PART I. **CHARACTERIZATION OF TYPE-1/TYPE-2 CYTOKINE AND IgE RESPONSES OF HIV-1 RESISTANT KENYAN WOMEN**

PART II. **CHARACTERIZATION OF NEONATAL TYPE-1/TYPE-2 CYTOKINE RESPONSES**

BY

HARSHA N. TRIVEDI

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Medical Microbiology
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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DEDICATION

Mummy-Pappa,

Gopal♥➔♥ and Sonu

I dedicate this thesis to you.

ACKNOWLEDGEMENTS

It is with a feeling of great joy, gratitude and indebtedness to many here and across the oceans that I am writing this page. Special thanks to my three supervisors - Dr. Francis A. Plummer, Dr. Kent T. HayGlass and Dr. Joanne E. Embree. - each of whom has brought their special skills and talents, and implanted in me the knowledge of three disciplines of expertise. This converted me into a real TRIVEDI (one who knows the three VEDAS -- the ancient Indian scriptures of knowledge). Meeting, communicating and knowing Frank at every instant during this program was a great pleasure. His overall approach towards science, and especially the human aspect of dealing with me, will always be a great treasure of mine. He always encouraged me and always made me feel important and confident in myself. His thought-provoking, and yet never compulsive, way of dealing with scientific matters was a real asset during my graduate training. Kent taught me the art of research and the science of its principles. His critical thoughts, meticulous laboratory training, and the overall concepts of both theory and practical immunology developed a systematic, rationalized approach in me. His modest nature always attracted me to ask even meagre questions which resulted in the research training that taught me some very important lessons which I am sure will be very useful in my scientific career ahead. Joanne's help during the work on neonates and especially in the last phase of writing of my thesis is greatly appreciated. Many, many thanks to each of you. Thanks to Dr. Marlis Schroeder for her comments on the thesis. Thanks are also due to Dr. James Allardice (since deceased) and the Labour Floor staff at the Health Sciences Centre for providing samples for the neonatal study.

I had a special privilege, as each day here was divided into two laboratories - Frank's and Kent's - thus enjoying the benefits of friends, parties and love of both the Medical Microbiology and Immunology departments. In my memory lanes, there are vivid pictures of many friends - Venu, Shirley, Anzala, Suzie, Zeena, Leslie, Joanna, Ian, Julie, Ian, Seema, Heyu, Melanie, Bill, Blake, John, Trevor and many more whom I may have not mentioned here. Their warmth, friendship, advice, talks and little tips for experiments and survival in Winnipeg winters is greatly appreciated. Special thanks to Kent and family, Leslie, our hosts, and numerous friends in both departments who participated and shared in some very special moments of my life. Mrs. Theresa Birkholz deserves a special mention for always being there with her warmth, and kind words and heart. Thanks to Carol Sigurdson, Yvonne, and Karen for the secretarial assistance. Finally, thanks to all my teachers in both departments for enlightening me with the course work, journal clubs and seminars, and all supporting staff for their friendly nature.

Friends in Nairobi - Grace, Rupert, Dorothy, Oyugi, Miriti, Kimani, Irene and Anne - ***Asante Sane*** to all of you. Grace and Frank will always remain in my heart for helping me to survive the **most** difficult days of my life.

I owe many people back home who have helped me directly or indirectly to achieve this goal. First, I pay tribute to my **Lord Almighty** for always being my key in the morning and lock in the evening. My loving parents, who enabled me to fulfill all of my dreams with their numerous sacrifices, encouragement, love and blessings, have always been the driving force of my life. My funding agency - The Canadian Commonwealth Scholarship Plan - deserves a special salute for the award and being my financial supporter during this study in Canada.

Back home, my gratitude to the Dean of NHL Medical College, Dr. H.S. Shah, who encouraged me to pursue a Ph.D., and to my little sister Sonu and family members of both sides, who showered their love and adoration for me and stimulated me throughout this study. To my friend, philosopher and guide (a 4th one!!), Venu, I have no words to express my feelings. His love, help, patience, understanding of the subject, critical thinking and his faith in me (more than myself) helped me to stand the challenges and grow day by day. In fact, he tilted me towards another passion - research. Thank you my Gopi for always being there for me.

ABSTRACT

PART I: CHARACTERIZATION OF TYPE-1/TYPE-2 CYTOKINE AND IgE RESPONSES OF HIV-1 RESISTANT KENYAN WOMEN

A group of HIV-1 resistant Kenyan female prostitutes has been previously identified who remain HIV-1 specific serum antibody (Ab) and polymerase chain reaction (PCR) negative despite chronic ongoing exposure to human immunodeficiency virus type 1 (HIV-1) (3-14 years). The overall objective of this study was to characterize the type-1/type-2 cytokine and IgE responses of these women. Toward this goal, the initial hypothesis that the resistant women exhibit enhanced HIV-1 driven type-1 and reduced type-2 cytokine responses was tested. Results revealed that the resistant women exhibited markedly enhanced virus driven IFN-γ and significantly reduced IL-4 responses compared to susceptible women. Resistant women also exhibited marginally enhanced IL-5 and similar IL-13 and IL-10 responses. Analysis of virus driven type-2:type-1 cytokine balance revealed a selective imbalance in IL-4:IFN-γ, but not in other type-2:type-1 ratios examined. Furthermore, recall Ag (streptokinase [SK] and purified protein derivative [PPD]) driven cytokine responses indicated that the resistant women exhibited similar levels of Ag driven IFN-γ and markedly reduced IL-4 responses compared to low risk Kenyan women. Subsequently, the hypothesis that HIV-1 resistant women exhibit enhanced responsiveness to IL-12 and IP-10 (both of which promote IFN-γ synthesis) was tested. Although the resistant women exhibited significantly enhanced IL-12 and IP-10 responsiveness on virus mediated activation compared to susceptible women, they did not differ from low risk women. Finally, the hypothesis that HIV-1 resistant women may exhibit deficient HIV-1 specific IgE Ab was

tested. Examination of their plasma confirmed this hypothesis. However, the resistant women did not exhibit a generalized defect of class switching to IgE isotype because their plasma levels of cat and dust mite allergen specific IgE antibodies and total IgE were similar to that of low risk women. In summary, this study has identified a potential cytokine mediated immune mechanism associated with the clinical resistance to HIV-1 infection among Kenyan women. These results may have implications in the prevention and treatment of HIV-1 infection and AIDS.

PART II: CHARACTERIZATION OF NEONATAL TYPE-1/TYPE-2 CYTOKINE RESPONSES

It is widely believed that enhanced vulnerability of human neonates to infections is due to the immaturity of their immune system. In the present study, two hypotheses were tested: 1) neonatal T cells are immature with regard to type-1/type-2 cytokine synthesis; and 2) neonatal antigen presenting cells (APC) have a defect in stimulating type-1/type-2 cytokine synthesis. Comparison of neonatal cytokine responses to phytohemagglutinin (PHA) indicated an impaired IFN-γ and similar IL-4, IL-5 and IL-10 responses compared to adults. In contrast, examination of a more physiologically relevant, alloantigen mediated response revealed that neonates have very similar IFN-γ, IL-4 and IL-10 responses but higher IL-5 responses compared to adults. Finally, the capacity of neonatal versus adult APC to induce cytokine synthesis was assessed. Results indicated that neonatal APC exhibit a selective defect in stimulating IFN-γ and IL-10, but not IL-4 and IL-5 synthesis. In summary, these data argue that, whereas neonatal T cells are functionally mature, their APCs exhibit a selective defect which may contribute to the vulnerability of neonates to pathogens.

ABBREVIATIONS

Ag	Antigen
Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
BMT	Bone marrow transplant
BSA	Bovine serum albumin
CBMC	Cord blood mononuclear cells
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DTH	Delayed hypersensitivity
EIA	Enzyme immunoassay
FCS	Fetal calf serum
HDM	House dust mite
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
MCH	Maternal & Child Health Study (code for antenatal project)
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemaglutinin
PMA	Phorbol myristate acetate
PNPP	P-nitrophenyl phosphate
PPD	Purified protein derivative
SAC	Staphylococcus A cowan strain
SK	Streptokinase
STI	Sexually transmitted infection
TCR	T cell receptor
TU	Tuberculin units

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PART-I: CHARACTERIZATION OF TYPE-1/TYPE-2 CYTOKINE AND IgE RESPONSES OF HIV-1 RESISTANT KENYAN WOMEN

INTRODUCTION

1. OVERVIEW

The first identification of acquired immunodeficiency syndrome, which we now know as AIDS, was reported in the early 1980's by the Centre for Disease Control (CDC), Atlanta, USA, among homosexual men (CDC,1981). Studies by three different groups later identified a retrovirus associated with this immunodeficiency syndrome (Barre-Sinoussie et al, 1983; Popovic et al, 1984; Levy et al, 1984). This virus was named as human immunodeficiency virus (HIV) by the International Committee on Taxonomy of Viruses in 1986 (Coffin et al, 1986). The virus was renamed HIV type 1 (HIV-1) after a second type of the virus was recovered in Portugal from patients with AIDS and subsequently designated as HIV type 2 (HIV-2) (Clavel et al, 1986; Marlink et al, 1994).

HIV-1 is transmitted horizontally through blood, blood products and genital secretions, and vertically from mother to child *in utero* during birth, or through breast milk (Goedert et al, 1988: Rossi 1992; Datta et al, 1995). Although the earliest cases of AIDS in the USA were predominantly found among homosexual men, the major route of transmission is now heterosexual contact (Devincenzi, 1994).

A large number of people are infected with HIV-1 worldwide. According to the UNAIDS annual survey, in 1997 alone, 5.8 million people were newly infected with HIV-1 (Bloom, 1998). Worldwide, ~2.3 million people will die of AIDS this year, and by the end of this decade, ~40 million people will have died from AIDS (Bloom, 1998; Greene, 1993).

Globally, sub-Saharan Africa has the largest number of HIV-1/AIDS cases (~12 million), followed by Southeast Asia (6 million), North America (860,000), Western Europe (50,000), and Latin America and South Asia (5.4 million in India) (Bloom, 1998)

Ninety percent of new HIV-1 infections occur in developing countries, where ultimately all infected people succumb to the disease or opportunistic infections (Bloom, 1998). The epidemiology of HIV-1 in developing countries has serious and powerful social and economic ramifications (Nzilambi et al, 1988; Carswell et al, 1989; Plummer, et al 1991; Simonsen et al, 1990; Bonacci et al, 1992). Since women in the childbearing age group are primarily affected (for instance, in some regions, >20% of women in the reproductive age group are HIV-1 seropositive) (Rwandan HIV-1 seroprevalence study group 1989; Temmerman et al, 1992), a large number of HIV-1 infected children will be born before the HIV-1 epidemic is controlled. The AIDS epidemic in Africa will lead to an estimated 10 million orphans (Anderson, 1988). Furthermore, the most economically productive portion of the population (ages 15-49) has the highest prevalence of HIV-1 infection (De cock et al, 1990). Deaths due to HIV-1 or AIDS will have dramatic social and economic effects (Lurie et al, 1995; Potts et al, 1991; Blattner, 1991; Merson, 1993; Peter, 1995). Thus, the potential impact of HIV-1/AIDS on the families and society at large is enormous.

Although advances in the treatment of HIV-1 have resulted in a reduced death rate from AIDS in developed countries, most developing countries cannot afford to implement the new anti-retroviral drug therapies because of high cost involved (currently about US $12,000-15,000 per patient each year in the USA). Furthermore, these drugs do not cure HIV-1 infection. The main public health measure available for control of HIV-1 infection is counselling against behaviours that increase the risk of the infection (Bloom, 1998).

