: 428-434.
143. Boue F, Wallon C, Goujard C, Barre-Sinoussi F, Galanaud P, Delfraissy JF. HIV induces IL-6 production by human B lymphocytes. Role of IL-4. *J Immunol* 1992; 148: 3761-3767.

144. Riekman P, PoliG, Fox CH, Kehrl JH, Fauci AS.
Recombinant gp120 specifically enhances tumor necrosis factor- α production and Ig secretion in B lymphocytes from HIV infected individuals but not from HIV seronegative donors.
J Immunol 1991; 147: 2922-2927.
145. Vendrell JP, Segondy M, Ducos J, .
Analysis of the spontaneous *in vitro* anti-HIV antibody secretion by peripheral blood mononuclear cells in HIV-1 infection.
Clin Exp Immunol 1991; 114: 678-693.
146. Pantaleo G, Fauci AS.
Tracking HIV during disease progression.
Curr Opin Immunol 1994; 6: 600-604.
147. Allen JB, Mc Carteney-Francis N, Smith PP, .
Expression of IL-2 receptors by monocytes from patients with AIDS and induction of monocyte IL-2 receptors by HIV .
J Clin Invest 1990; 192-199.
148. Ascher MS, Sheppard HW.
AIDS as immune system activation, a model for pathogenesis.
Clin Exp Immunol 1988; 73: 165-167.
149. Greene WC, Bohnlein E, Ballard DW.
HIV-1, HTLV-1 and normal T cell growth: Transcriptional strategies and surprises.
Immunol Today 1989; 10: 272-278.
150. Nobel G, Baltimore D.]
An inducible transcriptional factor activates expression of human immunodeficiency virus in T cells.
Nature 1987; 326: 711-713.
151. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS.
Cytokine induced expression of HIV-1 in a chronically infected promonocyte cell line.
Science 1987; 238: 800-802.
152. Clouse KA, Powel D, Washington I, .
Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone.
J Immunol 1989; 142: 431-438.

153. Mellors JW, Griffith BP, Ortiz MA, Landry ML, Ryan JL.
Tumor necrosis factor alpha/cachectin enhances human immunodeficiency virus type 1 replication in a primary macrophages.
J Infect Dis 1991; 163: 78-82.
154. Duh EJ, Maury WJ, Folks TM, Fausi AS, Rabson AB.
Tumor necrosis factor-alpha activates human immunodeficiency virus-1 through induction of nuclear factor binding to the NF- κ B sites in the long terminal repeat.
Proc Natl Acad Sci 1989; 86: 5974-5978.
155. Poli G, Kinter A, Justement JS .
Tumor necrosis alpha functions in an autocrine manner in the induction of human immunodeficiency virus expression.
Proc Natl Acad Sci 1990; 87: 782-785.
156. Poli G, Bressler P, Kinter A,
Interleukin 6 induces human immunodeficiency virus expression in monocyte cells alone and in synergy with tumor necrosis factor by transcriptional and post-translational mechanisms.
J Exp Med 1990; 172: 151-158.
157. Ramirez F, Fowell DJ, Puklavec M, Simmonds Manson D.
Glucocorticoids promote a TH2 cytokine response by CD4⁺T cells .
J Immunol 1996; 156: 2406-2412.
158. Brinkmann V, Kristofic C.
Regulation by corticosteroids of TH1 and TH2 cytokine production in human CD4⁺ effector T cells generated from CD45RO⁻ and CD45RO⁺ subset.
J Immunol 1995; 155: 322-3328.
159. Israel-Biet, Cadranel J, Beldjord K, Androeu JM, Jeffrey A, Even P.
Tumor necrosis factor production in HIV seropositive subjects. Relationship with lung opportunistic infections and HIV expression in alveolar macrophages. *J Immunol* 1991; 147: 490-494.
160. Araneo BA, Woods ML, Daynes RAI.
Reversal of the immunosenescent phenotype by dehydroepiandrosterone: hormone treatment provides an adjuvant effect on the immunisation of provides on adjuvant effect on the immunisation of aged mice with recombinant hepatitis B surface antigen.
J Infect Dis 1993; 167: 830-840.
161. Araneo BA, Do Well T, Terui T, Diegel M, Danynes RA. Dihydro testosterone exerts a depressive influence on the production of IL-4, IL-5 and IFN- γ but not IL-2 by activated murine cells.
Blood 1991; 78: 688-699.

162. Daynes RA, Araneo Ba, Ershler WB, Maloney C, Li GZ, Ryu ST.
Altered regulation of IL-6 production with normal ageing. Possible linkage to the age associated decline in dehydroepiandrosterone and its sulphated derivative
J Immunol 1993; 150: 5219-5230.
163. Daynes RA, Araneo BA, Dowell TA, Huang K, Dudley D.
Regulation of murine lymphokine production *in vivo* III. The lymphoid tissue microenvironment exerts regulatory influences over T helper cell function.
J Exp Med 1990; 171: 979-996.
164. Wegmann TG, Lin H, Guibert L, Mossman TR.
Bidirectional cytokine reactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon?
Immunol Today 1993; 14: 353-356.
165. Ren H, Bradley C, Saloga J, Loader J, Gelfand EW.
T cells expressing specific V β elements regulate immunoglobulin E production and airways responsiveness .
J Exp Med 1993; 177: 1175-1180.
166. Ho DD, Hartshorn KK, Rota TR, Andrews CA, Chaplain JC, Hirsute MS.
Recombinant human infection alpha A suppression HTLV III replication .
Lancet 1985; 1: 602-604.
167. Shirazi T, Pith PM.
Alpha interferon inhibits early stages of the human immunodeficiency virus type-1 replication cycle.
J Viral 1992; 60: 1321-1328.
168. Poli G, Orenstein JM, Kinter A, Folks TM, Fausi AS.
Interferon alpha but not AZT suppresses HIV expression in chronically infected cell lines.
Science 1989; 244: 575-577.
169. Koyanagi Y, O'Brien WA, Zhao JQ, Golde DW, Gasson JC, Chen IS.
Cytokines alter production of HIV-1 from primary mononuclear phagocytes.
Science 1988; 241: 1673-1675.

170. Biswas P, Poli G, Kinter AL, .
Interferon gamma induces the expression of human Immunodeficiency virus in persistently infected promonocytic cells (U1) and redirects production of virions to intracytoplasmic vacuoles in PMA -differentiated U1 cells.
J Exp Med 1992; 176: 739-750.
171. Peterson PK, Gekker G, Chao CC, Schut R, Molitor TW, Balfour HH Jr.
Cocaine potentiates HIV 1 replication in human blood mononuclear cell co-cultures involvement of transforming growth factor- beta.
J Immunol 1991; 146: 81-84.
172. Lazdins JK, Klimkait T, Woods-Cook K, .
effect of transforming growth factor-beta on HIV1 infection in primary mononuclear phagocytes.
J Immunol 1991; 147: 1201-1207.
173. Kazaki F, Mathij S, Change J, *et al.*
Recombinant interleukin 4 stimulates human immunodeficiency virus production by infecte dmonocytes and macrophages.
J Gen Virol 1992; 73: 941-949.
174. Clerici M, Roilides E, Butlerk
Changes in T helper cell function in human immunodeficiency virus infected children during didanosine therapy as a measure of anti retroviral activity.
Blood 1992; 80: 2196-2202.
175. Van Noesel CJ, Gruters RA, Terpsta FA, Miedema F.
Functional and phonotypic evidence for a selective loss of memory T cells in asymptotativ human immunodeficiency virus infected men.
J Clin Invest 1990; 86; 293-299.
176. Meyaard L, Schuitmaker H, Midema F.
T cell dysfunction in HIV infection: anergy due to defective antigen presenting cell function.
Immunol Today 1993; 14: 161-164.
177. Weinhold KJ, Lylerly HK, Stanley SD Austin AA, Matthews TJ, Bolognesi DP.
HIV-1 gp120 mediated immunw suppression and lymphocyte destruction in the ansence of viral infection.
J Immunol 1989; 142: 3091-3097.
178. Viscidi RP, Mayur K, Lederman HM, Frank AD.
Inhibition of antigen induced lymphocyte proliferation by Tat protein from HIV-1. *Science* 1989; 246:1606-1608.

179. Laurence J, Gottlids A, Kunkel HG.
Soluble suppressor factors in patients with acquired immunodeficiency syndrome and its prodrome. Elaboration in vitro by T lymphocyte.
J Clin Invest 1983; 72: 2072-2081.
180. Shearer GM, Clerici M.
T helper immune dysfunction in asymptomatic, HIV-1 seropositive individuals: the role of Th1-TH2 dysregulation.
Prog Chem Immunol 1992; 54: 21-43.
181. Clerici, Stocks NI, Zajac RA.
Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus immunodeficiency virus seropositive patients: independence of Cd4 clinical staging.
J Clin Invest 1989; 84: 1892-1899.
182. Clerici M, Shearer GM.
A Th1-TH2 switch is critical step in the etiology of HIV infection.
Immunol Today 1993; 14(3): 107-111.
183. Clerici M, Giogi JV, Chau CC, .
Cell mediated immune response to human immunodeficiency virus (HIV) type 1 in seronegative homosexual men with recent exposure to HIV.
J Infect Dis 1992; 165: 1012-1019.
184. Clerici M, Hakim FT, Venzon DJ, .
Changes in interleukin 2 and interleukin 4 production in asymptomatic human immunodeficiency virus seropositive individuals.
J Clin Invest 1993; 262: 1721-1724.
185. Clerici M, Lucey DR, Berzofsky JA, .
Restoration of HIV-specific cell-mediated immune responses by interleukin-12 *in vitro* .
Science 1993; 262: 1721-1724.
186. Clerici M, Wynn TA, Berzofsky JA *et al*.
Role of interleukin 10 in T helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus.
J Clin Invest 1994; 93: 768-775.
187. Collette Y, Change H-L, Cerdan C, .
Specific Th1 cytokine down-regulation associated with primary clinically derived human immunodeficiency virus type 1 Nef gene induced expression.
J Immunol 1996; 156: 360-370.

188. Maggi E, Mazzetti M, Ravina A .
Ability of HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells.
Science 1994; 265: 248-252.
189. Hyjek E, Lischner HW, Hyslop T, .
Cytokine patterns during progression to AIDS in children with perinatal HIV infection.
J Immunol 155: 4060-4071.
190. Meyaard L, Otto SA, Keet IPM, van Lier RAW, Miedema F.
Changes in cytokine secretion patterns of CD4⁺T cell clones in human immunodeficiency virus infection.
Blood 1994; 34: 4262-4268.
191. Poli G, Fauci AS.
The effect of cytokines and pharmacological agents on chronic HIV infection.
AIDS Re Hum Retrovir 1992; 8: 191-197.
192. Chemini J, Star SE, Frank I, .
Impaired interleukin-12 production in human immunodeficiency virus infected patients.
J Exp Med 1994; 179: 1361-1366.
193. Paganin C, Frank I, Trinchieri G.
Priming for high interferon- γ production induced by interleukin-12 in both CD4⁺ and CD8⁺T cell clones from HIV infected patients.
J Clin Invest 1995; 96: 677-1682.
194. Wenner C, Guler ML, Macatonia SE, O'Garra A, Murphy KM.
Role of IFN- γ and IFN- α in IL-12 induced TH1 development.
J Immunol 1996; 156: 1442-1447.
195. Parronchi P, De Carli , Manetti R, .
IL-4 and IFN(s) (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by TH1 or TH2 human T cell clones.
J Immunol 1992; 149: 2977-2982.
196. Vyakarnam A.
Mechanisms for an opposite role for TH1/TH2 cells in AIDS.
Res Immunol 1994; 145: 618-624.