However, despite wide spread public health education and control programs, the rate of viral spread worldwide has continued to increase (Blattner et al, 1991; Merson, 1993). Thus, alternative approaches, such as the development of suitable vaccines to prevent and control HIV-1, are highly desirable.

The concept that HIV-1 infection inevitably leads to AIDS and death has changed over the past few years. It is clear that the clinical outcome of HIV-1 infection in the general population is heterogeneous. Based on the onset of AIDS after HIV-1 infection, individuals have been classified as progressors, rapid progressors or, for approximately 10% of infected Caucasian populations, longterm non-progressors (Pantaleo and Fauci, 1996). Also, a small percentage of exposed individuals exhibit sustained "resistance" to HIV-1 infection and remain seronegative, PCR-negative and clinically healthy despite chronic, high level exposure (Shearer and Clerici, 1996; Rowland-Jones, 1997; Heeney et al, 1997). Since there is no suitable animal model for HIV-1/AIDS, it is widely believed that studying longterm non-progressors and/or resistant individuals may represent natural models of immunity to HIV-1 infection, and therefore would provide direction for the development of an effective vaccine.

In Nairobi, Kenya, a group of prostitutes have been identified who, despite intense sexual exposures, have remained HIV-1 uninfected for periods of >13 years. These women have been designated as resistant to HIV-1 infection based on the following definition. They have remained persistently seronegative and PCR negative for 3-14 years, have remained healthy with no clinical evidence of HIV-1 infection or immunodeficiency, and have normal CD4 T cell counts (Fowke et al, 1996). These women have evidence of cell-mediated immune responses to HIV-1 and mucosal, but not plasma, IgA to HIV-1. It seems possible

that these responses are mediating protection against HIV-1. A basic question of why these individuals develop these responses remains. This thesis describes experiments which characterize the type-1 and type-2 cytokine and IgE responses, and examines their differential role in resistance versus susceptibility to HIV-1 infection.

2. REVIEW OF LITERATURE

2.1 Replication Cycle of HIV-1

HIV-1 is a member of the lentiviridae subfamily of retroviridae. It has a characteristic cone shaped core and is composed of four nucleocapsid proteins (P24, P17, P9 and P7) with an envelope outside. The P24 protein forms the main component of the inner shell of the nucleocapsid. The nucleocapsid proteins (P9 and P7) are tightly associated with the viral genome. The envelope is composed of two glycosylated proteins- gp120 and gp41. The inner portion of the envelope is surrounded by myristylated P17 protein that provides the matrix for the viral structure and is important for the integrity of the virion. The viral surface is characteristically made of 72 knobs containing trimers or tetramers of the envelope glycoprotein (Levy, 1993).

Inside the capsid is the HIV-1 genome which has two identical RNA strands with the polarity of mRNA. The RNA genome (approximately 9.8 kb) contains *gag, pol* and *env* genes encoding for viral structural proteins; *tat, rev* and *nef* for regulatory proteins;, *vif, vpr vpu vpt*, and *tev/tnv* for accessory proteins; and long terminal repeats at 5' and 3' ends.

HIV-1 uses the CD4 receptor as the major cellular receptor (Levy, 1993) and, recently, several chemokine receptors (CCR5, CCR3, CCR2B, CXCR4, CCR8, CX3CR, US28) have been identified as co-receptors for viral entry into cells (Cairns and D'Souza,

1998). HIV-1 binds to the CD4 molecule through the high affinity binding of the envelope gp120 to a specific region of the CD4 molecule (McDougal, 1986). The V1 region of the CD4 molecule interacts with the 4th conserved portion near the C-terminal end of the HIV-1 envelope gp120. Once bound to CD4, HIV-1 is internalized into the cytoplasm of the cell. The exact mechanism of internalization is still under intense study but is thought to involve fusion of the cell membrane with another envelope gp41 that is non-covalently associated with gp120. The CD4 molecule is essential for initiating HIV-1 binding, but is not sufficient for efficient viral entry and infection. Recently, chemokine receptors have been shown to be coreceptors for HIV-1. GP120 interacts with these coreceptors to facilitate HIV-1 entry (Alkhatib et al, 1996; Feng et al, 1996). The family of chemokine receptors are variably expressed on lymphocytes (CXCR4) and macrophages (CCR5), and, in part, select for viral strains specific for each of these cell types (Feng et al, 1996; Cocchi et al, 1995). Additionally, CCR3 and CCR2B have also been shown to be fusion coreceptors for HIV-1 entry into monocytes (Doranz et al, 1996). Interaction of gp120 with these coreceptors has been mapped to the V3 loop of the gp120 molecule.

After the virus has gained entry into the cell, the virion associated reverse transcriptase, in conjunction with ribonuclease-H, transcribes the viral RNA into double stranded DNA. The double stranded DNA is then translocated into the nucleus where it is randomly inserted into the host cell genome via the action of viral integrase. At this point in the replication cycle, the HIV-1 genome is called a pro-virus (Varmus, 1988).

After proviral incorporation, both cellular and viral factors are required to initiate expression of viral genes and viral progeny (Varmus, 1988). The cellular factors may be constitutively expressed by the cell, or may be induced by a variety of activating signals:

Ags, mitogen, heterologous gene products, cytokines, ultraviolet light and heat (Levy, 1994). Following activation of HIV-1 provirus, the first viral genes to be expressed are those that encode non-structural proteins involved with regulatory functions (Rosenberg, 1989; Greene, 1991). One of these regulatory proteins is *tat*, which is a powerful transactivator of HIV-1 gene expression and exerts its effect by stimulating production of full length RNA transcripts. These are then multiply spliced and translocated to the cytoplasm where regulatory proteins are expressed. At this phase in the viral life cycle, another regulatory protein, *rev*, is important. It affects the transport of unspliced and single spliced mRNAs, which encode the structural and enzymatic proteins of HIV-1 required for the assembly of the infectious virion at the cell surface, from the nucleus to the cytoplasm. The progeny virus is released from the host cell by budding at the cytoplasmic membrane.

Several reports have documented that peripheral blood mononuclear cells (PBMC) from different donors are not equally infectible with HIV-1 *in vitro* (Wainberg et al, 1987; Cloyd and Moore, 1990; Williams and Cloyd, 1991; Spira and Ho, 1995). Human cells can theoretically resist infection by HIV-1 if there are alterations in host molecules, which HIV-1 uses as receptor (i.e., CD4) or coreceptor (i.e., chemokine receptors), to gain entry into a cell. *In vitro* experiments have shown that alterations in the protein sequence of the CD4 molecule can have dramatic effects on the ability of HIV-1 to infect the cell (Fleury et al, 1991). Although the CD4 molecule in humans is known to be polymorphic (Ghabanbasani et al, 1994), there is no known natural polymorphism in the CD4 molecule which confers resistance to HIV-1 infection. However, there are reports of *in vitro* resistance of host cells to HIV-1 infection attributable to polymorphism in CCR5 (Paxton, 1996). Thus, CD4+ T cells from such individuals do not express a functional cell surface CCR5. They therefore

do not permit HIV-1 entry and thus resist infection *in vitro* (Paxton, 1996). Furthermore, manipulation of the CD28 signal transduction pathway has been shown to result in resistance of cells to HIV-1 infection by downregulating CCR5 expression (Levine et al, 1996; Winkler et al, 1998). In a seminal paper, Walker et al (1986) demonstrated that soluble factors derived from CD8+ T cells are capable of suppressing the replication of HIV-1 in PBMC. Beta chemokines, RANTES, MIP-1α and MIP-1β (the natural ligands for CCR5) have been reported to inhibit HIV-1 infection (Cocchi et al, 1995). Recently, another chemokine (monocyte derived chemokine) was shown to exhibit similar effects on HIV-1 infection of cells (Pal et al, 1997). Baier et al (1995) reported dose dependent inhibition of HIV-1 replication by IL-16. Notably, whereas Levy's factor exerts it's action by inhibition of RNA transcription, the beta chemokines and IL-16 act at a pre-transcriptional level (Clerici, 1996). In summary, chemokine receptor polymorphism can protect cells from HIV-1 infection *in vitro*, and a number of soluble factors, including chemokines, can modulate HIV-1 replication *in vitro*. The relevance of such *in vitro* observations to clinical resistance versus susceptibility to HIV-1 infection is reviewed below.

2.2 The Paradigm of Resistance to HIV-1 Infection

The concept that humans are not uniformly susceptible to HIV-1 infection has evolved over the past five years. This is due to the documentation of a number of individuals who do not show evidence of infection (remaining persistently seronegative and PCR negative), despite evidence for continuous ongoing exposure to HIV-1. However, these individuals exhibit some immunological evidence, which indicates previous exposure and/or replication of HIV-1. For the purpose of this thesis, the group of individuals examined here will be referred to as "HIV-1 resistant" subjects. However, when reference to other studies

is made, the terminology used will be as described in the referenced report. Also, it should be noted that this section discusses evidence of resistance to HIV-1 infection rather than disease progression.

2.2.1 Examples of resistance to HIV-1 infection in humans

The documented cases of resistance to HIV-1 infection in humans may be classified into two types: 1) inherent resistance to HIV-1 infection; and 2) acquired resistance to HIV-1 infection.

2.2.1.1 Inherent resistance to HIV-1 infection. Paxton et al (1996) reported several individuals who had remained free from HIV-1 infection despite repeated exposure through sexual intercourse with HIV-1 positive partners. When HIV-1 was added to leucocytes from these subjects, it was found that cells from two of them were highly resistant to infection with macrophage trophic strains of virus, the strains believed to be important in person to person transmission. This remarkable observation dovetailed nicely with the subsequent discovery of chemokine receptors as cofactors that cooperate with the CD4 molecule to permit entry of HIV-1 into cells (Feng et al, 1996; Deng et al, 1996; Dragic et al, 1996; Doranz et al, 1996; Choe et al, 1996; Alkhatib et al, 1996). These findings were brought together by Liu et al (1996), who showed that the two exposed uninfected subjects harboured identical mutations on both chromosomal copies of CCR5. Subsequently, Samson et al (1996) identified the same mutation and described its prevalence in HIV-1 infected and uninfected populations in Northern Europe. This mutant allele contains a 32 bp deletion that results in truncated protein

which fails to be expressed on the cell surface. It is found in Caucasians of European descent (but very rare in Asians or Africans) with an allele frequency of 0.092-0.098. About 15-20% of Caucasian are heterozygous, while ~1% are homozygous. Only homozygosity for this mutation confers protection from infection in these individuals. However, this protection from infection is not absolute. Four HIV-1 infected men who were homozygous for the CCR5 32 bp mutation have been reported (Biti R et al 1997). Another much rarer mutation, CCR5-m303 with similar effects to 32 bp deletion, was reported earlier this year (Quillent et al, 1998). These mutations are the most likely explanation for how the exposed uninfected individuals resisted the virus and also provide clues as to the mechanism by which HIV-1 gains a foothold in those who are exposed to it. In response to the CC chemokines (RANTES, MIP-1α and MIP-1β), this receptor transduces signals in leucocytes that result in their chemotaxis towards areas of inflammation. Cochii et al (1995) had shown that these chemokines could also inhibit infection by several primary macrophage tropic strains of HIV-1.