197. Israle Biet D, Labrousse F, Tourani J-M.
Elevation of IgE in HIV infected children and its correlation with the progression of disease.
J Allergy Clin Immunol 1995; 95: 627-632.
198. Bentwich Z, Kalinkovic A, Weisman L.
Immune activation is a dominant factor in the pathogenesis of African AIDS.
Immunol Today 1995; 16: 187-191.
199. Smith CA, Gruss HJ, Davis T *et al.*
CD30 antigen, a marker of Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF.
Cell 1993; 73: 1349-1360.
200. Del Prete GF, De Carti M, Amerigogna F, *et al.*
Preferential expression of Cd30 by human CD4 T cells producing TH2 type cytokines.
FASEB J 1995; 9: 81-86.
201. Maggi E, Giudizi MG, Biagiotti R *et al.*
Th2 like CD8 cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency type 1 infection.
J Exp Med 1994; 180: 489-495.
202. Pizzolo G, Vinante F, Morosat OL, *et al.*
High serum levels of the soluble form of CD30 molecule in the early phase of HIV-1 infection as an independent predictor of progression to AIDS.
AIDS 1994; 8: 741-745.
203. Biswas P, Smith CA, Goletti D, Hordy EC, Jackson RW, Fauci AS.
Crosslinking of CD30 induces HIV expression in chronically infected T cells.
Immunity 1995; 2: 587-596.
204. Mc Donald PP, Cassatella MA, Bald A *et al.*
CD30 ligation induces nuclear factor κ B activation in human T cell lines.
Eur J Immunol 1995; 25: 2870-2876.
205. Maggi E, Annunziato F, Manetti R, *et al.*
Activation of HIV expression by CD30 triggering in CD4 T cells from HIV infected individuals.
Immunity 1995; 3: 251-255.

206. Romagnani S, Annunziato F, Manetti R, *et al.*
Role for CD30 in HIV expression.
Immunol Lett 1996; 51: 83-88.
207. Beutler B, van Huffel C.
Unravelling function in the TNF ligand and receptor families.
Science 1994; 264: 667-668.
208. Katsikis PD, Wunderloch ES, Smitch CA, Herzenberg LA.
Fas antigen stimulation induced marked apoptosis of T lymphocytes in human immunodeficiency virus infected individuals.
J Exp Med 1995; 181: 2029-2036.
209. Han S, Stadler T, Wernil M, *et al.*
Downmodulation of CD4+ T helper type 2 and type0 cells by T helper type 1 via Fas/Fas L interaction.
Eur J Immunol 1995; 16: 569-574.
210. Lynch DH, Ramsdell F, Anderson MR.
Fas and Fas L in the homeostatic regulation of immune responses.
Immunol Today 1995; 16: 569-574.
211. Clerici M, Sarin A, Coffman RL, *et al.*
Type1/Type cytokine modulation of T cell programmed death as a model for HIV pathogenesis.
Proc Natl Acad Sci 1994; 91: 11811-11815.
212. Oyaizu N, Pahwa S. Role of apoptosis in HIV disease pathogenesis.
J Clin Immunol 1995; 15: 217-231.
213. Meyaard L, Otto SA, Jonker RR, Mijster MJ, Keet RPM, Midema F.
Programmed death of T cells in HIV-1 infection.
Science 1992; 257: 217-219.
214. Lewis DE, Ng Tang DS, Adu Oppong A, Schober W, Rodgers JR.
Anergy and apoptosis in CD8+ T cells from HIV infected persons.
J Immunol 1994; 153: 412-420.
215. Meyaard L, OttoSA, Keet IPM, Roos MTL, Miedema F.
Programmed death of T cells in human immunodeficiency virus infection.
J Clin Invest 1994; 93: 982-988.

216. Ramkrishna T, Chandrasekhar P.
The control of tuberculosis: a continuous game of snakes and ladders.
J Biosci 1999; 24(2): 143-152.
217. Steel JH, Ranney AF.
Animal tuberculosis.
Am Rev Tuberc 1958; 77: 908-922.
218. Formicola V, Milanesi Q, Scarsini C.
Evidence of spinal tuberculosis at the beginning of the forth millennium BC from Al Arene Candide Cave (Liguria, Italy).
Am J Phys Anthropol 1987; 72:1-6.
219. Koch R.
Die Aetiologie der Tuberculose.
Am Rev Tuberc 1932; 25: 285-323. [Translated from the original 1882 article by Berna Pinner and Max Pinner].
220. Bloom BR and Murray CJL.
Tuberculosis : Commentary on a reemergent killer.
Science 1992; 257: 1055-1064.
221. Canetti G.
The tubercle bacillus in the pulmonary lesion of man. Springer Publishing Co. Inc New York 1955, p130.
222. Dannenberg Am Jr. Tomashefski JF Jr.
Pathogenesis of pulmonary tuberculosis p 1821-1842. In: Ed. Fishman AP, pulmonary diseases and disorders, 2nd Ed, Vol 3, mc Graw Hill Book Co, New York. 1988, p 1821-1842.
223. Pool JCF and Florey HW.
Chronic inflammation and tuberculosis In: Flowery HW (ed), General pathology, 4th edition. The WB Saunders CO, Philadelphia. 1970; p1183-1224.
224. Hemsworth GR and Kochan I.
Secretion of antimicrobial fatty acids by normal and activated macrophages.
Infect Immune 1978; 19: 170-177.
225. Rook GAW, Bloom BR. In: Tuberculosis :pathogenenesis, protection and control. Blomm BR, editor. Mechanism of pathogenesis in tuberculosis. ASM Press; Washington DC, 1994 pg 485-502.

226. Yammamura Y, Ogawa Y, Maeda H, Yammamura Y.
Prevention of tuberculosis cavity formation by desensitisation with tuberculin active peptide.
Am Rev Respir Dis 1974; 109: 594-601.
227. Dannenberg Am Jr and Sugimoto.
Liquefaction of caseous foci in tuberculosis.
Am Rev Respir Dis 1976; 113: 257-259.
228. Orme Im, Collin FM.
Protection against *Mycobacterium tuberculosis* infection by adoptive immunotherapy.
J Exp Med 1983; 74-83.
229. May ME, Sapgnuolo.
Evidence for activation of a respiratory burst in the interaction of human neutrophils with *Mycobacterium tuberculosis*
Infect Immunity 1987; 55: 2304-2307.
230. Bermudez LEM and Young LS.
Natural killer cell dependent mycobacterio static and mycobacteriocidal activity in Human macrophages.
J Immunol 1991; 146-265-270.
231. Castro AG, Esaguy N, Macedo PM, Aguas AP, Silva MT.
Live but not heat killed mycobacteria cause rapid chemotaxis of large numbers of eosinophils *in vivo* and are ingested by the attracted granulocytes.
Infect Immun 1991; 59: 3009-3014.
232. Barnes PF, Chatterjee D, Abrams JS, .
Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan relationship to chemical structure.
J Immunol 1992; 307: 1593-1597.
233. Toosi Z, Young T, Gogate P, Ellner JJ.
Expression of transforming growth factor β in lung granulomas and peripheral blood manocytes of patients with tuberculosis.
Twenty Sixth Joint Conf. Tuberc Lepr.1991; Abstr; p 57-62.
234. Valone SE, Rich EA, Wallis RA, Ellner JJ.
Expression of tumor necrosis factor *in vitro* by human mononuclear phagocytes
stimulated with whole *Mycobacterium bovis* BCG and mycobacterial antigens.
Infect Immun 1988; 56: 3313-3315.

235. Dinarello C.
Interleukin-1 and pathogenesis of the acute-phase response.
N Engl J Med 1984; 311: 1412-1418.
236. Hirano T, Akira S, Taga T, Kishimoto T.
Biological and clinical aspects of interleukin 6.
Immunol Today 1990; 11: 443-449.
237. Ding AH, Nathan CF, Stuehr PJ.
Release of reactive nitrogen intermediate and reactive oxygen intermediate, and reactive oxygen intermediates from mouse peritoneal macrophages comparison of activating cytolines and evidence for independent production.
J Immunol 1988; 141: 2407-2412.
238. Bermudez LEM and Young LS.
Tumor necrosis factor, alone or in combination with IL-2 but not IFN- γ is associated with macrophage killing of *Mycobacterium avium* complex.
J Immunol 1988; 140: 3006-3013.
239. Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P.
The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection.
Cell 1989; 56: 731-740.
240. Beutler B, Cerami A.
Cachectin more than a tumor necrosis factor.
N Engl J Med 1987; 316: 379-385.
241. Mossman TR and Moore KW.
The role of IL-10 in crossregulation of TH1 and TH2 responses.
Immunol Today 1991; 12: A 49-A53.
242. Ding AH, Nathan C, Graycar J, Dernyck R, Stuhr DJ, Srima S.
Macrophage deactivating factor and transforming growth factors- β 1, β 2 and β 3, inhibit macrophage nitrogen oxide synthesis by interferon γ .
J Immunol 1990; 145: 940-944.
243. Ellner JJ.
Suppressor adherent cells in human tuberculosis.
J Immunol 1978; 121: 2573-2578.
244. Twardy DJ, Schacter DZ, Ellner JJ.
Association of altered dynamics of monocyte expression of human leukocyte antigen- DR with immunosuppression in tuberculosis.
J Infect Dis 1984; 149: 31-37.

245. Fujiwara H, Kleinhens ME, Wallis RS, Ellner JJ.
Increased interleukin-1 production and monocyte suppressor cell activity associated with human tuberculosis.
Am Rev Respir Dis 1986; 133: 73-77.
246. Toosi Z, Sedor JR, Lapurge JP, Ondash RJ, Ellner JJ.
Expression of functional interleukin 2 receptors by peripheral blood monocytes from patients with active pulmonary tuberculosis.
J Clin Invest 1990; 85: 1777-1784.
247. Takashima S, Veta C, Tsuyuguchi I, Kishimoto S.
Production of tumor necrosis factor by monocytes from patients with pulmonary tuberculosis.
Infect Immune 1990; 58: 3286-3292.
248. Ogawa T, Uchida H, Kusumoto Y, Mori Y, Yamamura Y, Hamada S.
Increase in tumor necrosis factor alpha and interleukin 6 secreting cells in peripheral blood mononuclear cells from subjects infected with *Mycobacterium tuberculosis*.
Infect Immune 1991; 59: 3021-3025.
249. Barnes PF, Lu S, Arms JS, Wang E, Yamamura M, Modlin RL.
Cytokine production at the site of disease in human tuberculosis.
Infect Immun 1993; 61: 3482-3489.
250. Moreno C, Mehler A, Lamb J.
The inhibitory effects of effects of mycobacterial lipoarabinomannan and polysaccharides upon polyclonal and monoclonal human T cell proliferation.
Clin Exp Immunol 1988; 74: 206-210.
251. Flesch IE, Kauffman CHE. Mechanisms involved in mycobacterial growth inhibition by gamma interferon activated bone marrow macrophages: role of reactive nitrogen intermediates.
Infect Immune 1991; 39: 3213-3218.
252. Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages.
J Exp Med 1992; 1111-1112.
253. Rook GAW, Steele J, Fraher L, Barker S, Karmali R, O'Riordan J.
Vitamin D3, gamma interferon and control of proliferation of *Mycobacterium tuberculosis* by human monocytes.
Immunology 1986; 57: 159-163.

254. Denis M, Gregg ED, Ghandirian E.
Cytokine modulation of *Mycobacterium tuberculosis* growth in human macrophages.
Int J Immunopharmacol 1990; 12: 721-727.
255. Blanchard DK, Michelini-Norris MB, Djeu JY.
Interferon decreases the growth inhibition of *Mycobacterium avium intracellulare* complex by fresh human monocytes but not by culture derived macrophages.
J Infect Dis 1991; 164: 152-157.
256. Chan J, Fan X, Hunter SW, Brennan PJ, Bloom BR.
Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages.
Infect Immune 1991; 59: 1755-1761.
257. Chatterjee D, Roberts AD, Lowell K, Brennan PJ, Orme JM.
Structural basis of capacity of lipoarabinomannan to induce secretion of tumor necrosis factor.
Infect Immun 1992; 60: 1249-1253.
258. Wallis RS, Paranjpe R, Phillips M.
Identification by two dimensional gel electrophoresis of a 58 kilodalton tumor necrosis factor inducing protein of *Mycobacterium tuberculosis*.
Infect Immun 1993; 61: 627-632.
259. Pwdrazzini T, Hug K, Louis JA.
Importance of L3T4+ and Lyt-2+ cells in the immunologic control of infection with *Mycobacterium bovis* strain bacillus Calmette-Guerin in mice.
J Immunol 1987; 139: 2032-2037.
260. Muller I, Cobbold SP, Waldman H, Kaufman SHE. Impaired resistance to M. tuberculosis infection after selective *in vivo* depletion of L3T4+ and Lyt 2+T cells.
Infect Immun 1987; 55: 2037-2041.
261. Barnes PF, Mistry SD, Cooper Primez C, Rea TH, Modlin RL.
Compartmentalization of a CD4T lymphocyte subpopulation in tuberculosis pleuritis.
J Immunol 1989; 142; 1114-1119.
262. Barnes PF, Bloch AB, Davidson PT, Snider DE Jr.
Tuberculosis in patients with human immunodeficiency virus infection.
N Engl J Med. 1991; 324: 1644-1650.