2.2.1.2 Acquired resistance to HIV-1 infection. There are a number of reports describing seronegative uninfected individuals despite significant HIV-1 exposure. These include individuals who have unprotected sexual intercourse with HIV-1-seropositive individuals (Paxton et al, 1996; Demoyers et al, 1994; Clerici et al, 1992; Ranki, 1989), intravenous drug users with a known history of needle sharing (Barcellini et al, 1995; Baretta et al, 1996), prostitutes (Rowland-Jones et al, 1995; Fischl et al, 1987;

Travers et al, 1995; Fowke et al, 1996), newborn infants of HIV-1 seropositive mothers (Rich et al, 1997; Gesner et al, 1994; Cheynier et al, 1994; De-Maria, 1994; Clerici et al, 1993; Rowland-Jones et al, 1993), recipients of blood or blood products contaminated with HIV-1 (Shearer and Clerici, 1996), and healthcare workers who have sustained parental exposure to HIV-1 infected blood or body fluids (Clerici et al, 1994; Pinto et al, 1995). Several publications have reported the presence of HIV-1 specific T cell responses in the aforementioned cohorts of exposed seronegative individuals. These reports include T cell proliferation (Rank et al, 1989; Borkowski et al, 1990; Kelker et al, 1992), IL-2 production induced by HIV-1 peptides (Clerici et al, 1996, 1991, 1992, 1993, 1994 and 1989; Jehuda-Cohen et al, 1993; Beretta et al, 1996), and generation of HIV-1 specific cytotoxic T lymphocytes (CTL) responses (Cheynier, 1992; Rowland-Jones et al, 1993 and 1995; De-Maria, 1994; Langlade-Demoyen et al, 1994; Pinto et al, 1995) and are reviewed below.

2.2.1.2.1 Kenyan sex workers. Some of the strongest epidemiological evidence for resistance to HIV-1 infection comes from studies on a group of Kenyan sex workers (Rowland-Jones et al, 1997). Since 1985, these women have been followed as part of a cohort of prostitutes in Pumwani, Nairobi (Plummer et al, 1991; Simonsen et al, 1990). The prevalence of HIV-1 infection in their clients is as high as 25% and they have an estimated minimum average of 64 unprotected sexual exposures to HIV-1 per year

(Fowke et al, 1996). These women have been followed up for 3-13 years, and have remained persistently HIV-1 Ab and PCR negative. Many of these women exhibit a CTL response to HIV-1_{IIIB} *env, gag* and *pol* gene products, and increased IL-2 production to HIV-1 peptides (Fowke et al, 1997). Notably, resistance in these women is not associated with either enhanced levels of CC chemokines or 32 bp deletion in CCR5 gene (Fowke et al, 1998). Resistance or susceptibility in these women correlates with human leukocyte antigen (HLA) class I and class II alleles. The HLA allele A2/A6802 supertype and HLA DR*01 correlates with protection against infection. HLA-A2301 correlates with susceptibility to infection (MacDonald S et al, 1998). It also appears unlikely that HIV-1 receptor or coreceptor mutations are responsible for resistance in these women as their PBMC are readily infected with many different T cell and macrophage tropic HIV-1 isolates (Fowke et al, 1998).

2.2.1.2.2 Gambian sex workers. A total of six Gambian sex workers who remained seronegative for at least seven years have been described who are uninfected with HIV-1 (Rowland-Jones et al, 1995). Five of these six women exhibit CTL response to cross reactive epitopes of HIV-1 and HIV-2. It is noteworthy that, like the Nairobi women, the Gambian women also do not have the mutated CCR5 gene (Rowland-Jones et al, 1997).

2.2.1.2.3 Seronegative homosexual men with exposure to HIV-1. Clerici et al (1992) compared IL-2 production to HIV-1 peptides in seronegative homosexual men who had recent sexual exposure to HIV-1, with that of seropositive homosexual men and seronegative heterosexual men. They reported IL-2 production to HIV-1 peptides in 5/5 test subjects, as well as in 8/11 seropositive homosexual controls and 3/13 seronegative heterosexual controls. Based on this data, they concluded that IL-2 production to HIV-1 may be a more sensitive test for examining exposure to HIV-1 than specific HIV-1 Ab producion, lymphoproliferation, or PCR tests. Notably, this study did not address the role of IL-2 production in resistance versus susceptibility to HIV-1 infection, and there was no significant difference in frequency of IL-2 production between seropositive and seronegative homosexual men. Other cytokines were not examined.

2.2.1.2.4 Health care workers. Clerici et al (1994) examined HIV-1 peptide specific IL-2 production among seronegative healthcare workers exposed to HIV-1 positive blood in comparison with healthcare workers exposed to HIV-1 negative blood. They found IL-2 production in response to HIV-1 peptides in 6/8 of test subjects compared to 1/9 control subjects. Other cytokines were not examined. In a later study, Pinto et al (1995) extended these findings and reported the presence of HIV-1 specific CTL responses

in healthcare workers exposed to HIV-1 positive blood. Notably, these studies did not examine HIV-1 infected (i.e., susceptible) individuals, and hence did not address the role of IL-2 in resistance versus susceptibility to HIV-1 infection.

2.2.1.2.5 Children born to HIV-1 positive mothers. Three uninfected children born to HIV-1 infected mothers were described who lost maternal Ab and who were PCR negative, but in whom HIV-1 specific CTLs could be detected up to 3 years of age described (Cheynier et al, 1992). Subsequently, three other children with similar features were described. In this second group of children, CTLs to HIV-1 were transient and observed only in the first year of life (Aldhous et al, 1994; Rowland-Jones et al, 1995). Clerici et al (1993) examined IL-2 response to HIV-1 peptides in children born to seropositive mothers and found the presence of IL-2 response to HIV-1 peptides in 8/23 cord blood samples. Other cytokines were not examined. Based on this data, they suggested that HIV-1 specific T helper response (i.e., IL-2 response) may be protective in newborns.

2.2.1.2.6 Uninfected partners of seropositive individuals. A recent study examined the immune mechanisms of resistance to infection, possibly involving the CC chemokine pathway, in discordant couples expressing the wild type CCR5 allele (Furci et al, 1997). They derived high frequency of HIV-1 gp120 specific CD4+ T cell clones from the exposed uninfected partners.

Stimulation of these T cell clones with the specific HIV-1 derived peptide or with PHA, but not with the control peptide, induced the release of high amounts of all three CC chemokines (RANTES, MIP-1α and MIP-1β) that inhibited HIV-1 replication. However, they did not study type-1/type-2 cytokine responses in these subjects.

Mazzoli et al (1997) reported the presence of HIV-1-specific IgA in urogenital tract secretions and IL-2/IL-10 production by PBMC to HIV-1 *env* peptides in exposed uninfected subjects. Remarkably, they found the presence of HIV-1-specific IgA in both exposed uninfected as well as HIV-1 infected subjects. However, HIV-1 specific IgG was present in HIV-1 positive, but not in exposed uninfected subjects. Notably, both exposed uninfected and HIV-1 positive subjects exhibited similar frequency and intensity of HIV-1-specific IgA. Thus, secretory HIV-1-specific IgA is not associated with resistance versus susceptibility to HIV-1 infection in these subjects. Furthermore, IL-2 and IL-10 production to HIV-1 peptides was examined in these subjects. They found significantly enhanced IL-2 and lower IL-10 production by PBMC among exposed uninfected subjects compared to their seropositive partners. Other cytokines were not examined. The exposed uninfected subjects examined in this study did not differ in either CC chemokine secretion or the CCR5 gene mutation from the positive partners.

In essence, diverse mechanisms seem to be operating in these various groups of subjects who appear to be resistant to HIV-1 infection. Such subjects often exhibit the presence of HIV-1 specific CTLs. However, the protective role of HIV-1 specific CTL is still largely debated (Mathieson, 1995; Rowland-Jones et al, 1997). It remains

to be established whether they are actually associated with protection from future infection rather than simply markers of past exposure (Rowland-Jones et al, 1997). Notably, in a recent study, a significant inverse correlation was reported between HIV-1 specific CTL frequency and plasma RNA viral load (Ogg et al, 1998). The detection of CTLs in the Nairobi cohort, for whom there is the most convincing evidence of resistance to HIV-1 infection in the face of intense exposures, may point towards a protective role. However, the immune mechanisms to explain the development of such potentially protective CTLs in these individuals remains to be defined.

2.3 Type-1 and Type-2 Model of Cytokine Responses

Cytokines are molecular messengers with extremely important immunobiological functions ranging from haematopoiesis, inflammation, and tissue repair to the regulation of immune responses. Essentially, they orchestrate the immune system as they initiate, steer, amplify and, where necessary, downregulate immune responses.

2.3.1 Origins of the TH1/TH2 and type-1/type-2 concepts

To explain the regulatory role of cytokines, a model was proposed by Mosmann and Coffman a decade ago (Mosmann et al, 1986). They observed that murine T cell clones produced a package of cytokines. Based on the cytokine profile, they named these clones as TH1 (which produced IFN-γ, IL-2, lymphotoxin and IL-3) and TH2 (which produced IL-4, IL-5, IL-6 and IL-3, later IL-10 and IL-13) (Mosmann and Coffman, 1989). Later, Romagnani and colleagues (1991) demonstrated that human T cell clones can also be classified functionally into TH1 and TH2 subsets. Besides the above two sets, they described a third subset, named

TH0, which secreted overlapping sets of cytokines. Later studies by other investigators indicated that IL-2 and IL-10 in humans are unreliable markers for TH1 and TH2 subsets respectively (Del Prete et al, 1993; Abbas et al, 1996; Romagnani 1997). IL-2 can be produced by naive T cells as well sa TH0 and TH2 cells, while IL-10 can be produced by TH1 and TH0 cells and non-T cells, such as monocytes and macrophages (Del Prete et al, 1993; Abbas et al, 1996; Romagnani, 1997).

The regulatory mechanisms underlying the development of TH1- and TH2-like cells from naive T cells have been widely investigated. IL-12, produced by antigen presenting cells (APC), promotes development of naive T cells into TH1-like cells (Manetti et al, 1993; Trinchieri, 1996). In addition, IL-18 produced by APCs can promote TH1 like activity by acting on pre-differentiated TH1 cells (Khono et al, 1998; O'Garra, 1998; Murphy, 1998). Recently, it was demonstrated that human IP-10, a CXC chemokine, selectively promotes TH1 (IFN-γ) cytokine response, although its mechanism of action remains to be determined (Gangur et al, 1998). IL-4 promotes the differentiation of naive T cells into TH2-like cells. However, the determination of the source of initial IL-4 required for this process remains an intensely investigated area (O'Garra, 1998).

The initial classification of TH1/TH2 subsets identified CD4+ T helper cells as the source of these cytokines. However, the later studies showed that, in addition to CD4 T cells, other cell types (such as monocytes, macrophages, natural killer [NK] cells, B cells and CD8 T cells) can contribute to the production of these cytokines (Trinchieri, 1989; Ferrick et al, 1995; Seder et al, 1991; Mosmann and Sad, 1996). Therefore, a new terminology of type-1 (for TH1) and type-2 (for TH2) has been

proposed (Bloom, 1992; Clerici and Shearer, 1994; Abbas et al, 1996). Notably, type-1 and type-2 immune responses are often used to include not only cytokine responses, but also the associated effector responses, such as delayed type hypersensitivity (DTH)/CTL responses and IgE Ab responses, respectively (Abbas et al, 1996). The new type-1/type-2 nomenclature will be used for describing experiments conducted as part of this thesis.

2.3.2 Strengths of the model

Since the original findings by Mosmann and Coffman, the TH1/TH2 model of cytokine regulation has become widely used by many investigators in different systems. The immense popularity of this model is because it explained, under a unified framework, a number of observations previously described in the literature. For instance, in the mouse, IgG2a versus IgE isotypes of Ab produced in a given immune response can be associated with TH1 or TH2 type of cytokine production respectively (Mosmann et al, 1996). Similarly, a number of studies had identified a reciprocal relationship between humoral and cell mediated immune responses (Mosmann et al, 1996; Romagnani, 1997; Abbas et al, 1996). The TH1/TH2 model provides a rational basis for this because, in general, TH1 cytokines promote cell mediated responses and TH2 cytokines promote Ab responses.

More interestingly, the clinical phenotypes of resistance versus susceptibility to a number of diseases as opposed to infection can now be associated with TH1 or TH2 dominated responses. For example, in the widely studied mouse model of Leishmaniasis, *L. major* evokes a dominant DTH response in C57BL/6 mice which is associated with protection from disease and production of TH1 cytokines. In

contrast, disease susceptibility of Balb/c mice is associated with humoral immune response and TH2 cytokine response (Sadick et al, 1990; Scott et al, 1998). Similar examples of other infectious diseases in mouse models are presented in Table 1. In humans, a clear demonstration in TH1 versus TH2 cytokine bias has been described in leprosy (Yamamura et al, 1991). The disseminated form of the disease (lepromatous leprosy) is associated with the TH2 type of cytokine response. In contrast, the contained form of the disease (tuberculoid leprosy) is associated with TH1 type of cytokine responses.

A clearer association between the disease versus tolerance (resistance) and TH1/TH2 cytokine response has been extensively described in human allergic diseases (Romagnani, 1996; HayGlass, 1995). Initial studies by Romagnani et al (1996), using T cell clones derived from allergic and non-allergic subjects, associated TH2 dominated cytokine response with allergic disease. Using allergen mediated activation of fresh human PBMC directly *ex vivo*, Imada et al (1995) elegantly demonstrated that, whereas TH2 dominated cytokine response is associated with the expression of allergic disease, clinically non-allergic subjects exhibited a TH1 dominated cytokine response to the same environmental Ag (allergen).

2.3.3 Weakness of the model

As with any model, the TH1/TH2 paradigm has its own limitations. Since humans are genetically outbred, as apposed to inbred mice, many immune responses in humans are not as strongly polarised as one would see in murine models. Kelso (1995) studied keyhole lymphet haemocyanin and allo Ag primed T cell clones and found that the frequency distribution of cytokine producing T cell clones was

Table 1. TH1/TH2 response and resistance versus susceptibility to infectious diseases in murine models

Agent	Disease	Response	Evidence/Comments
Virus	Influenza	TH1 protects	TH1, but not TH2, anti-influenza clones confer *in vivo* protection (Graham et al, 1994)
Bacteria	*Borrelia burgdorferi*	TH1 protects	Specific TH1 cell lines confer resistance to infection in susceptible mice (Pride et al, 1998)
	Chlamydia	TH1 protects	IFN-γ produced during resistant response; anti-IFN-γ Ab exacerbates; IFN-γ protects (Rank et al, 1992; Perry et al, 1997)
Fungi	*Candida albicans*	TH1 protects	TH1 and TH2 responses correlate with resistance and susceptibility; anti-IL-4 Ab, anti IL-10 Ab and soluble IL-4 receptor induces resistance (Romani et al, 1994a,b,c)
Protozoa	*Leishmania major*	TH1 cures	TH1 response in resistant mice, TH2 in susceptible mice; anti-IL-4 Ab protects, anti IFN-γ exacerbates;TH1 cells protect, TH2 cells exacerbates (Scott et al, 1998)
	Trypanasoma cruzi	TH1 protects	TH1 cells transfer protection (Nickell et al, 1993)
	T. equiperdium	TH1 protects	Successful immunization is associated with a TH1 response (Perito et al, 1992)

unimodal rather than bimodal. She noted that cytokine producing T cells display a spectrum of cytokine secretion profiles of which TH1 and TH2 cells may represent the two possible extreme phenotypes (Kelso, 1995).

Additional arguments have been made that the sensitivity of the assay used to determine the presence of a cytokine may influence the conclusions drawn (Kelso, 1995). Also, the experimental conditions employed by the investigators may result in significantly different results. For example, Imada et al (1995) demonstrated that different mitogens (PHA versus anti-CD3 Ab) can skew the cytokine responses towards TH1 or TH2 dominance. Allen and Maizels (1997) recently concluded that it is important to avoid immediate categorization of immune responses observed in infectious diseases into TH1 or TH2 type, but that the immune responses should be assessed properly by the individual cytokines and the effector pathways that are induced.

The original description of TH1/TH2 hypothesis was based on studying cell clones, which were secreting a package of cytokines. Clones ideally represent a single or few T cells, which are specific for one or few epitopes of a given Ag. The results from such studies do not necessarily reflect the immune response exhibited by the diverse T cell repertoire of a given individual against a complex microbe. Therefore, one has to be cautious in extrapolating the results from such studies. Studying the cytokine response using whole PBMCs may be a better alternative as it includes the cytokines produced by multiple cell types, involves both autocrine and paracrine cytokine regulation, and analyzes a broad T cell repertoire.

2.4. Type-1/Type-2 Paradigm and HIV-1/AIDS

2.4.1 Type-1/type-2 cytokines and HIV-1 replication

There are several reports on modulation of HIV-1 replication *in vitro* by type-1 and type-2 cytokines (Levy, 1993; Fauci, 1993; Foli et al, 1995). However, the modulatory effect of cytokines on HIV-1 replication seems to depend upon the cell type examined and the conditions under which the studies were undertaken. Levy et al (1993) reported that IFN-γ inhibited HIV-1 replication in T cell lines while it enhanced HIV-1 replication in PBMC or in primary macrophages. However, Fauci et al (1993) reported variable effects of IFN-γ on HIV-1 replication in macrophages. Similarly, whereas Levy (1993) reported IL-4 enhanced HIV-1 replication in primary macrophages and CD4+ cells, Fauci (1993) reported variable effects in T cell lines or macrophages. Recently, Jourdan et al (1998) reported that IL-4 induces functional cell surface expression of CXCR4 on human T cells. Thus, IL-4 may play an important role in rendering CD4+ T cells susceptible to infection with HIV-1 via CXCR4, which acts as a co-receptor for virus entry (Jourdan et al, 1998). Also, IL-5 was reported to enhance HIV-1 replication in CD4+ cells (Levy, 1993) and IL-13 inhibited HIV-1 replication in T cells and macrophages (Fauci, 1993). On the other hand, IL-10 was reported to have variable effects on HIV-1 replication (Fauci, 1993). Interestingly, examining the replication pattern of HIV-1 in TH1 and TH2/TH0 clones, Maggi et al (1994) reported that HIV-1 preferentially replicates in TH2 clones than in TH0 clones. Finally, others have reported that HIV-1 replication was inhibited by TH1 but not TH0 CD4+ T cell clones specific for HIV-1 gag p24 (Vyakaranam et al, 1995).

2.4.2 Type-1/type-2 immune response and disease progression in HIV-1/AIDS

2.4.2.1 Type-1/type-2 cytokines and disease progression. It is well known from the experimental and clinical observations that AIDS is associated with (i) increased B cell activity, serum immunoglobulin (Ig) levels and spontaneous *in vitro* IgG production (Pahwa et al, 1986; Clerici and Shearer, 1993); and (ii) decreased T cell proliferation and IL-2 production (Clerici et al, 1989). Based on these observations, Clerici and Shearer (1993) proposed a hypothesis that a TH1 to TH2 switch in cytokine synthesis may play a critical role in the progression of HIV-1 infection to AIDS.

To test this hypothesis Clerici et al (1993) examined THE cytokine response of asymptomatic HIV-1 positive subjects in comparison with a group of HIV-1 infected subjects in various stages of disease progression. Thus, PBMCs from these subjects were cultured with influenza Ags, allogenic cells, or PHA. IL-2 was measured as a TH1 marker cytokine and IL-4 as a TH2 marker cytokine. They found that as patients progressed to disease, their IL-2 response to influenza Ags was lost first, followed by loss of response to alloantigen. Concomitantly, they found enhanced IL-4 responses to PHA in individuals with advanced disease stage compared to asymptomatic subjects. From this data, they concluded that as patients progressed towards AIDS, a switch from TH1 to TH2 cytokine response occurs.

The limitations of this study are: 1) IL-2 was used as a marker of TH1 cytokines. IL-2 is a poor representative of the TH1 subset because, as discussed previously, it is shown to be produced by TH1, TH0 and TH2 clones as well as by naive T cells (Romagnani, 1997; Abbas, 1996; O'Garra, 1998); 2) they correlated IL-2 production to influenza Ags with IL-4 production to PHA for deriving the switch conclusion; and 3) HIV-1 specific cytokine responses were not examined.

Fauci and coworkers (1994) also examined the switch hypothesis in HIV-1 positive individuals in various stages of diseases. They examined PBMC and lymph nodes for constitutive mRNA expression for IL-2, IL-4, IL-10 and IFN-γ directly *ex vivo*. In PBMCs, they found no detectable cytokine message in CD4 T cells. However, CD8 T cells showed message for IL-10 and IFN-γ. Similar results were obtained from lymph node studies. From these findings, they concluded that there was no evidence for TH1/TH2 dichotomy in HIV-1 infection.

This study raises the following concerns. Messenger RNA for cytokine genes in general has a very short half-life and is usually not constitutively expressed. Cytokine gene expression usually requires cellular activation. However, this study did not involve activation of cells *ex vivo* prior to cytokine messenger RNA analysis. Furthermore, the presence of messenger RNA for a cytokine does not necessarily mean protein production (Bamford et al, 1996). Therefore, presence or absence of cytokine messenger RNA is inconclusive evidence of the type of cytokine responses.

Maggi et al (1994) examined the switch hypothesis in seronegative controls and seropositive groups of subjects with different CD4 counts. They examined polyclonally driven (phorbol myristate acetate (PMA) + anti CD3) cytokines IL-4, IL-10 and IFN-γ production. They found that, as disease progressed, both IL-4 and IFN-γ production decreased. They later derived T cell clones from skin biopsies of HIV-1 positive and HIV-1 seronegative subjects, and patients with atopic dermatitis. They obtained the same number of clones producing IFN-γ and IL-4 from HIV-1 positive patients from the other groups. Therefore, they suggested that these HIV-1 positive subjects are in the TH0 stage. Subsequently, they carried out infectivity experiments using TH1, TH0, and TH2 clones. They found that HIV-1 replicates better in TH2 and TH0 than in TH1 clones. Thus, they concluded that while there is no evidence for TH1 to TH2 switch, HIV-1 can favour a shift to TH0 phenotype.

The limitations of this study are: 1) cytokines were examined with a polyclonal stimulator which may not necessarily reflect physiologically relevant Ag driven cytokine responses as has been reported (Imada et al, 1995); 2) this study involves analysis of T cell clones. As alluded to earlier, extrapolating results from T cell clones needs to be done with caution.

There are several experimental differences which may have contributed to the conflicting results among these three studies: 1) type of cells used (PBMC, clones versus skin biopsies); 2) length of the time of activation of cells (0, 4 hours, 6 days); 3) different stimulators used (nothing,

influenza Ag, allo, PHA, anti-CD3 mAb+PMA); 4) different assays used to measure cytokines (mRNA, T cell clones, biopsies, ELISA); and 5) criteria of patient selection (Maggi et al [1994] selected patient groups on the basis of CD4 counts whereas Clerici et al [1993] selected patients on the basis of presence or absence of response to recall and alloantigens).

In addition to these studies, there are several reports testing the TH1/TH2 hypothesis in relation to HIV-1 disease progression. These studies have also employed similar approaches of examining either cytokine protein levels (Meyaard et al, 1996; Clerici et al, 1994 and 1996), mRNA expression (Fan et al, 1993), plasma cytokine levels (Agarwal et al, 1998) or frequency of cytokine secreting cells (Hagiwara et al, 1996). These studies examined *in vivo* levels of cytokines (Agarwal SK et al 1998), directly *ex vivo* (Fan et al, 1993), polyclonal activation (Clerici et al., 1996; Alonso et al, 1997; Meyaard et al, 1996; Fakoya et al, 1997; Fan et al, 1993) or HIV-1 peptide mediated activation (Clerici et al, 1997). Some find evidence for a switch to TH2 phenotype during progression (Clerici et al, 1996 and 1997; Meyaard et al, 1996), others find evidence for TH2 cytokine production in the early phases of HIV-1 infection itself (Meroni et al, 1996). In contrast, some others reported no evidence for TH1/TH2 dysregulation (Alonso et al, 1997; Hagiwara et al, 1996; Fan et al, 1993).