263. Del Prete GF, De Carli M, Falgiani P, .
Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand *in vitro* human T cells with stable and opposite (type 1 T helper or type 2 helper) profile of cytokine production.
J Clin Invest 1991; 88:346-350.
264. Lorget F, Kerran MM, Lukey PT, Ress SR.
Evidence for *in vivo* generation of cytotoxic T cells: PPD stimulated lymphocytes from tuberculosis pleural effusions demonstrated enhanced cytotoxicity with accelerated kinetics of induction.
Am Rev Respir Dis 1992; 145: 418-423.
265. Kaufman SHE.
CD8 T lymphocytes in intracellular microbial infections.
Immunol Today 1988; 9: 168-174.
266. Orme IM, Miller ES, Roberts SK, *et al*.
T lymphocytes mediating protection and cellular cytolysis during the course of *Mycobacterium tuberculosis* infection.
J Immunol 1992; 148: 189-196.
267. Dannenberg AM.
Delayed type hypersensitivity and cell mediated immunity in the pathogenesis of tuberculosis.
Immunol Today 1991; 12: 228-233.
268. Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR.
Major histocompatibility complex class II restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection.
Proc Natl Acad Sci 1992; 89: 12013-12017.
269. Ottenhoff THM and Mutis T.
Specific killing of cytotoxic T cells and antigen-presenting cells by CD4 cytotoxic T cell clones.
J Exp Med 1990; 171: 2011-2024.
270. Barnes PF, Mistry SD, Cooper CL, Rea TH, Modlin RL.
Compartmentalization of a CD4 T cell lymphocyte subpopulation in tuberculosis pleuritis.
J Immunol 1989; 142: 1114-1119.

271. Jones BE, Young MM, Antomiskis D, Davidson PT, Kramer F, Barnes PF.
Relationship of the manifestations of tuberculosis to CD4 cell counts in patients with human immunodeficiency virus infection.
Am Rev Respir Dis 1993; 148: 1292-1297.
272. Kabelitz D, Bender A, Schondelmaier S, Schole B, Kaufmann SHE.
A large fraction of human peripheral blood gamma/delta T cells is activated by
Mycobacterium tuberculosis But not by its 65 KD heat shock protein.
J Exp Med 1990; 171: 667-679.
273. Follows GA, Munk ME, Gatrill AJ, Conradt P, Kaufmann SHE.
Gamma interferon and interleukin 2, but not interleukin 4 are detectable in gamma
delta T cell cultures after activation with bacteria.
Infect Immun 1992; 60: 1229-1231.
274. Munk ME, Gatrill AJ, Schoel B, *et al.*
Immunity to mycobacteria and gamma/delta T lymphocytes.
Acta Pathol Microbiol Immunol 1990; 98: 669-673.
275. Barnes PF, Grisso CL, Abrams JS, Band H, Rea T, Modlin RL..
 δ T lymphocytes in human tuberculosis.
J Infect Dis 1992; 165: 506-512.
276. Barnes PF, Abrams JS, Lu S, Sieling PA, Rea T, Modlin RL.
Patterns of cytokine production by mycobacterium reactive human T cell clones.
Infect Immun 1993; 61: 197-203.
277. Pfeffer KB, Schole H, Gulle H, Kaufmann SHE.
Primary responses of human T cells to mycobacteria: a frequent set of
 $\gamma\delta$ T cells are stimulated by protease resistant ligands.
Eur J Immunol 1990; 20: 1175-1179.
278. Tsuguguchi I, Kawasumi H, Ueta C, Yano I, Kishimoto S.
Increase of T cell receptor gamma/delta bearing T cells in cord blood of newborn
babies obtained by *in vitro* stimulation with mycobacterial chord factor.
Infect Immune 1991; 59: 3053-3059.
279. Modlin RL, Pirmez C, Hofman FM.
Lymphocytes bearing antigen specific $\gamma\delta$ T cell receptors accumulate in human infectious disease lesions.
Nature 1989; 339: 544-548.

280. Cooper AM, Daltob DK, Stewart TA, Griffith JP, russel DC, Orme IM.
Disseminated tuberculosis in interferon γ gene disrupted mice.
J Exp Med 1993; 178: 2243-2247.
281. Cohn ZA, Kaplan G.
Hansen's disease cell mediated immunity and recombinant lymphokines.
J Infect Dis 1991; 163: 1195-1200.
282. Haanen JBAG, de Waal Malefijt R, Res PM, .
Selection of human T helper type 1 like T cell subste by Mycobacteria.
J Exp Med 1991; 174: 583-592.
283. Tsicopoulos A, Hamid Q, Varney Y, .
Preferential messenger RNA expression of the TH1 type cells (IFN- γ + IL-2+) in
classical delayed type (tuberculin) hypersensititivity reactions in human skin.
J Immunol 1992; 148: 269-274.
284. Sander B, Skansen-Saphir U, Damm O, Hakansson L, Andersson J, Andersson U.
Sequential production of Th1 and Th2 cytokines in response to live bacillus Calmette-
Gurine.
Immunology 1995; 86: 512-518.
285. Zang M, Gately MK, Wang E, .
Interleukin 12 at the site of disease in tuberculosis.
J Clin Invest 1994; 93: 1733-1739.
286. Orme IM.
The kinetics of emergence and less of mediator T lymphocytes acquired in
response to infection with *Mycobacterium tuberculosis*.
J Immunol 1987: 293-298.
287. Barnes PF, Fong SJ, Twomwy PF, Mazumdar A, Modlin RL.
Local production of tumor necrosis factor and IFN- γ in tuberculosis patients.
J Immunol 1990; 145: 149-154.
288. Sanchez FO, Rodriguez JI, Agudelo G, Garcia LF.
Immune responsiveness and lymphokine production in patients with tuberculosis and healthy controls.
Infect Immun 1994; 62: 5673-5678.

289. Toosi Z, Gogate P, Shiratsuchi h, Yong T, Ellner JJ.
Enhanced production of TGF- β by blood monocytes from patients with active tuberculosis and presence of TGF- β in tuberculous granulomatous lung lesions.
J Immunol 1995; 154: 465-473.
290. Modlin RL, Barnes PF.
and the human immune response to mycobacteria.
Res Immunol 1995; 146: 526-530.
291. Flynn JL, Goldstein MM, Triebold KJ, Sypek J, Wolf S, Bloom BR.
Increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection.
J immunol 1995; 155: 2515-2524.
292. Misra N, Murtaza A, Walker B, .
Cytokine profile of circulating T cells of leprosy patients reflects both indiscriminate and polarized T helper subsets: T helper phenotype is stable and uninfluenced by related antigens of *Mycobacterium leprae*.
Immunology 1995; 86: 97-93.
293. Bates JH.
Tuberculosis: susceptibility and resistance.
Am J Rev Respir Dis 1982; 125: 20-24.
294. Dolin PJ, Raviglione MC, Kochi A.
Global tuberculosis incidence and mortality during 1900-2000.
Bull WHO 1994; 72: 213-220.
295. Di Perri G, Cruciani M, Danzi MC, *et al*.
Nosocomial epidemiology of active tuberculosis among HIV infected patients.
Lancet 1989; 2: 1502-1504.
296. Selwin PA, Hartel O, Lewis VA, *et al*.
A prospective study of the risk of tuberculosis among intravenous drug users with HIV.
N Engl J Med 1989; 320: 545-550.
297. Lane HC, masur H, Gelman EP, *et al*.
Correlation between immunologic function and clinical subpopulations patients with the acquired immune deficiency syndrome.
Am J Med 1985; 78: 417-422.
298. Krowka JF, Stites DP, Jain S, *et al*.
Lymphocyte proliferative responses to human immunodeficiency virus antigens *in vitro*.
J Clin Invest 1989; 1198-1230. 160

299. Midema F, Petit A, Terpsta FG, *et al.*
Immunologic abnormalities in human immunodeficiency virus infected asymptomatic homosexual men. HIV affects the immune system before CD4⁺T helper cell depletion occurs.
J Clin Invest 1988;82: 1908-1914.
300. Kumararatne DS, Pithie A, Bassi DE, Barlett R.
Mycobacterial immunity and mycobacterial disease in relation to HIV infection. In: Bird G, *et al.* Immunology of HIV infection. Boston: Kluwer Academic Publishers; 1992; 113-154.
301. Rose P.
Immunology of the lung in HIV infection: the pathophysiologic basis for the development of tuberculosis in the AIDS setting.
Bull Int Union
Tuberc lung Dis 1991; 66: 15-20.
302. Orme IM, Anderson P, Boom WH.
T cell responses to Mycobacterium tuberculosis.
J Infect Dis 1993; 167: 1481-1497.
303. Fauci AS, Schittman SM, Poli G, Kowing S, Pantaleo G.
Immunopathogenesis of mechanisms in human immunodeficiency virus (HIV) infection.
Ann Intern Med 1991; 66: 15-20.
304. Chaisson RE, Schechter GF, Theuer CP, *et al.*
Tuberculosis in patients with acquired immunodeficiency syndrome: clinical features, response to therapy and survival.
Am Rev Respir Dis 1987; 136: 570: 574.
305. Whalen C, Horshurgh CR, Hom D, *et al.*
Accelerated course of human immunodeficiency virus infection after tuberculosis.
Am J Respir Crit Care Med 1995; 151: 129-135.
306. Ellner JJ.
Tuberculosis in the time of AIDS (editorial)
Chest 1990; 98: 1051.
307. Vjecja m, Okwera A, Byekwaso F, *et al.*
Predictors of mortality and drug toxicity in HIV infected patients from Uganda treated for pulmonary tuberculosis (Abstract). In: program and abstracts: VIII international conference on AIDS (Amsterdam) 1992.

308. Shattock RJ, Friedland JS, Griffin GE.
Modulation of HIV transcription in release from monocyctic cells following phagocytosis of *Mycobacterium tuberculosis*.
Res Virol 1993; 144: 7.
309. Lederman MM, Georges D, Kusner DJ, Mudido P, Giam CZ, Toossi Z.
Mycobacterium tuberculosis and its purified protein derivative activates expression of the human immunodeficiency virus.
J AIDS 1994; 7: 727-733.
310. Zang Y, Nakata K, Weiden M, Rom WN.
Mycobacterium tuberculosis enhances human immunodeficiency virus 1 replication by transcriptional activation of long terminal repeat.
J Clin Invest 1995; 95: 2324-2331.
311. Schauf V, Rom WN, Smith KA, *et al*.
Cytokine gene activation and modified responsiveness to interleukin 2 in the blood of tuberculosis patients.
J infect Dis 1993; 168: 1056-1059.
312. Osborn LS, Kunkel S, Nabel GJ.
Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κ B.
Proc Natl Acad Sci 1989; 86: 2336-2340.
313. Rosenberg ZF, Fauci AS. Immunopathogenesis of HIV infection.
FASEB J 1991; 5: 2382-2390.
314. Greene WC.
The molecular biology of human immunodeficiency virus type 1 infection.
N Engl J Med 1991; 324:308-317.
315. Tong-Starksen SE, Luciw PA, Peterlin PM.
Human immunodeficiency virus long terminal repeat responds to T cell activation signals.
Proc Natl acad Sci 1987; 84: 6845-6849.
316. Goletti D, Weissman D, Jackson RW, *et al*.
Effect of *Mycobacterium tuberculosis* on HIV replication: role of immune activation.
J Immunol 1996; 157:1271-1278.