Since IL-12 promotes the type-1 response, several studies have examined IL-12 production in relation to HIV-1 infection with varying conclusions. Chehimi et al (1994) reported impaired IL-12 production to

Staphylococcus aureus (Cowans strain) in HIV-1 infected patients compared to healthy controls which was not due to hyperproduction of IL-10. Daftarian et al (1995) studied mitogen stimulated IL-12 synthesis among HIV-1 positive and control subjects and found significantly reduced mitogen stimulated IL-12 synthesis among HIV-1 infected patients. Meyaard et al (1997), examined IL-12 production by PBMC from HIV-1 positive subjects. They found that where LPS stimulated IL-12 production was normal, the staphylococcus A Cowan (SAC) strain stimulated IL-12 p40 and p70 production was decreased in HIV-1 infected persons. Chougnet et al (1996) reported reduced IL-12 production in HIV-1 infected subjects and attributed this as secondary to hyperproduction of IL-10 in their studies. However, Alonso et al (1997) found no significant difference in PHA stimulated IL-12 production between AIDS patients and normal healthy volunteers.

In summary, as evident from these studies, no consensus has been reached on the issue of the switch hypothesis in HIV-1 disease progression. The key question pf whether the switching reported in some studies is the cause or effect of overt disease remains open for investigation. In contrast to the widely examined role of type-1/type-2 cytokines in relation to disease progression, there are only a limited number of studies examining type-1/type-2 cytokines in seronegative uninfected subjects with a history of exposure to HIV-1. As stated earlier, while there is evidence for the presence of T helper cell responses in exposed uninfected subjects, examination of cytokines has been limited mostly to IL-2 and, in some cases, both IL-2 and

IL-10 synthesis. Thus, a detailed study on the role of Type-1/Type-2 cytokine responses, especially virus driven cytokine responses, in relation to clinical resistance versus susceptibility to HIV-1 infection (as opposed to progression) remains to be conducted.

2.4.2.2 IgE antibody response (a type-2 immune response) and disease progression. In view of the proposed TH1/TH2 switch hypothesis in HIV-1 disease progression and because IgE represents a prototypic product of type-2 immune response, several groups have examined the IgE Ab responses and serum IgE levels in relation to disease progression.

The current model of IgE synthesis in humans is as follows (Bacharier et al, 1998). Entry of exogenous Ag into the immune system leads to its processing and presentation by APC such as macrophages and B cells, to the T cell via the T-cell receptor in the context of the major histocompatibility complex (MHC) class II molecule. After this step, two signals are critical in class switching of B cells to the IgE isotype: i) interaction between the CD40 ligand on the activated T cell and it's receptor CD40 on the B cells; this signal is required for class switching in general and is not specific to IgE; and ii) IL-4 as well as IL-13 produced by type-2 cells (TH2 cells, mast cells/basophils) act as switch factors for class switching of B cell to IgE isotype production; this signal is IgE isotype specific (Pene et al, 1988; Snapper et al, 1988). IL-5 and IL-6 enhance IgE production induced by IL-4/IL-13 and IL-10 promotes B cell growth (Banchereau et al, 1991). Type-1 cells are important in inhibiting IgE synthesis by the production of IFN-γ.

This cytokine inhibits the action of IL-4 on B cells and also inhibits T cell production of IL-4 (Pene et al, 1988).

Multiple reports demonstrate that elevated serum IgE levels can be found in HIV-1 infected patients (Ammann et al, 1983; Israel-Biet et al, 1992; Lucey et al, 1990; Shor-Posner et al, 1995; Wright et al, 1990). Three studies have been performed in an attempt to correlate increased IgE levels in HIV-1 infected subjects with HIV-1 disease progression. Wright et al (1990) found that in a subset of their study cohort of 67 HIV-1 infected men, those with CD4+ T cell counts of less than 200/mm3 had a higher mean IgE level than did controls. Lucey et al (1990) reported a similar finding in their study of 622 HIV-1 infected men. Israel-Biet et al (1992) found a relationship between IgE levels and prognosis in HIV-1 infected patients. Thus, higher rates of AIDS occurred with those patients with AIDS related complex and higher IgE levels than with patients with AIDS related complex and lower IgE levels.

In several studies, atopy, as measured by clinical history or measures of skin prick test or specific IgE Ab has not been found to correlate with elevated IgE levels in HIV-1 infected patients (Ellurie et al, 1995; Israel-Biet et al, 1992; Koutsonikolis et al, 1996; Vigano et al, 1995). However, Sample et al (1990) found increased incidence of allergic disease by ~2-fold after seroconversion in a group of HIV-1 infected males. Maggi (1989) reported the presence of increased serum IgE Abs to fungal Ags in patients with advanced disease. In contrast, in a recent report, Goetz (1997) studied

aeroallergen (dust mite, cat, grass and mold) specific IgE Ab changes in subjects with rapid disease progression. They found reduction in aeroallergen specific IgE Ab as the disease progressed, while total IgE increased from a median of 69 to 116 IU/ml. Thus, they suggested that levels of aeroallergen specific IgE Ab levels do not account for the elevated plasma IgE as seen in patients with advanced disease.

In the previous studies, HIV-1 specific IgE Ab was not examined. Secord et al (1996) reported the presence of HIV-1 specific IgE Ab (as assessed by Western blotting) in the sera of three children who were free from opportunistic infections despite being HIV-1 positive. A recent study reported the presence of HIV-1-specific IgE Ab in children born to HIV-1 infected mothers (Miguez-Burbano et al, 1997). Since IgE Abs do not cross the placenta, they suggested that detection of HIV-1 specific IgE Ab may be useful in the early diagnosis of neonatal infection.

In summary, elevated IgE levels are found in HIV-1-infected subjects and these elevated levels are often not associated with atopy. Elevated IgE levels are more frequently found in the HIV-1 infected patients with low CD4 cell counts or AIDS than in asymptomatic patients. However, aeroallergen specific IgE Ab decreases with disease progression. To date, HIV-1 specific IgE Ab has not been widely examined.

3. HYPOTHESIS AND RATIONALE

Central to the hypotheses tested in this thesis is that an immunological bias towards

a type 1 response is protective against HIV-1 infection. If so, then this can be shown by studying type 1 and type 2 cytokine profiles among HIV-1 resistant prostitutes compared with those who are infected with HIV-1 (ie., susceptible to infection) and unexposed uninfected women. Thus, four specific hypotheses were tested.

3.1 Epidemiologic Resistance to HIV-1 Infection is Associated with Virus Driven Type-1 Dominated Cytokine Response and Susceptibility to HIV-1 Infection is Associated with Virus Driven Type-2 Dominated Cytokine Response

This hypothesis was tested by characterizing inactivated HIV-1 and PHA driven type-1 and type-2 cytokine responses of HIV-1 resistant and susceptible Kenyan women. Comparison was made between resistant and susceptible women based on the rationale that both of these subject groups are chronically exposed to HIV-1 due to their profession, and that only susceptible women are HIV-1 infected. Therefore, differences in the responses observed would reflect differential host response to the virus. HIV-1 positive women with CD4+ cells ≥500/ul were chosen so that recall cytokine responses are readily measurable *in vitro*. HIV-1 driven cytokine responses were also examined in low risk women to ensure that the responses were HIV-1 specific.

Inactivated HIV-1 virus was used in preference to a live infectious virus so that cytokine analysis was not influenced by the infectious virus mediated death of the cells. Also, whole virus was chosen in preference to peptides or proteins so that potential limitation of HLA restriction influencing the immune response was avoided. Since the immune system of a person ultimately decides which Ags are processed and presented to T cells, it was left to the cells and virus to decide which peptides are presented and which cells mediate the cytokine response. The viral strain used in this study is HIV-1_{IIIB} , a laboratory adapted clade

B virus which is widely used for experiments. In Nairobi, the currently dominant Kenyan isolates belong to clades A, C and D. Thus an ideal choice would have been the use of local isolates from Kenya. However, preliminary data suggest the presence of clade B virus (Melanie Murray, personal communication). This viral preparation was used to readily allow others to replicate our work in other populations. Moreover, resistant women in this study have been documented to exhibit a high frequency of CTL to HIV-1 peptide epitopes that are highly conserved between HIV-1 viral clades. Hence, resistance in these women appears to be broadly cross protective.

Whole unfractionated PBMCs were used in preference to purified subsets of T lymphocytes or clones because it has been well shown that many non-T cells, such as NK cells and basophils, can contribute to the cytokine responses (Fearon et al, 1996; Mosmann and Sad, 1996). Furthermore, the idea was to examine the cytokine response of the intact immune repertoire of the host to the intact pathogen.

The cytokines which were measured included IFN-γ, IL-4, IL-5, IL-13 and IL-10 as these cytokine responses can clearly identify type 1 and type 2 responses.

3.2 HIV-1 Resistant Women Exhibit Enhanced Recall Ag Driven Type-1 And Reduced Type-2 Cytokine Responses Relative To Low Risk Kenyan Women

This was tested by characterizing recall Ag (PPD and SK) and mitogen (PHA and anti-CD3 mAb) driven type-1 and type-2 cytokine responses of HIV-1 resistant and presumably low risk HIV-1 negative Kenyan women. This was based on the following rationale: Both groups are HIV-1 negative (seronegative and PCR negative). However, resistant women are commercial sex workers who are continuously exposed to HIV-1 but have remained clinically resistant. In contrast, low risk women are not sex workers and hence

are presumably at low risk for exposure to HIV-1. Therefore, comparison of these two groups of healthy subjects would provide insights into how the HIV-1 resistant women might be potentially different from other healthy subjects with regard to their immune responses.

Recall Ags SK and PPD were used with the following rationale: streptococcal infections are ubiquitous and hence it is unlikely that a given individual may not have been exposed, at least once, to this very common pathogen. Therefore, SK was used in the present study. PPD was chosen as the second recall Ag because tuberculosis is endemic in Kenya and the Kenyan immunization policy recommends bacilli cealmette guerrin (BCG) vaccination at birth. Thus, most individuals have been previously exposed to this Ag as well.

3.3 HIV-1 Resistant Women Differ From Susceptible As Well As Low Risk Kenyan Women In Their Responsiveness To Type-1 Promoting Agents

This was tested by characterizing the IFN-γ responses to type-1 promoting agents (viz., IL-12 and IP-10) on HIV-1 mediated activation among resistant and susceptible women, and IFN-γ responses to IL-12 and IP-10 on SK and PPD Ag mediated activation among resistant and low risk women. IL-12 was chosen as it has a key role in the development of Th1 responses while IP-10, a CC chemokine, has recently been shown to promote IFN-γ responses.

3.4 HIV-1 Resistant Women Differ From Susceptible As Well As Low Risk Kenyan Women In Their Plasma IgE Profiles

This was tested by measuring plasma levels of HIV-specific and allergen specific IgE as well as total plasma IgE among the above three groups of subjects. The allergens chosen were house dust mite (HDM) and cat Ag. Both of these allergens are ubiquitous and can induce and bind specifically to IgE.

MATERIALS AND METHODS

1. SUBJECTS

1.1 HIV-1 Resistant Women

A community based cohort of prostitutes was established in a slum area of Nairobi, Kenya (Simonsen 1990; Plummer et al, 1991) in 1981 for studying sexually transmitted infections (STIs) and later studies focussed on the risk factors for HIV-1 seroconversion. Since then, 1730 women have been enrolled and followed in the study cohort. At enrollment, women were asked in a standard interview about demographic information, sexual behaviour, duration of prostitution, number of sex partners per day, number of regular partners, condom use, and reproductive history. A physical examination, including a genital examination, was also done. Endocervical swabs were obtained to test for gonococcal and chlamydial infections, and samples of peripheral blood were taken for HIV-1 and syphilis serology, T cell subset determination, and isolation of PBMCs. These women were seen routinely every six months (Plummer et al, 1991) but were free to attend the study clinic for any acute condition. At each visit, information on sexual behaviour, contraception, and condom use during the previous six months was collected, and a physical examination and laboratory studies for STIs and HIV-1 were repeated.

All women in the study were tested for the presence of HIV-1 Abs with commerical enzyme immunoassays (EIA) (HTLV-III ELISA [Dupont] from 1985-88; VIRONOSTIKA [Organon Technika] from 1988-90; Detect HIV-1 [IAF Biochem] from 1990-92 and ENZYGNOST HHIV-1/2 EIA [Behring] from 1991 onwards). All seroconversions were confirmed by immunoblot (Novopath Immunoblot, BIORAD) until 1991 and then by RECOMBIGEN HIV-1/2 EIA (Cambridge Biotech). All women who were negative by EIA

were confirmed negative by immunoblot on at least one occasion (Plummer et al, 1991). PBMC from persistently HIV-1 seronegative women were tested on one or more occasions with PCR assay (Dawood et al, 1992). The limit of detection of this assay is 3.8 viral copies per 150,000 cells. Seroconversion was defined as a positive HIV-1 EIA, and a positive immunoblot or confirmatory EIA for women who were previously seronegative. HIV-1 resistant women were defined as those who worked as prostitutes for three or more years during the study period and who remained seronegative and PCR negative for HIV-1 (Fowke et al, 1996).

At enrollment, 1059 (62.8%) of the 1730 women were HIV-1 positive. The remaining 671 women, initially HIV-1 seronegative, were enrolled in a nested study of risk factors for HIV-1 seroconversion of whom 439 women were followed up for 1-12 years. Of these 439 seronegative women, 277 seroconverted to HIV-1 despite prevention efforts (Ngugi et al, 1988). The overall HIV-1 seroincidence was 42 per 100 person-years. In 1986, 12% of clients were HIV-1 seropositive (Simonsen et al, 1988). The prevalence has since increased substantially to ~25%. The average minimum number of unprotected sexual exposures of HIV-1 per year for women in the cohort was estimated to have increased from 24 in 1984 to 64 in 1994. Women followed for the entire period of the study would have experienced ~500 unprotected exposures to HIV-1.

A group of 85 women enrolled in this study between 1985 and the end of 1990 have been identified as resistant to HIV-1 as defined previously. All have remained healthy with no clinical evidence of HIV-1 infection or immunodeficiency,and have normal CD4 T cell counts (>500/ml). They do not differ from other prostitutes who seroconvert to HIV-1 with

respect to sexual behaviours (eg., condom use) or susceptibility to transmission cofactors such as other STI (Fowke et al, 1996).

1.2 HIV-1 Susceptible Women

HIV-1 susceptible women included in this study were prostitutes in the cohort described above, who had tested HIV-1 Ab positive by ELISAs and had been confirmed positive by Western blot and PCR. All the susceptible women included in this study had CD4 counts of ≥500/ml (CDC stage I).

1.3 Low Risk Unexposed HIV-1 Negative Women

HIV-1 seronegative individuals (negative controls) included in this study were Kenyan mothers at low risk for acquiring HIV-1 infection. These women were non-prostitutes and were attending an antenatal clinic as a part of an ongoing Maternal and Child Health study (MCH study) which was initiated in 1986. These women are examined physically, screened for HIV-1 Ab by ELISA (Datta et al, 1994), and routinely followed up. Those women who were non-pregnant at the time of this study were included as low risk; presumably unexposed HIV-1 negative subjects.

2. MATERIALS

2.1 Chemicals and Reagents

Hydrochloric acid, ethyl alcohol, sodium azide, sodium phosphate monobasic, sodium carbonate, sodium bicarbonate and paraformaldehyde were purchased from CanLab (Winnipeg, MB); bovine serum albumin (BSA), P-nitrophenyl phosphate (PNPP), histopaque-1077 and EDTA from Sigma Chemical Co. (St. Louis, MO); sodium chloride, Tween 20 and sodium hydroxide from Mallinckrodt Canada Inc. (Missisuaga, ON);

glutaraldehyde and 2-Mercaptoethanol from Kodak (Rochester, NY); trypan bBlue from Matheson Coleman and Bell (Ohio, USA). Hank's balanced salt solution (HBSS), L-glutamine, penicillin, streptomycin and fungizone from Flow Laboratries (Mclean, VI); RPMI 1640 from Gibco Laboratories Life Technologies, Inc. (Grand Island, NY). DMSO and 2-propanol from Fisher Scientific (Ottawa, ON); and streptavidin-alkaline phosphatase from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.2 Antigens

Psoralene inactivated HIV-1_{IIIB} was purchased from Advanced Biotechnologies Inc., Maryland, USA. According to the manufacturer's specifications, the virus was propagated into H9 cell line, direct pelleted, and suspended into culture medium (RPMI with 10% fetal calf serum [FCS] containing gentamycin). The stock viral particle count was 1.27×10^{10} virus particles./ml and $TCID_{50}$ was $10^{7.5}$/ml. This virus was titrated in H9 cells over a four week period with end point determination by HIV-1 P24 capture assay. The viral titre was calculated by Karber's method.

SK (a kind gift from Hoechst-Roussel Canada Inc., Quebec), PPD (Tubersol, Connaught Laboratories, Toronto, ON), HDM allergen extract (ALLERGENIC EXTRACT FROM *Dermatophagoides pteronissimus*, ALK, Allergologisk Laboratories, Denmark), and cat hair allergenic extract (containing *Fel d1* [the major cat allergen], ALK SQ, Denmark) were used.

2.3 Mitogens

PHA was purchased from Difco (Detroit, MI). Anti-CD3 mAb was OKT3 Ab purified from hybridomas obtained from the American Type Culture Collection, Rockville, MD. This Ab preparation was routinely used in Dr. HayGlass's Lab.

2.4 Recombinant Proteins

Recombinant human IL-4, IL-5, IL-10, IL-13 and IL-12 were purchased from Pharmingen. Human rIP-10 was purchased from PeproTech (Rocky Hill, NJ).

2.5 Antibodies

Capture Abs for IFN-γ, IL-4, and IL-5, as well as biotinylated Abs for IFN-γ, IL-4, IL-5, IL-10 and IL-13 were purchased from Pharmingen Canada. Coating Abs for IL-10 and IL-13 were hybridoma Abs grown and tested in Dr. HayGlass's laboratory.

For IgE ELISA, mouse anti-human IgE (IgG isotype), and biotinylated goat anti-mouse IgG + M (H+L) (Jackson Immunoresearch Laboratories, Inc.) were used.

2.6 Plasma Samples

The plasma samples collected from Kenyan women were frozen at -20°C and shipped to Winnipeg. The samples were thawed at the time of Ab measurements.

2.7 ELISA Buffers

The coating buffer was a bicarbonate buffer of pH 9.6. After coating, plates were blocked with a phosphate buffered saline (PBS) containing 1% BSA, and NaN_3 0.02%. All samples, standards and developing reagents were diluted in PBS containing 0.5% BSA, 0.05% tween 20 and NaN_3 0.02%, with a final pH of 7.4. The substrate PNPP was dissolved in a buffer containing $MgCl_2$.6H2O and dissolved in diethanolamine at pH 9.8. ELISAs were developed with streptavidin alkaline phosphatase conjugate and were used at the optimized concentration of 1:4000. All ELISA results were obtained by reading the absorbance at dual wavelength 405-690 nm.

3. METHODS

3.1 Development of Sensitive Cytokine Specific and IgE ELISAs

Sensitive cytokine assays based on the sandwich ELISA principle were established for measuring cytokine protein production.

3.1.1 IFN-γ ELISA

The capture and biotinylated anti-human IFN-γ Abs (Pharmingen) were used for establishing the ELISA. For this, ELISA plates were coated with different concentrations of the capture Ab (purified mouse anti-human IFN-γ) and incubated overnight at 4°C. The plates were blocked at 37°C for three hours with blocking buffer. IFN-γ containing standard was calibrated against human IFN-γ reference reagent Gg23-901-530 (specific activity 7 x 10^5 U/mg, 1 NIH unit=115 pg provided by Dr. C. Laughlin, NIAID, Bethesda, MD) added at a concentration from 20-0.31 U/ml (seven 2-fold dilutions). No samples were added to two wells in each plate to measure the background colour. Each coated concentration of capture Ab was then developed individually with different concentrations of the biotinylated mouse anti-human IFN-γ (Pharmingen). After overnight incubation at 4°C, the plates were developed with streptavidin alkaline phosphatase conjugate for 45 minutes at 37°C followed by the addition of PNPP substrate tablets. Optical density was measured at the 405-690 filter at different time points (30, 60, 90 and 120 minutes). The maximal window was determined for each combination at different time points by taking into consideration the linearity of curve, low background and maximum range of absorbance obtained. The optimized conditions were then used for assaying IFN-γ production throughout this study.

3.1.2 IL-4 ELISA

An ultrasensitive sandwich ELISA, established in Dr. HayGlass's laboratory, (Gangur et al, 1998) was used in this study to measure Ag driven IL-4 production. This assay typically had a sensitivity of 0.45-0.9 pg/ml. A recombinant human IL-4 standard of specific activity equivalent to that of the WHO standard 88/656 (Mire-Sluis et al, 1996) was used in this ELISA.

3.1.3 IL-5 ELISA

The capture and biotinylated anti-human IL-5 Abs (Pharmingen) were used for establishing this ELISA. ELISA plates were coated with different concentrations of the capture Ab (purified rat anti-human IL-5 mAb; clone TRFK5) and incubated ovenight at 4 °C. The plates were blocked at 37 °C for three hours with blocking buffer. The sample was rIL-5 (Pharmingen) added at a concentration from 1000-7.8 pg/ml (seven 2-fold dilutions). No samples were added to two rows to measure the background absorbance. Each coated concentration was then developed individually with different concentrations of the biotinylated rat anti-human IL-5 mAb. After overnight incubation at 4°C, the plates were developed with streptavidin alkaline phosphatase conjugate for 45 minutes at 37 °C, followed by the addition of PNPP substrate. Optical density was measured at 405-690 nm filter at different time points. Optimal conditions were deduced as described above.

3.1.4 IL-10 ELISA

The IL-10 ELISA was established using the combination of a capture mAb (obtained from a hybridoma clone JES3-19F1.1.1) and a biotinylated rat anti-human IL-10 mAb (Pharmingen). Anti-human IL-10 mAb was coated overnight at 4°C.

Recombinant IL-10 (Pharmingen), in 2-fold dilutions from 500 pg/ml to 3.8 pg/ml, was added as the sample and the plate incubated at 37 °C for 5 hours. Biotinylated anti-human IL-10 mAb was then added. After overnight incubation at 4°C, the plate was developed with streptavidin alkaline phosphatase followed by the addition of substrate. The plates were read at 30, 60 and 90 minutes. Optimal conditions were determined as described previously.