317. Vanham G, Edmonds K, Qung L, *et al.*
Generalised immune activation in pulmonary tuberculosis co-activation with HIV infection.
Clin Exp Immunol 1996; 103:30-34.
318. Garritt V, Cadranet J, Esvant H.
Tuberculosis generates a microenvironment enhancing the productive infection of local lymphocytes by HIV.
J Immunol 1997; 159: 2824-2823.]
319. Toossi Z, Sierra Madero JG, Blinkhorn RA, Meuter MA.
Enhanced susceptibility of blood monocytes from patients with pulmonary tuberculosis to productive infection with human immunodeficiency virus.
J Exp Med 1993; 177: 1511.
320. Hirsch CS, Hussain R, Toossi Z, Dawood G, Shahid F, Ellner JJ.
Crossmodulation by transforming growth factor β in human tuberculosis suppression of antigen driven blastogenesis and interferon gamma production.
Proc Natl Acad Sci 1996; 93: 3193-3198.
321. Gong G, Zang M, Modlin RL, *et al.*
IL-10 downregulates Mycobacterium tuberculosis induced Th1 responses and CTLA-4 expression.
Infect Immun 1996; 64: 913-918.
322. Hirsch CS, Yoneda T, Averill L, Ellner J, Toossi Z.
Enhancement of intracellular growth of Mycobacterium tuberculosis in human monocytes by Transforming growth factor β .
J Infect Dis 1994; 170: 1229-1237.
323. Naif HM, Chang J, Ho-Shon M, Cunningham AL.
Inhibition of human immunodeficiency virus replication in differentiating monocytes by interleukin10 occurs in parallel with inhibition of cellular RNA expression.
AIDS Res Hum Retroviruses 1996; 12: 1237-1245.
324. Weissman D, Poli G, Fauci AS.
IL-10 synergizes with multiple cytokines in enhancing HIV production in cells of monocytic lineage.
J Acquir Immune Defic Syndr Hum Retrovirol 1995; 9: 442-449.
325. Hohnson BJ, McMurray DN.
Cytokine gene expression by culture of human lymphocytes with autologous Mycobacterium tuberculosis infected monocytes.
Infect Immun 1994; 62: 1444-1450.

326. Lin Y, Zang M, Hofman FM, *et al.*
Absence of prominent Th2 cytokine response in human tuberculosis.
Infect Immune 1996; 64: 1351-1356.
327. Surcel HM., Troye-Blomberg M, Paulie S, *et al.*
Th1/Th2 profiles in tuberculosis based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens.
Immunology 1994; 81: 171-176.
328. Wallis RS, Vjecja M, Amir Tahamasseb A, *et al.*
Influence of tuberculosis on human immunodeficiency virus (HIV-1) enhanced cytokine expression and elevated β 2 microglobulin in HIV-1 associated tuberculosis.
329. Zang M, Gong J, Iyer DV, *et al.* T cell cytokine responses in patients with tuberculosis and human immunodeficiency virus infection.
J Clin Invest 1994; 94: 2435-2442.
330. World Health Organization Surveillance of HIV infection.
recommendations in training module. Annex D.P. B3. Draft Dec 1992.
331. Hattikudur S and Kamat RS.
Polymorphism of mycobacterial antigens, particularly in cell mediated immunity.
J Med Microbiol 1985; 19: 69-75.
332. Maluish AE, Strong DM.
Lymphocyte proliferation. In: Manual of clinical laboratory Immunology. 3rd edition, Rose NR, Friedman H, Fahey JL, editors. Washington DC, 1986; 274-281.
333. Gillis S, Fern MM, Winny OU, Kendall SA.
T cell growth factor parameters of production and a quantitative microassay for activity.
J Immunol 1978; 120: 2027-2037.
334. Armitage P. Statistical methods in medical research Oxford London: Blackwell Scientific Publications, 1997.
335. Jannossy G, Greaves MF,.
Lymphocyte activation response of T and B lymphocytes to phytoimitogens.
Clin Exp Immunol 1971; 9: 483-489.

336. Farrar JJ, Benjamin WR, Hilfiker ML, *et al*.
The biochemistry, biology and role of interleukin 2 in the induction of cytotoxic T cell and antibody forming B cell responses.
Immunol Rev 1982; 63: 129-166.
337. Stiles CD.
Autocrine control of growth.
Nature 1984; 311: 604-605.
338. Waldman TA.
The structure and expression of interleukin receptors on normal and malignant lymphocytes.
Science 1986; 232: 727.
339. Efrat S and Kaempfer K.
Control of biologically active interleukin 2 messenger RNA formation in induced human lymphocytes.
Proc Natl Acad Sci 1984; 81: 2601-2605.
340. UNAIDS. Tuberculosis and AIDS. October 1997; pg1-8.
341. Reichman LB, O'Day R.
Tuberculosis infection in a large urban population.
Am Rev Respir Dis 1978; 117: 705-712.
342. Styblok EDA.
The impact of infection with HIV on tuberculosis. In: Mitchell DM.editor. Recent advances respiratory medicine. London: Churchill Livingstone pub. 1991; 5: 147-162.
343. Comstock GW and Cauthen GM.
Epidemiology of tuberculosis. In: Reichman LB and Hershfield ES. Editors: tuberculosis: A comprehensive international approach, New York, Marcel Dekker Inc Pub; 1993: 23-48.
344. Mangtani P, Jolley DJ, Watson JM and Rodrigues L.
Socioeconomic deprivation and notification rates for tuberculosis in London during 1991-1992.
BMJ 1995; 310: 963-966.
345. Friedman LN, Sullivan GM, Bevilacqua RP, Loscos R.
Tuberculosis screening in alcoholics and drug addicts.
Am Rev Respir Dis 1987; 136: 1188-1192.
346. Alcaide J, Altet MN, Plans P.
Cigarette smoking as a risk factor for tuberculosis in young adults: A case control study.
Tubercle Lung Dis 1996; 77: 112-116.

347. Decock KM, Soro B, Cocilibaly IM, Lucas SB.
Tuberculosis and HIC infection in sub Saharan Africa
JAMA 1992; 268: 1581-1587.
348. Elliot AM, Nalwiindi B, Hayes RJ, *et al.*
The impact of Human Immunodeficiency virus on presentation and
diagnosis of tuberculosis in a cohort study in Zambia.
J Trop Med Hyg 1993; 96: 1-11.
349. Chum NJ, O'Bries RJ, Chonde TM, Graf D, Reider HL.
An epidemiological study of tuberculosis and HIV infection in Tanzania
1991-1993.
AIDS 1996; 10: 299-309.
350. Houston S, Ray S, Mahari M, *et al.*
The association of tuberculosis and HIV infection in Harare.
Tuberc Lung Dis 1994; 75: 220-226.
351. Mohanty KC, Nair S, Saharstrabudhe T.
Changing trend of HIV infection and tuberculosis in Bombay area since
1988.
Ind J Tuberc 1995; 42: 117-120.
352. Bhawe GG, Shetty K.
Study of drug resistance of tuberculosis in HIV positive and HIV
negative cases of pulmonary tuberculosis. Msc thesis 1995, University
of Bombay.
353. Sambasiva Rao R, Amarnath SK.
HIV infection in Pondicherry.
Ind J Med Virol 1996; 94: 331-333.
354. Paranjape RS, Tripathe SD, Menon Pd *et al.*
Increasing trend of HIV seroprevalence among pulmonary
tuberculosis patients in Pune India.
Ind J Tuberc 1997; 106: 207-211.
355. Kulshrestha R, Nigam M, Sarin S, Mathur A.
Prevalence of AIDS among tuberculosis patients in Lucknow.
Ind J Med Microbiol 1997; 15(2) 79-81.
356. Talib SH, Bansal MP, Kamble HN.
HIV-I seropositivity in pulmonary tuberculosis (study of 340 cases from
Marathwasa)
Ind J Pathol Microbiol 1993; 36: 383-388.

357. Centres for Disease control and prevention.
National centre for Health statistics. Monthly vital statistics Report
1997; 46: no.1 supp 2.
358. Quinn TC.
AIDS Africa An epidemiological paradigm.
Science 1986; 234: 955-963.
359. Anuradha S, Soloman S, Rahasekaran S.
HIV seropositivity in patients with respiratory disease.
Ind J Tuberc 1993; 40: 13-15.
360. Hazarika A, Chelleng DK.
HIV infection among the high risk groups of upper Assam.
J Com Dis 1995; 27: 15-18.
361. Segal S.
HIV epidemic in Punjab.
Ind J med Res 1995; 101: 47-49.
362. Parulkar N.
Study of association of HIV infection and pulmonary tuberculosis. Msc
Thesis submitted to the University of Mumbai, 1995.
363. Piot P, Laga M.
The global epidemiology of HIV infection continuity, heterogeneity and
change.
J AIDS 1990; 3: 403-412.
364. Dodd CL.
Oral candidiasis in HIV infection: pseudomembranous and
erythematous candidiasis show similar rates of progression to AIDS.
AIDS 1991; 5: 1339-1343.
365. Mann SL.
Prevalence and incidence of herpes virus infections among
homosexually active men.
J Infect Dis 1984; 149: 1026.
366. Kovacs JA.
Cryptococcus in the acquired immunodeficiency syndrome.
Ann Intern Med 1985; 103: 533.
367. Mc Hugh S, Deighton J, Rifkin L *et al.*
Kinetics and functional implications of Th1 and Th2 cytokine
production following activation of peripheral blood mononuclear cells
in primary culture.
Eur J Immunol 1996; 26: 1260-1265.

368. Seiling PA, Chatterjee D, Dorcelli SA, *et al.*
CD1 restricted T cell recognition of microbial lipoglycan antigens.
Science 1995; 269:227.
369. Porcelli S, Morita CT and Brenner MB.
CD1b restricts the response of human CD4⁺CD8⁻ T lymphocytes to a
microbial antigen.
Nature 1992; 360: 593.
370. Pinching AJ, Mc Manus JJ, Jeffree DJ *et al.*
Studies of cellular immunity in male homosexuals in London.
Lancet 1983; 16
371. Roos MT.
T cell function *in vitro* is an independent progression marker for AIDS
in human immunodeficiency virus infected symptomatic subjects
J infect Dis 1995; 171: 531-537.
372. Lucey DR.
Human immunodeficiency virus infection in US air force:
seroconversions clinical staging and assessment of T helper cell
functional assay to predict change in CD4⁺ T cell count.
J Infect Dis 1991; 164: 631-637.
373. Lane HC.
Qualitative analysis of immune function in patients with AIDS
syndrome evidence for a selective defect in soluble antigen
recognition.
N Engl J Med 1985; 313:79-84.
374. Fauci AS.
AIDS: Immunopathogenesis mechanism and research strategies.
Clin Res 1987; 35: 503-510.
375. Harper MC.
Detection of lymphocytes expressing human T lymphotropic virus type
III in lymph nodes and peripheral blood from infected individuals by in
situ hybridization.
Proc Natl Acad Sci 1986; 83: 772-776.
376. Maggi E, Macchia D, Parronchi P *et al.*
Reduced production of interleukin-2 and interferon gamma and
enhanced helper activity for IgG synthesis by cloned CD4⁺ T cells from
patients with AIDS.
Eur J Immunol 1987; 17: 1685-1690.

377. Estaquier J, Idziorek T, Zou W *et al.*
T helper 1/T helper 2 type cytokines and T cell death: preventive effects of interleukin 12 on activation-induced and CD95 (Fas/APO) mediated apoptosis of CD4⁺T cells from human immunodeficiency virus infected persons.
J Exp Med 1995; 182: 1759-1767.
378. Brinkmann V, Kristotic C.
Regulation by corticosteroids of Th1 and Th2 cytokine production in human CD4⁺ effector T cells generated from CD45 Ro⁻ and CD45RO⁺ subset.
J Immunol 1995; 155: 3222-3228.
379. Miller F, Ankrust P, Lien E, Hanes W, Frelund SS.
Enhanced interleukin 10 production in response to *Mycobacterium avium* products in mononuclear cells from patients with human immunodeficiency virus infection.
J Infect Dis 1998; 177: 586-594.
380. Lane HC, Fauci AS.
Immunologic abnormalities in the acquired immunodeficiency syndrome.
Ann Rev Immunol 1985; 3: 477-500.
381. Garry RF.
Potential mechanisms of cytopathic properties of HIV.
AIDS 1989; 3: 683-694.
382. el Ghazali GE, Paulie S, Anderson G *et al.*
Number of interleukin 2 and interferon gamma secreting human T cells reactive with tetanus toxoid and mycobacterial antigen PPD or phytohaemagglutinin show distinct response profiles depending on the type of antigen used for activation.
Eur J Immunol 1993; 23: 2740-2745.
383. Graziosi C, Pantaleo G, Grant KR, *et al.*
Lack of evidence for the dichotomy of Th1 and Th2 predominance in HIV infected individuals.
Science 1994; 265: 248-252.
384. Barcelline W, Rizzardi GP, Borghi MO, Fain C, Lazzarin A, Meroni PL.
TH1 and TH2 cytokine production of peripheral blood mononuclear cells from HIV infected patients.
AIDS 1994; 8: 757-762.