3.1.5 IL-13 ELISA

An ELISA established in Dr. HayGlass's laboratory was used in this study to assay Ag driven IL-13 production. The capture Ab was hybridoma grown (clone JES10-5A2, ATCC), biopilot purified anti-human IL-13, and the development Ab was biotinylated rabbit anti-human IL-13 (Pharmingen). This assay routinely had a sensitivity of 1.05 pg/ml. Recombinant human IL-13 (PeproTech) was used as a standard in this ELISA.

3.1.6 IgE ELISA

Of all the immunoglobulins (Igs) in the plasma, IgE is generally present in the lowest level (pg to ng/ml). Furthermore, Ag specific IgE Ab is present in much lower concetrations. Therefore, ultrasensitive IgE assays were established based on the principle of sandwich ELISA as described below.

3.1.6.1 Antigen specific IgE Ab ELISA

3.1.6.1.1 HIV-1 specific ELISA: HIV-1 whole virus lysate precoated plates (Genetic Systems, Inc.) were used for this assay. Plates were blocked at 37 °C for 3 hours with blocking buffer. Plasma sample was then added and serially diluted (four 2-fold

dilutions each) and incubated overnight at 4°C. Monoclonal mouse anti-human IgE Ab (HB121 hybridoma Ab prepared in Dr. HayGlass's laboratory; IgG isotype) was added and incubated overnight at 4°C. The following day, biotinylated anti-mouse IgG Ab (biotinylated goat anti-mouse IgG + IgM [H+L], Jackson Immunoresearch Laboratories, Inc.) was added. After overnight incubation at 4°C, the plates were developed with streptavidin alkaline phosphatase for 45 minutes at 37°C followed by the addition of PNPP substrate. Optical density was measured at 405-690 nm at different time points (30 minutes and then every hour to a maximum of 6 hours). The assay was repeated several times to obtain a proper initial dilution of plasma for measuring the IgE Ab and avoiding non-specific background. Positive control serum samples, supplied with the kit and also from previously characterized HIV-1 positive patients, were always included in the assays. Negative control serum, supplied with the kit and the serum samples from known seronegative volunteers, or plasmas from subjects with allergic disease, were included in the assays to ensure the Ag specificity as well as the isotype of the Ab measured.

The plasma sample giving the best window was determined and used as an internal standard for all HIV-1 specific IgE measured in this study. For determining the maximal window, the optical densitiy obtained with each combination of the capture Ag at

different time points was compared by taking into consideration the linearity of curve, low background and maximum range of absorbance. The data is expressed as the actual optical density values obtained at the dual wavelength of 405-690 nm. These optimized conditions were then used for assaying HIV-1 specific IgE Ab levels in plasma throughout this study.

3.1.6.1.2 Cat allergen specific IgE ELISA. Plates were coated with a standardized cat hair extract containing *Fel d1* (Nordic Merrel Dow Research, Alk SQ, Denmark) at two different concentrations. Plates were incubated overnight at 4°C. The following day, plates were blocked at 37°C for 3 hours with blocking buffer and plasma samples (n=5) from previously characterised cat allergic subjects were serially diluted (seven 2-fold dilutions each) and incubated overnight at 4°C. The ELISA was carried out and optical coating allergen concentration was deduced as described for the HIV-1-IgE ELISA procedure. These optimized conditions were then used for assaying cat specific IgE Ab levels in the plasma. An internal standard containing cat specific IgE Ab (1000 units/ml) was included in each assay. Cat specific IgE Ab levels in each plasma sample were determined from quadruplicate estimations falling on the linear part of the standard curve generated using the internal standard in each plate and expressed as arbitrary units/ml.

3.1.6.1.3 HDM specific IgE ELISA. Standardized mite *Dermatophagoides pteronyssimus* exudate (ALK Allergologisk Laboratories, Denmark) was coated at two different concentrations. Plates were incubated overnight at 4°C. The following day, plates were blocked at 37°C for 3 hours with blocking buffer and plasma samples (n=5) from previously characterised allergic subjects was added and serially diluted (seven 2-fold dilutions each) and were incubated overnight at 4°C. The ELISA was carried out and optical coating allergen concentration was deduced as described for the HIV-1-IgE ELISA procedure. These optimized conditions were then used for assaying HDM specific IgE Ab levels in the plasma. An internal standard containing HDM specific IgE Ab (1000 units/ml) was included in each assay. Mite specific IgE Ab levels in each plasma sample was determined from quadruplicate estimations falling on the linear part of the standard curve, generated using the internal standard in each plate and expresssed in arbitrary units/ml.

3.1.6.2 Total plasma IgE (TIgE) ELISA. ELISA plates were coated with a monoclonal mouse anti-human IgE Ab (Serotec, Canada). Plates were incubated overnight at 4°C. The following day, plates were blocked at 37°C for 3 hours with blocking buffer and plasma samples were added and diluted (seven 2-fold dilutions each). An internal standard IgE (whose IgE concentration was kindly determined and reported by Johns Hopkins University, USA) was included in each assay. The first developing Ab

(sheep anti-human epsilon chain Ab) was added and incubated overnight at 4°C. The following day, a second Ab [biotinylated goat anti-mouse IgG + IgM (H+L), Jackson Immunoresearch Laboratories, Inc.] was added at six different final concentrations. After overnight incubation at 4°C, the plates were developed with streptavidin alkaline phosphatase conjugate for 45 minutes at 37°C, followed by the addition of PNPP substrate. Optical density was measured at 405-690 nm at different time points (30, 60 and 120 minutes). Optimal concentrations of developing Abs were deduced as described before. These optimized conditions were used in all assays. Total IgE levels in each plasma sample was determined from quadruplicate titration, falling on the linear part of the standard curve generated on each plate using the internal standard and expressed in ng/ml.

3.2 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Whole blood (25 ml) was collected into citrate tubes. Blood was layered on histopaque-1077 (Sigma) for 30 minutes at 1600 rpm. Plasma samples were collected from the top layer and sent for serological test for HIV-1. An aliquot of plasma was frozen at -20°C. Immidiately following the plasma collection, PBMC were collected from the interface, washed three times with 0.15 N NaCl, counted and viability determined by the trypan blue exclusion method. Cells were either used immediately or frozen under liquid nitrogen.

3.3 Long-Term Storage of PBMC

3.3.1 Freezing of PBMC

PBMC to be frozen were counted on a hemocytometer and then pelleted by centrifuging at 400xg (1500 rpm) for 5 minutes. The supernatant was discarded and

the cell pellet was resuspended in ice cold freezing medium to yield a final cell concentration of 10 million/ml. The resuspended cells were transferred to prechilled, labelled cryovials with silicon gasket (Nalgene) in 1 ml aliquots. The lid of the cryovials was tightened and immediately transferred to a prechilled cell freezing apparatus (Nalgene), where cells were stored overnight at -80°C. Cryovials were then transferred to liquid nitrogen tanks. The freezing solution contained 50% FCS, 42.5% RPMI 1640 and 7.5% DMSO and was cooled on ice before use. The frozen cells were kept in liquid nitrogen until they were transported to Winnipeg. They were thawed when required.

3.3.2 Thawing of frozen PBMC

The cryovials containing cells of interest were removed from the liquid nitrogen and lowered into a 37°C waterbath until some ice remained. The vial was taken into a laminar flow hood where it was opened while wrapped in an ethanol swab. The contents of the cryovial were gently resuspended and transferred to a tube containg ice cold culture medium (13 ml) and mixed. The cells were pelleted by centrifuging at 1500 rpm for 5 minutes. The supernatants were poured off and cells were gently resuspended in to 2-3 ml of culture medium. A small aliquot was stained with trypan blue for viability estimations. PBMC with a viability of >92 % were used in the experiments.

3.4 Cell Culture

3.4.1 Establishment of a short-term cell culture system to measure Ag driven cytokine responses

A short-term bulk cell culture system was established using PBMC isolated

from Kenyan subjects. Collected fresh blood was subjected to density gradient centrifugation to isolate mononuclear cells as described before. Isolated PBMC were either used fresh, or frozen and later thawed when required. PBMC were assessed for viability using trypan blue dye exclusion and were used for culture only when the viability was >92%. Cultures were set up following standard human cell culture protocols. The optimal cell concentration of 1.5 x 10^6/ml, established in Dr HayGlass's laboratory was used.

For assessing cytokine responses, the following culture conditions were optimized: 1) concentration of each Ag and the polyclonal activator used; and 2) days of cell culture to harvest supernatant

3.4.2 Estimation of cytokine protein concentration in culture supernatant

Cytokine levels in various culture supernatants were measured following titrations of 4-8 dilutions falling on the linear part of the standard curve generated on each plate for the cytokine-specific ELISAs employed. The data presented represent means (±SEM) of quadruplicate determinations for each cytokine.

3.4.3 Optimization of culture conditions for assaying HIV-1 specific cytokine responses

Initial experiments were carried out with different virus concentrations to determine the optimal concentration for use in later assays. For this, PBMC from three resistant women were used. Three sets of replicate cultures were set up for each concentration and supernatants were harvested on different days from each set to determine the kinetics of cytokine responses following virus stimulation. PBMC were cultured at 1.5 x 10^6/ml in 200 ul complete tissue culture medium (RPMI 1640

supplemented with 2 mM L-glutamine, 50 uM 2-mercaptoethanol, 100 units/ml penicillin, 100 ug/ml streptomycin, 100 units/ml fungizone, and 10% heat-inactivated FCS) in the absence and presence of different concentrations of inactivated HIV-1_{IIIB} (1.25×10^6, 10^5, 10^4, 10^3, and 10^2 VP (viral particles)/ml). Cultures were incubated at 37°C and supernatants were harvested from respective sets on days 4, 6 and 8. The supernatants were analyzed for IFN-γ, IL-4, IL-5, IL-10 and IL-13 by ELISA. The concentration of virus and days of culture for optimal cytokine response were determined, taking into consideration peak virus driven cytokine synthesis and days of culture, and minimum background cytokine production.

In the case of IL-4, no detectable HIV-1 driven IL-4 synthesis was evident among resistant women. Hence, to determine optimal virus concentration for IL-4 production, PBMC from two HIV-1 susceptible Kenyan women were used. These experiments were essentially set up in the same way as above, and optimal conditions were determined.

3.4.3.1 Determination of the effect of psoralene on cytokine production: Since psoralene was used for HIV-1_{IIIB} inactivation, its effect on cytokine production was examined. For this, a sample of the same brand of psoralene as that used for HIV-1_{IIIB} inactivation, was obtained from the manufacturer and added to SK at the same concentration as present in the supplied product (0.00001 ug/ml). Cultures were set up with SK, without SK, and with psoralene plus SK. Supernatants were harvested on the previously preoptimized day and cytokine production was determined. Effect of psoralene on Ag driven cytokine production was assessed.

3.4.3.2 Determination of the specificity of virus induced cytokine responses: To determine that the cytokine response induced is indeed specific to HIV-1, virus driven cytokine responses of known HIV-1 seronegative and PCR negative, presumably unexposed low risk Kenyan women, were examined. PBMC, stimulated with the optimized concentration of viral preparation and culture supernatants, were harvested on the optimized day. Supernatants obtained were analyzed for various cytokines and specificity of cytokine response to HIV-1 was assessed.

3.4.4 Optimization of culture conditions for SK Ag driven cytokine responses

To determine the optimal concentration and kinetics of cytokine responses following *in vitro* stimulation with SK Ag, PBMC from two known HIV-1 seronegative and PCR negative, presumably HIV-1 unexposed, Kenyans were cultured in the absence or presence of different concentrations of SK (at 1000, 5000, 25000 and 50,000 U/ml). Four sets of replicate cultures (for each different day of harvest) were set up with the above concentrations of SK. Culture supernatants were collected on days 1, 2, 4 and 6 from the representative set. Various cytokines were assayed by ELISA and the optimal conditions deduced.