385. Diaz-Mitoma F, Kumar A, Karimi S, *et al.*
Expression of IL-10 IL-4, and interferon γ in unstimulated and mitogen stimulated peripheral blood lymphocytes from HIV seropositive patients.
Clin Exp Immunol 1995; 9: 484-489.
386. Daftarian MP, Diaz-Mitoma F, Creery WD, Cameron W, Kumar A.
Dysregulated productin of interleukin-10 (IL-10) and IL-12 by peripheral blood lymphocytes from human immunodeficiency virus infected individuals is associated with altered proliferative responses to recall antigens.
Clin Diag Lab Immuol 1995; 2: 712; 718.
387. Ameglio E, Fei PC, Solmone M, *et al.*
Serum IL-10 levels in HIV positive subjects: correlation with CDC stages.
J Biol regul Homeost Agents 1994; 8: 48-52.
388. Salvaggio A, Balotta C, Galli M, Clerici M.
CD4 count in HIV infection is positively correlated to interferon γ and negatively correlated to interleukin 10 *in vitro* production.
AIDS; 10: 449-451.
389. Vicenxi E, E, Poli G.
Regulation of HIV expression by viral genes and cytokines.
J leuko Biol 1994; 56: 328-334.
390. Fan J, Bass HZ, Fahey JL.
Elevated IFN- γ and IL-2 gene expression are associated with HIV infection.
J Immunol 1993; 154: 5031-5040.
391. Sanchez FO, Rodriguez JI, Agudelo G *et al.*
Immue responseiveness and lymphokine production in patients with tuberculosis and healthy controls.
Infect Immune 1994; 62: 5673-5678.
392. Silvieira H, Ordway D, Dockrell H, Jackson M, Ventura F.
Cell mediated immune responses to mycobacterial antigens in patients with pulmonary tuberculosis and HIV infection.
Clin Exp Immunol 1997; 110: 26-34.
393. Shiratsucyi H, Tsuguyuchi I.
Analysis of T cell sunsets by monoclonal antibodies in patients with tuberculosis after *in vitro* stimulation with tuberculosis after *in vitro* stimulation with purified protein derivative of tuberculin.
Clin Exp immunol 1984; 57: 271-178.

394. Beck JS, Potts RC, Kardjito T, Grange M.
T4 lymphopenia in patients with active pulmonary tuberculosis.
Clin Exp Immunol 1985; 60: 49-54.
395. Onwubalili JK, Scott GM, Robinson JAR.
Deficient immune interferon production in tuberculosis.
Clin Exp Immunol 1985; 59: 405.
396. Vileek J, Klion A, Henriksen-deSteffano D *et al.*
Defective γ interferon in peripheral blood leukocytes of patients with
acute tuberculosis.
J Clin Immunol 1986; 6: 146
397. Mc Dyer JF, Hackey MN, Walsh TE, Cook JL, Seder RA.
Patients with multidrug resistant tuberculosis with low CD4 T cell
counts have impaired Th1 responses.
J Immunol 1997; 158: 492-500.
398. Sodhi A, Gong J, Silva C, Quin D, Barnes PF.
Clinical correlates of interferon γ production in patients with
tuberculosis.
399. Law KF, Jagirdare J, Weiden MB, Bodkin M, Rom WN.
Tuberculosis in HIV positive patients: Cellular response and immune
activation in the lung.
Am J Respir Crit Care Med 1996; 153: 1377-1384.
400. Schauf V, Rom WN, Smith KA, *et al.*
Cytokine gene activation and modified responsiveness to interleukin 2
in the blood of tuberculosis patients.
J Infect Dis 1993; 168: 1056-1059.
401. Nunn P, Brindle R, Carpenter L *et al.*
Cohort study of human immunodeficiency virus infection in patients
with tuberculosis in Nairobi, Kenya. Analysis of early (6 month)
mortality.
Am Rev Respir Dis 1992; 146: 849-854.
402. Potts BJ, Murray W, Martin MA.
Replication of HIV 1 in primary monocytic cultures.
J Virol 1990; 175: 465-476.
403. Folks TM, Justement J, Kinter A.
Characterisation of a promonocyte clone chronically infected with HIV
and inducible by 13-phorbol-12 myristate acetate.
J Immunol 1988; 140:1117-1122.

404. Latham PS, Lewis AM, Varesuo L, *et al.*
Expression of human immunodeficiency virus long terminal repeat in human promonocytic cell line U937: effect of endotoxin and cytokines.
Cell Immunol 1990; 129: 513-518.
405. Tadmori WD, Mondal D, Tadmori I, Prakash D.
Transactivation of human immunodeficiency virus type 1 long terminal repeats by cell surface tumor necrosis factor α
J Virol 1991; 65: 6425
406. Vyakarnam A, Mc Keating AJ, Meager A, Beverly PC.
Tumor necrosis factors ($\alpha\beta$) induced by HIV 1 in peripheral blood mononuclear cells potentiate virus replication.
AIDS 1990; 4:21.
407. Schuitmaker H, Kootstra NA, Koppelman MH, *et al.*
Proliferation dependent HIV-1 infection of monocytes occurs during differentiation into macrophages.
J Clin Invest 1992; 89: 1154.
408. Griffin GE, Leung K, Folks TM, Kunkel S, Nabel GJ.
Activation of HIV gene expression during monocytes differentiation by induction of NF- κ B.
Nature 1989; 339: 70
409. Garrait V, Cadranel J, Esvant H.
Tuberculosis generates a microenvironment enhancing the productive infection of local lymphocytes by HIV.
J Immunol 1997; 159: 2824-2823.
410. Pape JW, Jean SS, Ho JL, Hafner A, Johnson WDJ.
Effect of isoniazid prophylaxis on incidence of active tuberculosis and progression of HIV infection.
Lancet 1993; 342: 268-272.
411. Atlas RM.
Handbook of microbiological media. Parks LC, editor, USA, CRC press; 1993, p967.
412. Chadwick MV.
In: Baker FJ, editor, Mycobacteria: Monographs in medical laboratory service, England, John Wright and Sons Ltd., 1982.
413. Lowry OH, Rosenberg NJ, Forr AL, Kendall RJ.
Protein measurement with the Folin phenol reagent.
J Biol Chem 1951; 193: 265-275.

414. ◇Uniform requiriments for manuscripts.
Ann Intern Med 1997 (1st Jan)

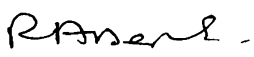
◇ The references quoted in the Bibliography have been cited in accordance to the guidelines of the Vancouver group, which evolved into the international Committee of Medical Journal of editors (ICMJE) developed by the national Library of Medicine

J

SYNOPSIS

**SYNOPSIS OF THE THESIS
TO BE SUBMITTED
TO
THE UNIVERSITY OF MUMBAI
FOR
THE DEGREE OF
MASTER OF SCIENCES
IN MICROBIOLOGY**

TITLE OF THE THESIS	Study of cytokine profile (IL-2, IL-4, IL-10, IFN- γ , TNF- α) in Pulmonary tuberculosis patients co-infected with HIV.
NAME OF THE CANDIDATE	Ms. Smita S. Patil.
NAME AND DESIGNATION OF THE GUIDE	Dr.(Mrs)R.A.Deshmukh. Officer-in-Charge, Department of Virology Haffkine Institute, Parel, Mumbai -12.
PLACE OF RESEARCH	Department of Virology, Haffkine Institute, Parel, Mumbai -12.
DATE OF REGISTRATION	20-11-96
NUMBER OF REGISTRATION	24


SIGNATURE OF THE GUIDE


SIGNATURE OF THE STUDENT

INTRODUCTION:

The acquired immune deficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) has reached epidemic proportions world-wide since the first cases were reported in 1981 [1]. After 17 years, 30 million men, women and children are alive with HIV with 6 million new infections occurring this year [2]. There are diseases for which natural infection and recovery leads to long lasting protective immunity. However, in many parasitic diseases e.g. malaria, leishmaniasis, schistosomiasis, leprosy, tuberculosis, syphilis and some rickettsial diseases, infection persists despite an immune response to pathogen. HIV infection falls in to this category. There are many parallels of the immunobiology between AIDS and other intracellular pathogens in which role of prototypes of T helper cells (TH1 and TH2) are important.

There is considerable evidence to suggest the existence of functionally polarised responses by CD4⁺T helper cell and CD8⁺T cytotoxic cell subsets depending on the cytokines they produce. TH1 cells produce Interferon -gamma (IFN- γ), Interleukin-2 (IL-2), Tumor necrosis factor-beta (TNF- β), and responsible for both humoral and cell mediated immune responses(antibody production, macrophage activation, antibody dependent cell cytotoxicity and delayed type hypersensitivity). TH2 cells produce IL-4, IL-5, IL-6, IL-10, IL-13 and provide optimal help for humoral immune responses. These include mucosal immunity through production of mast cells and eosinophil growth and differentiation and facilitation to IgA synthesis. CD4⁺ T cells with less differentiated cytokine profiles than TH1 or TH2 cells are designated as TH0. They mediate intermediate effects depending on the ratio of lymphokines produced and nature of responding cells [3,4].

It is now well established that the lymphokines produced by TH1 and TH2 cells can exert mutual regulatory interactions. In particular IL-4 and IFN- γ , the principal products of the TH2 and TH1 cells respectively, oppose one another's action. IL-4 prevents priming of naive TH cells to become IFN- γ producers. IL-4 is able to inhibit IL-2 production by naive T cells in response to anti-CD3 antibody and

antigen presenting cells (APC) [5]. IL-4 also inhibits IFN- γ mediated positive feed back stimulation of IL-12 production (a TH1 inducer) by macrophages[6].IL-10 is common to all types of human T helper cells [7]. and exerts negative regulatory effects on some TH1 cell functions. It selectively inhibits IFN- γ mediated feedback stimulation of IL-12 production by macrophages [8]. It suppresses co-stimulatory molecules such as TNF- α and IL-1 and thereby inhibits the stimulatory effects of IL-12 on T and natural killer (NK) cells. IL-10 inhibits secretion of IL-2 production by TH1 cells. IL-2 causes proliferation of CD8⁺T cells. Thus TH2 and other cells that secrete IL-10 regulate clonal expansion of those subsets that produce IFN- γ [9].

IFN- γ can inhibit IL-4 dependent B cell differentiation [10] and the proliferation of human TH2 clones is also inhibited by IFN- γ [7].

The fundamental abnormality in HIV infected individuals is decrease in CD4⁺T lymphocytes. CD4⁺T helper cells secrete factors that affect growth and differentiation of lymphoid cells, haematopoietic cells and then functions of non lymphoid cells as well. Thus the qualitative and quantitative abnormality in CD4⁺T helper cell population in an HIV infected individual have profound effects on immune system [11].

Therapeutic intervention in regulation of the balance between TH1 and TH2 cells is becoming relevant in number of diseases, including AIDS.

A shift away from the dominant production of immune protective type 1cytokines to a dominant type 2 cytokine profile is observed in the progression to AIDS [12]. As HIV infection progresses along with loss of production of IL-2, there is concomitant increase in production of IL-4 in response to mitogen. This suggests the possibility of shift towards TH2 cytokine profile as a part of progression of the disease. The possibility that TH2 cells, while allowing higher virus replication, favour at the same time their own killing and further spread of HIV is supported by *in vivo* observation that HIV infected individuals with elevated IgE levels in serum at the time of infection, progresses more rapidly to full blown AIDS as compared to those HIV infected patients exhibiting normal or low serum IgE [13] .

It has been shown that the multiply exposed HIV seronegative individuals who are virus free, exhibit strong HIV specific cellular immune responses [14]. Thus type 1 cytokines and strong cellular immune responses elicited by them are important in protection against HIV infection and progression to AIDS [15].

Thus, it is possible that the individuals with a low type 1 or a dominant type 2 cytokine profile will be susceptible to infection following low doses of HIV. Other infections, such as with helminthic infestations [16], certain mycobacterial diseases [17] or sexually transmitted diseases (syphilis) [18] could reverse the balance from type 1 to type 2 cytokines. Such infections therefore, could render host more susceptible to HIV infections and could be considered as cofactors which may contribute to the higher incidence of seroconversion and more rapid progression to AIDS in those areas of the world in which parasitic infections that induce type 2 profiles are endemic [19].

Co-infection with HIV and *M. tuberculosis* results in significant alterations in cellular immune responses to both the pathogens [20]. Both AIDS and tuberculosis have a prolonged latency and dysfunction of CMI is a common denominator in both. T lymphocytes are involved in immune response and host defence against *M. tuberculosis* [21] and it has been shown that tuberculosis is associated with increase in cellular activation [22]. Cellular activation is important *in vitro* to establish productive HIV infection in T cells [23]. *In vivo* cellular activation is associated with an acceleration of viral replication in HIV infected patients. It has been shown that *M. tuberculosis* increases HIV replication both at local tissue sites [24], and at the systemic level [25] and accelerated the rate of progression of HIV disease [26].

A complex network of proinflammatory cytokines is involved in the pathogenesis of *M. tuberculosis* infection [27]. TNF- α is critical in granuloma formation and is essential for immune control of tuberculosis [28]. However, it promotes HIV expression and replication in lymphocytes and macrophages *in vitro* [29]. *M. tuberculosis* and its components may activate HIV replication through enhanced transcription of the long terminal repeat via the NF κ B motif. This effect is

mediated through release of endogenous proinflammatory cytokines [24]. *M. tuberculosis* increases the secretion of anti-inflammatory cytokines such as IL-10 [27,30] and TGF- β [31] which may contribute to immune suppression often observed during tuberculosis [32]. The anti-inflammatory cytokines have been reported to upregulate and down regulated HIV replication [33,34,35]. Cytokines produced during immune response to mycobacteria may result in both activation of latently infected cell and induction of virus infection, as well as increased viral replication from newly activated cells .

Immune activation in HIV and *M. tuberculosis* co-infected patients is associated with shortened survival [26]. Previous work done in the Department of Virology, Haffkine Institute has shown mortality of 40% among patients infected with pulmonary tuberculosis and HIV. The patients died within 6 to 18 months during follow up while the controls (age and sex matched pulmonary tuberculosis patients without HIV infection) survived longer [36].

India has more cases of HIV than any other country. [37]. There is no high profile national campaign against this huge speed spread among 970 million population. As HIV spreads tuberculosis will also increase as disease caused by these two are closely linked. Also, a difficult clinical problem has emerged as outbreaks of multidrug- resistant tuberculosis (MDRTB) in both HIV positive and HIV negative individuals world-wide. Incomplete or inappropriate treatment with available chemotherapy, deficiency in the host defence due to poor nutritional status or alcoholism and qualitative or quantitative immune deficiency are some of the mechanisms that might exist independently or together that would lead to the increase in the rate of development of MDRTB in HIV positive as well as HIV negative individuals. A study done in the Department of Virology, Haffkine Institute showed increase in the rate of MDRTB among the HIV seropositive pulmonary tuberculosis patients [36].

AIDS pandemic has stalled the elimination of tuberculosis and has reversed many of the hard won gains in tuberculosis control in developing world. WHO estimates

that, by the end of the century, HIV infection will annually produce at least 1.4 million active cases of tuberculosis that otherwise would not have occurred [38].

Tuberculosis being endemic in India is going to have devastating effect on spread of HIV in this country. In India, tuberculosis control is limited to BCG vaccination passive case findings and treatment. An additional impetus is needed for study and implementation of anti tuberculosis preventive therapy and active case finding in HIV infected persons.

The present study was undertaken to evaluate the *in vitro* cytokine profile and T cell proliferation in response to stimulation with mitogen and mycobacterial antigen in pulmonary tuberculosis patients co-infected with HIV as altered cytokine profiles due to other infections need to be considered before designing vaccination or therapy for population in developing countries.

AIMS AND OBJECTIVES :

1. Determination of HIV seroprevalence in clinically diagnosed pulmonary tuberculosis patients.
2. Quantitative estimation of IL-2, IFN-gamma (TH1) and IL-4 (TH2) cytokines in cell free supernatants of PHA and *Mycobacterium tuberculosis* whole sonicate (MTBs) stimulated peripheral blood mononuclear cells of pulmonary tuberculosis patients co-infected with HIV and comparison with cytokine secretion in healthy volunteers, AIDS patients without tuberculosis and asymptomatic HIV seropositive patients.
3. Quantitative assessment of production of TNF- α (pro-inflammatory) and IL-10 (anti-inflammatory) cytokines from PBMNCs stimulated with PHA and MTBs in HIV seropositive and HIV seronegative pulmonary tuberculosis patients and comparison with healthy volunteers and HIV seropositive cases without tuberculosis.
4. Study and comparison of ability of PBMNCs from patients in all the above mentioned groups, to proliferate in response to mitogen (PHA) and antigen (MTBs)

MATERIALS & METHODS

1. PATIENTS :

A total of 240 patients and 10 healthy volunteers were studied.

The patients were grouped as follows :

Group I : Out of the 210 radiologically and clinically diagnosed pulmonary tuberculosis patients, admitted in The Group of TB Hospitals, Sewri, Mumbai were tested for HIV I&II antibodies in the serum. 51 were found to be seropositive for HIV I & II antibodies on two consecutive ELISA, out of them 25 patients, donated blood for study of *in vitro* cytokine profile and lymphocyte proliferation.

Group II : Out of the 159/210 radiologically and clinically diagnosed pulmonary tuberculosis patients who were found to be HIV seronegative , 25 were included in this group for the study of *in vitro* cytokine profile and lymphocyte proliferation.

Group III : consisted of 14 patients admitted to the JJ group of Hospitals, Byculla, Mumbai. These patients were HIV seropositive by ELISA and confirmation of the same infection had been done by western blot. These patients were admitted to the hospital with at least one AIDS related opportunistic infection but were confirmed clinically and radiologically to be not infected with pulmonary or extra pulmonary *M. tuberculosis* infection.

Group IV : 16 HIV seropositive and Western blot confirmed asymptomatic cases visiting OPD of The JJ Group of Hospitals were included in this group. These individuals were apparently healthy and were not infected with any other opportunistic infection.

Patients from all these groups were of either sex, having similar socio-economic status and were in the age group of 20-50 years.

Controls : Ten healthy, age matched employees and students of The Haffkine Institute donated blood to the experiments. All were seronegative for HIV I & II by ELISA and had no apparent pulmonary or extra pulmonary tuberculosis infection.

2. DETECTION OF HIV SEROSTATUS :

Informed written consent from all patients and controls was taken. 5ml blood was collected by vein puncture, Sera were separated, labelled and stored at -20⁰ C until used for testing. Detection of anti-HIV antibodies were be carried out by commercially available ELISA kits (Gamma Belgium and Labsystems), according to the instructions given along with the kits.

3. LYMPHOCYTE PROLIFERATION :

Peripheral blood mononuclear cells (PBMNCs) were isolated on Ficoll-Hypaque density gradient. PBMNCs were washed and cultured in 96 well U-bottomed micro titre plates using RPMI 1640 and 10% foetal calf serum as culture medium. The cells were stimulated with Phytohaematogglutinin (PHA-M) and Mycobacterium tuberculosis whole sonicate and incubated in the presence of 5% CO₂. Assays were terminated with addition of 1µCi ³H thymidine per well for 18 hours before harvesting and thymidine incorporation were measured by Liquid Scintillation counter and expressed as mean counts per minute [39].

4. GENERATION OF CELL SUPERNATANTS :

PBMNCs from patients as well as controls were stimulated with PHA(SIGMA) and MTB whole sonicate for different duration. The cell free supernatants were collected and stored at -20⁰C till assayed [40].

5. BIOASSAY FOR IL-2 :

IL-2 in the cell culture supernatants were estimated using IL-2 dependent murine CTLL-2 cell line as per the method described by Gillis et al [41]. The cell free culture supernatants (50µl/well), in triplicates, were added to the washed CTLL-2 cells (10⁴/well) in a U bottom microtitre plate and incubated at 37°C in 5% CO₂ for 48 hours. 1µCi ³H thymidine per well was added after 24 hours of incubation and cells were harvested and incorporation of thymidine in the CTLL-2 cells was estimated using Liquid Scintillation counter and expressed as mean counts per minute. The mean cpm produced by a supernatant depends on the amount of IL-2 present in that supernatant.

6. ASSAY OF CYTOKINES IN CELL SUPERNATANTS :

Cytokines IL-4, IL-10, TNF-α and IFN-γ were assayed using commercially available ELISA kits (ENDOGEN, USA) according to instructions given in the kits.

The statistical analysis was carried out using SPSS package. The results were considered to be statistically significant only at the level 0.01 by ANOVA.



RESULTS

Out of the 210 pulmonary tuberculosis patients screened for HIV I&II antibodies, 51 (24.29%) were found to be seropositive.

LYMPHOCYTE PROLIFERATION

Controls showed the highest stimulation index (14.22 ± 1.54) on stimulation with MTBs as compared to any other group ($p < 0.01$).

Asymptomatic HIV seropositive patients showed significantly high proliferation as compared to Group I (2.79 ± 0.29) ($p < 0.01$) and Group III (2.55 ± 0.29) ($p < 0.01$) patients. Also there is a significant decrease in proliferation of AIDS patients with or without tuberculosis (Group I & III resp.) as compared to pulmonary tuberculosis patients without HIV infection (5.61 ± 0.77) ($p < 0.01$). [table 1]

CYTOKINE PRODUCTION

The amount of IL-2 in a supernatant was expressed as the mean CPM $\pm$ SE of the CTLL-2 cells.

IL-2 production in Group I (1947.88 ± 357.86) and III (1775.07 ± 346.26) is markedly reduced than all the other groups of patients as well as controls (20738.40 ± 1461.99) ($p < 0.01$). Asymptomatic HIV patients produced considerably decreased amount of IL-2 (8702.44 ± 759.96) ($p < 0.01$) and so also the HIV seronegative pulmonary tuberculosis (5701.36 ± 839.37) ($p < 0.01$) patients than the healthy controls [table 2].

Controls produced highest amount of IFN- γ (1763.75 ± 1530.03 pg/ml) as compared to all other groups; Group I (301.58 ± 47.68 pg/ml) ($p < 0.01$), Group II (653.85 ± 59.42 pg/ml) ($p < 0.01$) Group III (222.76 ± 50.34 pg/ml) ($p < 0.01$) Group IV (980.63 ± 165.82 pg/ml) ($p < 0.01$). Only 17/25 (68%) produced detectable levels of IFN- γ and in Group II 23/25 (92%) produced IFN- γ in response to MTBs [table 3].

No significant difference was observed among the patients and controls with respect to IL-4 production [table 4].

Amount of IL-10 produced in response to PHA as well as MTBs differs insignificantly among the controls and all the four patients groups [table 5].

Group I (1236.46 ± 103.61 pg/ml) showed highest production of $\text{TNF}\alpha$ in response to MTBs ($p < 0.01$). And Group II produced significantly higher $\text{TNF}\alpha$ as compared group III (138.62 ± 43.39 pg/ml) ($P < 0.01$) and Group IV (56.65 ± 9.88 pg/ml) ($P < 0.01$) [table 6].

DISCUSSION

A rising trend of HIV seroprevalence (20.1%) among pulmonary tuberculosis patients in India was reported in 1996 [42]. A rate of 24.29% HIV seroprevalence among pulmonary tuberculosis patients found in the present study confirms the high HIV seroprevalence.

Deficiencies in both the quantitative and qualitative aspects of the immune response have been shown to have an impact on clinical outcome in patients infected with tuberculosis as well as HIV. In several studies, proliferation and IL-2 production from PBMCs have been found to be decreased in patients with active tuberculosis and advanced disease [43], as well as in HIV patients [11,12].

In the present study, AIDS patients with or without tuberculosis showed the least proliferation in response to mitogen and antigen as compared to healthy controls as well as asymptomatic HIV infected patients and tuberculosis patients without HIV infection ($p < 0.01$).

Patients coinfecting with HIV and tuberculosis and AIDS patients without tuberculosis produced the least IL-2 in response to mitogen as well as antigen. HIV seropositive asymptomatic cases also showed significantly lowered IL-2 production as compared to healthy controls ($p < 0.01$) in response to mitogen and antigen stimulation. This shows early dysfunction of T cell immediate response. Also IL-2 production in Group I and III i.e. HIV patients with opportunistic infections showed markedly decreased IL-2 production as compared to symptomatic cases ($p < 0.01$) indicating progressive functional damage caused by HIV.

Recently , Zang *et al* showed that purified CD4⁺ cells from TB patients had decreased expression of IL-2 mRNA compared with healthy controls suggesting a qualitative defect in IL-2 production [44]. In the present study, we found significantly decreased IL-2 production in HIV seronegative patients with pulmonary tuberculosis (Group II) both in response to mitogen and mycobacterial sonicate, as compared to that in the healthy controls ($p<0.01$).

AIDS patients with or without tuberculosis show the least proliferation in response to mitogen and antigen as compared to healthy controls as well as asymptomatic HIV infected patients and tuberculosis patients without HIV infection.

Asymptomatic cases show a significant difference in IFN- γ production in response to antigen and is significantly reduced than healthy controls ($p<0.01$), but is significantly higher than those in symptomatic cases with opportunistic infections (Group I and Group III)($p<0.01$) again indicating progressive deterioration of TH1 responses during the course of HIV infection.

HIV seronegative PTB patients showed significantly lowered IFN- γ production in response to antigen as compared to healthy individuals ($p<0.01$). This is similar to IL-2 production.

There can be many potential mechanisms to explain IFN- γ deficiency in these patients :

1. Patients with low CD4⁺ T cell counts have a decrease in number of antigen specific cells capable of producing IFN- γ [45].
2. Presence of IL-2 is required for IFN- γ production *in vitro* [46]., which is clearly found to be decreased in both the diseases.
3. The presence of down regulatory cytokines such as IL-4, IL-10 or TGF- β could be involved in inhibiting IFN- γ production .

The cellular basis for this unresponsiveness is not fully understood. Antigen specific immune responses require that the CD4 molecule interact with class II MHC molecules on surface of antigen presenting cells during the presentation of processed antigen to CD4⁺ T cells. Binding of gp120 to CD4 molecule disrupts

the CD4 - class II MHC association and may cause qualitative decrease in CD4+ T cell function. Inappropriate signalling could render the cell anergic [47]. HIV nef protein induces disturbance of signal transduction and IL-2 production resulting in impaired T cell function [48]. Anergy would provide a systemic explanation for T cell abnormalities observed in HIV infection. Recently, the possibility that HIV related nef gene expression may be responsible for the downregulation of TH1 cytokines (IL-2 and IFN- γ) without affecting the production of TH2 cytokines has been suggested [48]. Our own observation, demonstrating decrease in IL-2, IFN- γ in short term PBMNC culture as compared to controls might be a consequence of anergy of TH1 cells.

In our study, we found no enhancement in TH2 type of cytokines viz. IL4 in HIV infected and HIV seronegative tuberculosis patients in response to antigen or mitogen.

So also, no significant difference was found in IL-10 production by PBMNCs among controls and patients in all the four groups. Zang *et al* have also found that there is no increase in type 2 cytokines in HIV patients with tuberculosis. However, addition of anti-IL-10 antibodies to cultures increases proliferation as well as IFN- γ production in co-infected patients. These patients produced IL-10 in response to specific antigen in similar amounts as did the HIV negative tuberculosis patients. Also antiIL-10 antibody had a minimal effect on increasing the proliferative response of these patients, suggesting that IL-10 is playing a more prominent role in the down regulation of responses in HIV infected tuberculosis patients [30]. Although the mechanism underlying this effect is uncertain, the above results suggest that the reduced proliferative and type 1 responses in HIV infected tuberculosis patients are mediated at least in part through IL-10. Probably, macrophages and/or T cells from HIV infected patients may express higher levels of IL-10 receptor and therefore be more sensitive to the effects of IL-10 than are patients without HIV infection.

In this study, we found that TNF- α production by PBMNCs from patients co-infected with HIV and tuberculosis, on stimulation with MTBs was highest as

compared to that in any other group ($p < 0.01$) or healthy controls ($p < 0.01$). TNF- α has been demonstrated to have an important role in immunity against tuberculosis. TNF- α is expressed by macrophages following direct interaction with *M. tuberculosis*. This is essential for inhibition of bacillary replication and for isolation of the remaining bacilli within granulomas and thus has a positive role in immune response against mycobacteria [49]. Also several lines of evidence suggest that HIV infection itself causes an increase in cytokine production that in turn can have a positive effect on viral replication. HIV infection of cells *in vitro* has been shown to cause increased production of TNF- α and also increase in TNF- α gene expression is dependent on the production of HIV tat protein [50]. TNF- α functions *in vivo* in an autocrine and paracrine fashion to upregulate HIV expression, TNF- α increases HIV expression via the induction of NF κ B proteins that in turn act as transcriptional activators of HIV [51]. A study carried out in Uganda showed 3-10 fold increase in PPD stimulated production of TNF- α in HIV positive tuberculosis patients as compared to controls [52]. Initial serum levels of TNF- α and soluble TNF receptor were inversely correlated with survival [53]. Such increased monocyte cytokine production must be occurring *in vivo* within granulomas and in infected body fluids [54]. It likely is the cause of fever and wasting that are prominent in HIV associated tuberculosis and that may persist despite adequate therapy [55].

Enhanced apoptosis is the major contributor to the depletion of CD4⁺ T cells in HIV infection [56]. TH2 cytokines IL-4 and IL-10 exert a favouring effect on apoptosis [57] whereas IL-2 and IL-12 (cytokine upregulating TH1 functions) are protective [58]. Finally production of TNF- α and IFN- γ in absence of IL-2, which occurs as a consequence of CD4 crosslinking, contributes apoptosis induction [59]. It is of note that in many studies including ours, it has been found that production of protective cytokines (IL-2, IL-12) is defective in HIV infected patients, whereas production of cytokines favouring apoptosis (IL-4, IL-10 TNF- α) is not reduced or even increased.

CONCLUSION

1. A high HIV seroprevalence (24.29%) was observed among pulmonary tuberculosis patients .
2. The data presented here indicate that the proliferation and type 1 (IL-2 and IFN- γ) response to *M. tuberculosis* sonicate in pulmonary tuberculosis patients co-infected with HIV are reduced without enhancement of type 2 (IL-4) responses.
3. TNF- α production in response to *M. tuberculosis* sonicate was highest in the HIV seropositive PTB patients.
4. HIV seronegative patients infected with tuberculosis showed reduced proliferative response, IL-2 and IFN- γ production (TH1) in response to *M. tuberculosis* sonicate antigen stimulation as compared to healthy controls. TNF- α production in pulmonary tuberculosis patients without HIV was higher as compared to controls.

TABLE 1
PBMNCs PROLIFERATION IN RESPONSE TO MTBs STIMULATION

	GROUP I	GROUP II	GROUP III	GROUP IV	CONTROLS
SI MEAN CPM ± SE	2.79 ± 0.29	5.61 ± 0.77	2.55 ± 0.29	6.36 ± 0.48	14.22 ± 1.54

COMPARISON OF GROUPS	SIGNIFICANCE
CONTROLS > GROUP I	P < 0.01
CONTROLS > GROUP II	P < 0.01
CONTROLS > GROUP III	P < 0.01
CONTROLS > GROUP IV	P < 0.01
GROUP IV > GROUP I	P < 0.01
GROUP IV > GROUP III	P < 0.01
GROUP II > GROUP I	P < 0.01

Group I : HIV seropositive pulmonary tuberculosis patients.
Group II : HIV seronegative pulmonary tuberculosis patients.
Group III : AIDS patients having opportunistic infections other than tuberculosis.
Group IV : HIV seropositive asymptomatic cases.
Controls : Healthy volunteers.

TABLE 2
IL-2 PRODUCTION IN RESPONSE TO MTBs

	GROUP I	GROUP II	GROUP III	GROUP IV	CONTROLS
CTLL-2 MEAN CPM ± SE	1947.88 ± 357.86	5701.36 ± 839.37	1775.07 ± 346.26	8702.44 ± 759.96	20738.40 ± 1461.99

COMPARISON OF GROUPS	SIGNIFICANCE
CONTROLS > GROUP I	P < 0.01
CONTROLS > GROUP II	P < 0.01
CONTROLS > GROUP III	P < 0.01
CONTROLS > GROUP IV	P < 0.01
GROUP IV > GROUP I	P < 0.01
GROUP IV > GROUP III	P < 0.01
GROUP II > GROUP I	P < 0.01
GROUP II > GROUP I	P < 0.01

Group I : HIV seropositive pulmonary tuberculosis patients.
Group II : HIV seronegative pulmonary tuberculosis patients.
Group III : AIDS patients having opportunistic infections other
than tuberculosis.
Group IV : HIV seropositive asymptomatic cases.
Controls : Healthy volunteers.

TABLE 3
IFN- γ PRODUCTION IN RESPONSE TO MTBs

	GROUP I	GROUP II	GROUP III	GROUP IV	CONTROLS
IFN- γ (PG/ML)	301.58 $\pm$ 47.68	653.85 $\pm$ 59.42	222.76 $\pm$ 50.34	980.63 $\pm$ 165.82	1763.75 $\pm$ 1530.03

COMPARISON OF GROUPS	SIGNIFICANCE
CONTROLS > GROUP I	P < 0.01
CONTROLS > GROUP II	P < 0.01
CONTROLS > GROUP III	P < 0.01
CONTROLS > GROUP IV	P < 0.01
GROUP IV > GROUP I	P < 0.01
GROUP IV > GROUP III	P < 0.01
GROUP II > GROUP I	P < 0.01
GROUP II > GROUP I	P < 0.01

Group I : HIV seropositive pulmonary tuberculosis patients. .

Group II : HIV seronegative pulmonary tuberculosis patients.

Group III : AIDS patients having opportunistic infections other than tuberculosis.

Group IV : HIV seropositive asymptomatic cases.

Controls : Healthy volunteers.

TABLE 4
IL-4 RESPONSE TO MTBs

	GROUP I	GROUP II	GROUP III	GROUP IV	CONTROLS
IL-4 (PG/ML)	1.26 ± 0.31	2.75 ± 1.51	0.69 ± 0.16	3.39 ± 1.86	0.033 ± 0.013

NO TWO GROUPS ARE SIGNIFICANTLY DIFFERENT AT 0.01 LEVEL.

TABLE 5
IL-10 PRODUCTION IN RESPONSE TO MTBs

	GROUP I	GROUP II	GROUP III	GROUP IV	CONTROLS
IL-10 (PG/ML)	646.12 ± 44.17	490.29 ± 46.25	500.94 ± 66.74	582.67 ± 66.74	535.84 ± 41.99

Group I : HIV seropositive pulmonary tuberculosis patients.

Group II : HIV seronegative pulmonary tuberculosis patients.

Group III : AIDS patients having opportunistic infections other than tuberculosis.

Group IV : HIV seropositive asymptomatic cases.

Controls : Healthy volunteers.

TABLE 6
TNF- α PRODUCTION IN RESPONSE TO MTBs

	GROUP I	GROUP II	GROUP III	GROUP IV	CONTROLS
TNF- α (PG/ML)	1236.46 $\pm$ 103.61	542.94 $\pm$ 81.60	56.65 $\pm$ 9.88	138.62 $\pm$ 43.39	136.64 $\pm$ 17.38

COMPARISON OF GROUPS	SIGNIFICANCE
GROUP I > CONTROLS	P < 0.01
GROUP I > GROUP II	P < 0.01
GROUP I > GROUP III	P < 0.01
GROUP I > GROUP IV	P < 0.01
GROUP II > GROUP III	P < 0.01
GROUP II > GROUP IV	P < 0.01

Group I : HIV seropositive pulmonary tuberculosis patients.

Group II : HIV seronegative pulmonary tuberculosis patients.

Group III : AIDS patients having opportunistic infections other than tuberculosis.

Group IV : HIV seropositive asymptomatic cases.

Controls : Healthy volunteers.

REFERENCES

1. Gottlieb MS, Schroff R, Schanker HM *et al.*
Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men : evidence of a new acquired cellular immunodeficiency.
N Engl J Med 1981; 305: 1425-1431.
2. Decock K.
Monitoring the epidemic of HIV/AIDS [abstract L2] In proceedings of the fifth conference on retroviruses and opportunistic infections. 1998 Feb 1-5; Chicago.
Edited by Richman DD *et al.* Alexandria, Virginia: The foundation of Retrovirology and Human Health; 1998 : 1.
3. Mossman TR, Coffman RL.
TH1 and TH2 cells : different patterns of lymphokine secretion lead to different functional properties.
Ann Rev Immunol 1989; 7: 145-173.
4. Abbas AK, Keneth MM, Sher A.
Functional diversity of helper T lymphocytes.
Nature 1996; 383: 787-793.
5. Tanaka T, Hu-Li J, Seder RA, de St Groth BF, Paul WE.
Interleukin 4 suppresses interleukin 2 and interferon gamma production by naive T cells stimulated by accessory cell dependent receptor engagement.
Proc Natl Acad Sci [USA] 1993; 90: 5914-5918.
6. Chensue SW, Ruth JH, Warmington K, Lincoln P, Kunkel SL.
In vivo regulation of macrophage IL-12 production during type 1 and type 2 cytokine mediated granuloma formation.
J Immunol 1995; 155: 3546-3551.
7. Del Prete GF, De Carli M, Almerigogna F, Giudizi M-G, Biagihi R, Romagnani S. Human IL-10 is produced by both type 1 helper [TH1] and type 2 helper [TH2] T cell clones and inhibits their antigen specific [proliferation and cytokine production].
J Immunol 1993; 150: 1-8.
8. Hino A, Nariuchi M.
Negative feed back mechanism suppresses interleukin 12 production by antigen presenting cells interaction with T helper 2 cells.
Eur J Immunol 1996; 26: 623-628.

9. Fitch FW, McKisic MD, Lancki DW, Gajeswski TP.
Differential regulation of murine T lymphocyte subsets.
Ann Rev Immunol 1995; 146: 423-431.
10. Abed NS, Chace JH, Cowdery JS.
T cell independent and T cell dependent B cell activation increases IFN-gamma receptor expression and renders B cells sensitive to IFN-gamma mediated inhibition.
J Immunol 1994; 153: 3369-3377.
11. Lane HC, Fauci AS.
Immunologic abnormalities in the acquired immunodeficiency syndrome
Ann Rev Immunol 1985; 3: 477-500.
12. Clerici M, Hakim FT, Venzon DJ *et al*.
Changes in interleukin 2 and interleukin 4 production in asymptomatic HIV seropositive individuals.
J Clin Invest 1993; 91: 759-765.
13. Israel-Biet D, Labrousse F, Tourani JM, Sors H, Andrieu JM, Even P.
Elevation of IgE in HIV infected subjects : a marker of poor prognosis.
J Allergy Clin Immunol 1992; 89: 68-75.
14. Clerici M, Berzofsky JA, Shearer GM, Tacket CO.
Exposure to human immunodeficiency virus type 1 indicated by T helper cell responses before detection of HIV infection by polymerase chain reaction and serum antibodies.
J Infect Dis 1991; 164: 178-182.
15. Clerici M, Giorgi JV, Chau CC *et al*.
Cell mediated immune response to human immunodeficiency virus type 1 in seronegative homosexual men with recent sexual exposure to HIV 1.
J Infect Dis 1992; 165: 1012-1019.
16. Kullberg MC, Pearce EJ, Hieny SE, Sher A, Berzofsky JA.
Infection with *Schistosoma Mansoni* alters TH1/Th2 cytokine responses to a non-Parasite antigen.
J Immunol 1992; 148, 3264-3270.
17. Yamamura M, Uyemura K, Deans RJ *et al*.
Defining protective responses to pathogens : Cytokine profiles in leprosy lesions
Science 1991; 254: 277-279.
18. Fitzgerald TJ.
The Th1/Th2 like switch in syphilitic infection : Is it detrimental?
Infect Immun 1992; 60(9): 3275-3479.

19. Clerici M, Sheares GM.
A Th1 to TH2 shift is a critical step in the etiology of HIV infection.
Immunol Today 1993; 14: 107-111.
20. Wallis RS Helfand MS, Whalen C *et al.*
Immune activation, allergic drug toxicity and mortality in HIV positive tuberculosis.
Tubercle Lung Dis 1996; 77: 516-523.
21. Barnes PF, Mistry SD, Cooper CL, Pirmez C, Rea TH, Modlin RL.
Compartmentalisation of a CD4⁺T lymphocyte subpopulation in tuberculosis pleuritis.
J Immunol 1989; 142: 1114-1119.
22. Vanham G, Edmonds K, Qing L *et al.*
Generalised immune activation in pulmonary tuberculosis : Co-activation with HIV infection.
Clin Exp Immunol 1996; 103: 30-34.
23. Ramilo O, Bell KB, Uhr JW, Vitetta ES.
Role of CD25⁺ and CD25⁻ T cells in acute HIV infection *in vitro*.
J Immunol 1993; 150: 5202-5208.
24. Zang Y, Nakata K, Weiden M, Rom WN.
Mycobacterium tuberculosis enhances human immunodeficiency virus 1 replication by transcriptional activation at the long terminal repeat.
J Clin Invest 1995; 95: 2324-2331.
25. Goletti D, Weissman D, Jackson RW *et al.*
Effect of Mycobacterium tuberculosis on HIV infection replication : role of immune activation.
J Immunol 1996; 157: 1271-1278.
26. Whalen C, Horsburgh CR, Hom D *et al.*
Accelerated course of human immunodeficiency virus infection after tuberculosis.
Am Respir Crit Care Med 1995; 151: 129-135.
27. Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, Modlin RL.
Cytokine production at the site of disease in human tuberculosis.
Infect Immun 1995; 61: 3482-3489.
28. Kindler V, Sappino AP, Grau Gf, Piguet PF, Vassalli P.
The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection.
Cell 1989; 56: 731-740.

29. Poli G, Kindler AL, Justement JS *et al.*
Tumor necrosis factor alpha functions in an autocrine manner in the induction of human immunodeficiency virus expression.
Proc Natl Acad Sci[USA] 1990; 87:782-785.
30. Zang M, Gong J, Iyer DV, Jones BE, Modlin RL, Barnes PF.
T cell cytokine responses in persons with tuberculosis and human immunodeficiency virus infection.
J Clin Invest 1994; 94: 2435-2442.
31. Toosi Z, Goggat P, Shiratsuchi H *et al.*
Induction of TGF- β by blood monocytes from patients with active tuberculosis and presence of TGF- β in tuberculosis granulomatous lung lesions.
J Immunol 1995; 14: 465-473.
32. Gong JH, Zang M, Modlin RL *et al.*
Interleukin 10 downregulates Mycobacterium tuberculosis induced TH1 responses and CTLA-4 expression.
Infect Immun 1996; 64: 913-918.
33. Poli G, Kinter AL, Justement JS *et al.*
Transforming growth factor β suppresses human immunodeficiency virus expression and replication in infected cells of monocyte/macrophage lineages.
J Exp Med 1991; 73: 589-597.
34. Weissman D, Poli G, Fauci AS.
Interleukin 10 blocks HIV replication in macrophages by inhibiting the autocrine loop of tumor necrosis alpha and interleukin 6 induction of virus.
AIDS Res Hum Retrovir 1994; 10: 1199-1206.
35. Weissman D, Poli G, Fauci AS.
IL-10 synergizes with multiple cytokines in enhancing HIV production in cells of monocytic lineage.
J Acquir Immun Defic Syndr Hum Retrovirol 1995; 9: 442-449.
36. R. A. Deshmukh.
Personal communication.
37. Bollinger RC.
[Http:// www.sph.jhu.edu/~d-paffrslepi.htm](http://www.sph.jhu.edu/~d-paffrslepi.htm).
38. Raviglione MC, Sinder DE, Kochi A.
Global epidemiology of tuberculosis : Mortality and morbidity of a world wide epidemic.
JAMA 1995; 273: 220-226.

39. Maluish AE, Strong DM.
Lymphocyte proliferation. In; Manual of clinical laboratory Immunology. 3rd edition, Rose NR, Friedman H, Fahey JL, editors. Washington Dc. 1986; 274-281.
40. Clerici M, Stocks NI, Zajac RA *et al*.
Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. Independence of CD4+ T cell numbers and clinical staging.
J Clin Invest 1989; 84: 1892-1899.
41. Gillis S, Fern MM, Winny OU, Kendall SA.
T cell growth factor : parameters of production and a quantitative microassay for activity.
J Immunol 1978; 120(6) : 2027-2032.
42. Paranjape RS, Tripathy SP, Menon PA *et al*.
Increasing trend of HIV seroprevalence among pulmonary tuberculosis patients in Pune, India.
Ind J Med Res 1997; 106: 207-211.
43. Toosi Z, Klinhenz ME, Ellner JJ.
Defective interleukin 2 production and responsiveness in human pulmonary tuberculosis.
J Exp Med 1986; 1631-1632.
44. Zang M, Lin Y Iyer DV, Gong J, Abrahams JS, Barnes PF.
T cell cytokine responses in human infection with Mycobacterium tuberculosis.
Infect Immun 1995; 63: 3231-3234.
45. McDyer JF, Hackley MN, Walsh TE, Cook JL, Seder RA.
Patients with multidrug resistant tuberculosis with low CD4+ T cell counts have impaired TH1 responses.
J Immunol 1997; 158: 492-500.
46. Seder RA, Grabstein KH, Berzofsky JA, McDyer JF.
Cytokine interactions in human immunodeficiency virus infected individuals : roles of interleukins (IL-2, IL-12 and IL-15).
J Exp Med 1995; 182: 1067-
47. Rosenstein Y, Burakoff SJ, Herrmann SH.
HIV -gp 120 can block CD4-classII MHC mediated adhesion.
J Immunol 1090; 144: 526-531.
48. Collette Y, Chang HL, Cerdan C *et al*.
Th1 cytokine downregulation associated with primary clinical derived immunodeficiency virus type 1 *nef* gene induced expression.
J Immunol 1996; 156: 360-370.

49. Wallis RS, Amir Tahmasseb M, Ellner JJ.
Induction of interleukin 1 and tumor necrosis factor by mycobacterial proteins; the monocyte western blot.
Proc Natl Acad Sci (USA) 1990 87: 3348-3352.
50. Buonaguro L, Barillari G, Chang HK *et al*.
Effects of the human immunodeficiency virus type 1 tat protein on the expression of inflammatory cytokines.
J Virol 1992; 66: 7159-7167.
51. Griffin GE, Leung K, Folks TM, Kunkel S, Nabel GJ.
Induction of NF-kappa B during monocyte differentiation is associated with activation of HIV gene expression in chronically infected promonocyte cells.
J Virol 1990; 64: 4306-4312.
52. Wallis RS, Vjecha M, Tahmasseb M *et al*.
Influence of tuberculosis on human immunodeficiency virus (HIV-1) enhanced cytokine expression and elevated beta-2 microglobulin in HIV-1 associated tuberculosis.
J Infect Dis 1993, 167: 43-48.
53. Wallis RS, Ellner JJ.
Cytokines and tuberculosis.
J Leukoc Biol 1994; 55: 676-681.
54. Barnes PF, Long SL, Brennan PJ, Twomey PE, Mazumder A, Modlin RL.
Local production of tumor necrosis factor and IFN- γ in tuberculosis pleuritis.
J Immunol 1990; 145: 149-154.
55. Masud T, Kemp E.
Corticosteroids in treatment of disseminated tuberculosis in patients with HIV infection.
BMJ 1988; 296: 464-465.
56. Meyaard L, Otto SA, Jonker RR, Mijster MJ, Keet RPM, Miedema F.
Programmed death of T cells in HIV-1 infection.
Science 1992; 257: 217-219.
57. Clerici M, Sarin A, Coffman RL.
Type 1/Type 2 cytokine modulation of T cell programme death as a model for HIV pathogenesis.
Proc Natl Acad Sci (USA) 1994; 91: 11811-11851.
58. Oyaizu N, Pahwa S.
Role of apoptosis in HIV disease pathogenesis.
J Clin Immunol 1995; 15: 217-231.