3.4.5 Optimization of culture conditions for PPD Ag mediated cytokine responses

To determine the optimal concentration and kinetics of cytokine responses following *in vitro* stimulation with PPD, PBMC from two known HIV-1 seronegative and PCR negative, presumably HIV-1 unexposed, Kenyan women (low risk women) were cultured in the absence or presence of different concentrations of PPD (at 5 and

50 tuberculin units [TU]/ml). Three sets of replicate cultures for each different day of harvest were set up. Culture supernatants were collected on days 4, 6 and 8, and various cytokines were measured by ELISA. Optimal conditions were deduced as described before.

3.4.6 Optimization of culture conditions for PHA driven cytokine responses

PBMC isolated from two healthy subjects were used for determining optimal PHA concentrations and the optimal day for the harvest of culture supernatant. A replicate set of cultures were set up with 1.5 x 10^6/ml PBMC in the absence or presence of two different concentrations of PHA-P (1% and 5%). Supernatants were harvested at two different time points (48 and 96 hours) after stimulation. Various cytokine levels were measured by ELISA, and the optimal concentration and the day of harvest were determined.

3.4.7 Optimization of culture conditions for anti-CD3 mAb driven cytokine responses

The conditions preoptimized in Dr. HayGlass's laboratory were used in all experiments done in this study (Gangur et al, 1998).

3.5 Analysis of IL-12 and IP-10 Responsiveness

3.5.1 Analysis of IL-12 responsiveness among Kenyan women

In the experiments presented, all conditions employed were similar to that described in the previous section. The concentrations of rIL-12 and rIP-10 used in these experiments were based on previous results from Dr. HayGlass's laboratory (HayGlass et al, 1997; Gangur et al, 1998).

For each subject, cultures were set up in the absence of stimuli, in presence

of rIL-12 alone (Pharmingen, Canada), in HIV-1_{IIIB} 1.25 x 10^2 and 1.25 x 10^3 VP/ml (ABI diagnostics, Maryland, USA)), in PPDat 5 tuberclin units/ml (Connaught Laboratories, ON) and in SK at 5000 U/ml (Streptase, Hoechst). To examine the IL-12 responsiveness, cultures were set up with and without these stimuli plus rIL-12 at 25 and 100 pg/ml. These concentrations were selected based upon experiments in Dr. HayGlass's laboratory. The concentrations selected are at or below the range of IL-12 synthesis typically observed following bacterial Ag (SAC) mediated stimulation of fresh PBMC (data not shown). Culture supernatants were collected on days 4 and 6. IL-12 responsiveness was measured as the capacity of fresh PBMC to mount IFN-γ responses to the above concentrations of exogenous rIL-12. Peak IFN-γ protein levels were determined as described before, and were used as a read out for these experiments.

3.5.2 Analysis of IP-10 responsiveness among Kenyan women

For each subject, cultures were set up in the absence of stimuli, in presence of rIP-10 alone (PeproTech, Rocky Hill, NJ, endotoxin 0.031 ng/mg), with HIV-1_{IIIB}. at 1.25 x 10^2 and 1.25 x 10^3 VP/ml (ABI-Diagnostics, Maryland, USA), with PPD at 5 TU/ml (Connaught Laboratories, ON), and with SK at 5000 U/ml (Streptase, Hoechst). To examine the IP-10 responsiveness, cultures were set up with and without these stimuli plus rIP-10 at 0.1 and 1 ng/ml. IP-10 responsiveness was measured as IFN-γ response to exogenous IP-10. Culture supernatants were collected on days 4 and 6. Peak IFN-γ protein levels were determined as described before, and were used as a read out for these experiments.

4. STATISTICAL ANALYSIS

The data are expressed as median and range for each of the groups. Medians were chosen instead of means because they are less influenced by the outlier values. Statistical significance between the groups was determined by a non-parametric Mann-Whitney U test. The difference in the frequency of responders between the groups was evaluated for significance by Fisher's exact test. The statistical significance cut-off value used for both Mann-Whitney and Fisher's test in this study was at a level of 0.05.

RESULTS

1. OPTIMIZATION OF EXPERIMENTAL CONDITIONS

1.1 Optimization of Cytokine Specific ELISAs

Optimization of the ELISA parameters for measuring IFN-γ protein levels was done as described in the Methods Section. The sensitivity of the assay was routinely ~0.3 U/ml (Figure 1). Optimization of the parameters for measuring IL-5 production was done as described in Methods. The detection limit of the assay was routinely ~4 pg/ml. Optimization of the parameters for measuring IL-10 production was done as described in Methods. The detection limit was routinely ~8 pg/ml. The sensitivity of the IL-4 ELISA (~0.45 pg/ml) used in this study is shown in Figure 2.

1.2 Establishment of Short-Term *In Vitro* Culture System to Measure Cytokine Responses

A short-term bulk culture system was established using PBMC isolated from Kenyan subjects as described in Methods. Isolated PBMC were either used fresh, or frozen and later thawed when required. PBMC were assessed for viability using trypan blue dye exclusion and were used for culture only when the viability was >92%. Cultures were set up following standard human cell culture protocols. For assessing cytokine responses, the concentration of each Ag, the polyclonal activator used, and days of cell culture were optimized.

1.2.1 Optimization of culture conditions for assaying HIV-1 specific cytokine responses

Preliminary experiments performed to optimize the virus concentration and days of culture were conducted using PBMC from HIV-1 resistant Kenyan women.

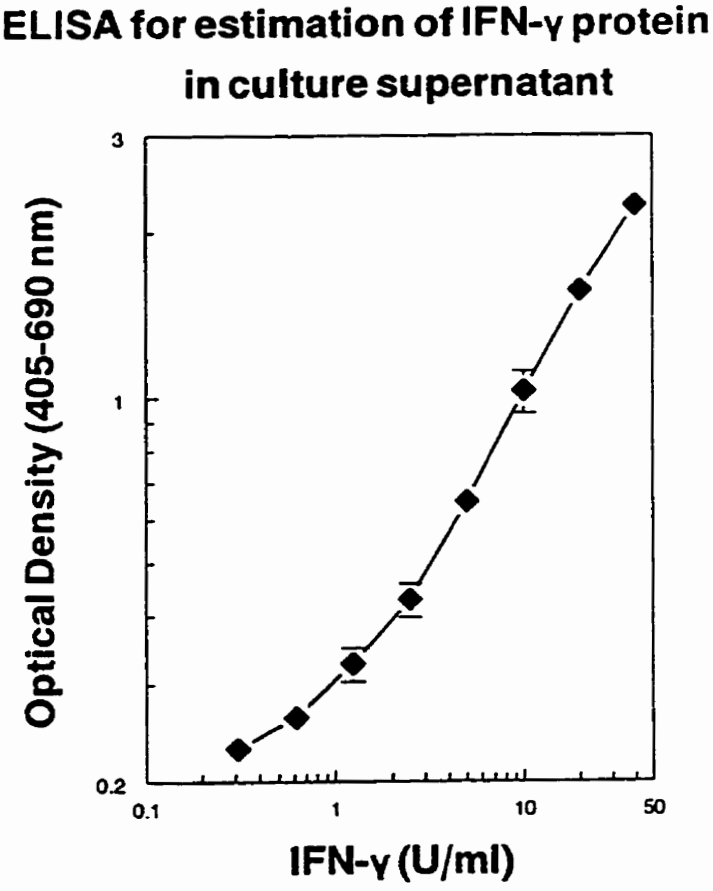

Figure 1. ***Development of a sensitive IFN-γ ELISA.*** This figure shows the standard curve generated on each ELISA plate for estimation of IFN-γ protein levels in the culture supernatants. The figure shows the sensitivity of assay (typically ~ 0.31 U/ml) used.The Y axis indicates the optical density at 405-690 nm. The X-axis indicates various concentrations of human IFN-γ in U/ml.

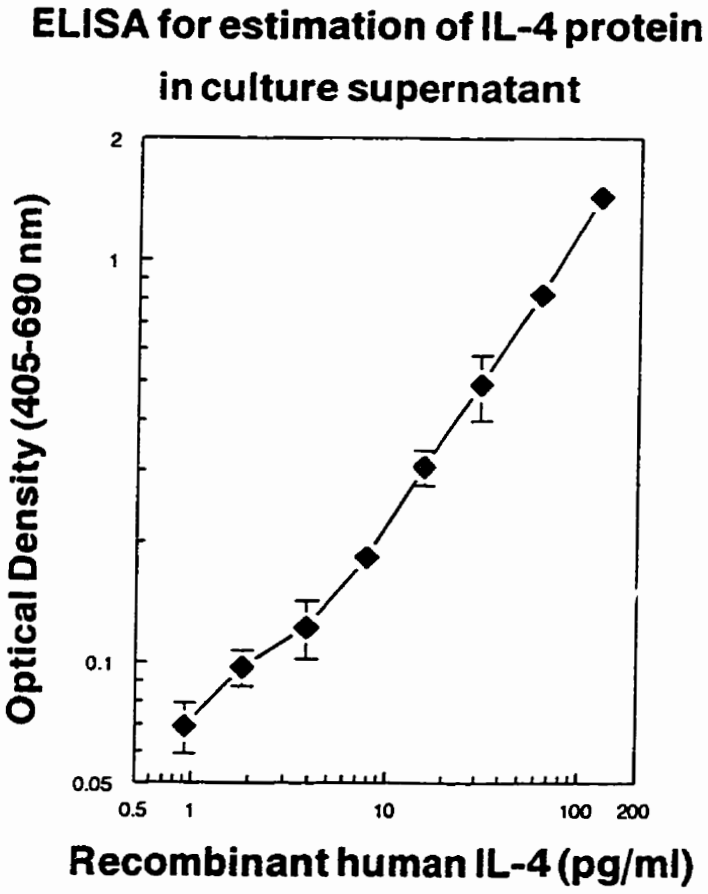

Figure 2. ***Sensitivity of IL-4 ELISA.*** This figure shows the standard curve generated on each ELISA plate for estimation of IL-4 protein levels in the culture supernatants. The figure shows the sensitivity of assay (typically ~ 0.45 pg/ml) used. The Y axis indicates the optical density at 405-690 nm. The X-axis indicates various concentrations of human rIL-4 (pg/ml).

1.2.1.1 IFN-γ: Optimization experiments done using PBMC from two resistant women (as described in the Methods Section) revealed that peak virus driven IFN-γ response was observed with $1.25x10^2$ or $1.25x10^3$ vp/ml on day 6 of the culture. Therefore, both concentrations of virus were used for all the experiments. Results from one representative subject of the three studied is shown in Figure 3.

1.2.1.2 IL-4: PBMC isolated from two resistant women were cultured with a range of concentration of HIV-1 ($0\text{-}1x10^6$ VP/ml). Culture supernatants were collected on days 4, 6, and 8, and assayed for IL-4. ELISA analysis revealed that there was no detectable IL-4 production. Hence, for evaluating the optimal conditions for this cytokine, PBMC from two HIV-1 susceptible Kenyan women were used. Peak virus driven IL-4 response was observed with a virus concentration of $1.25x10^2$ or $1.25x10^3$ VP/ml on day 6 of culture. Results from one representative subject of the two studied are shown in Figure 4.

1.2.1.3 IL-5: PBMC isolated from two resistant women were cultured with the virus as described. Peak virus driven IL-5 response was observed with a virus concentration of $1.25x10^2$ VP/ml or 10^3 VP/ml on day 6 of culture (Figure 5).

1.2.1.4 IL-10: PBMC isolated from two resistant women were cultured with the virus as described. Peak virus driven IL-10 response was observed with a concentration of $1.25x10^2$ and $1.25x10^3$ VP/ml on day 6. Figure 6 shows the results from one of the two representative subjects studied.

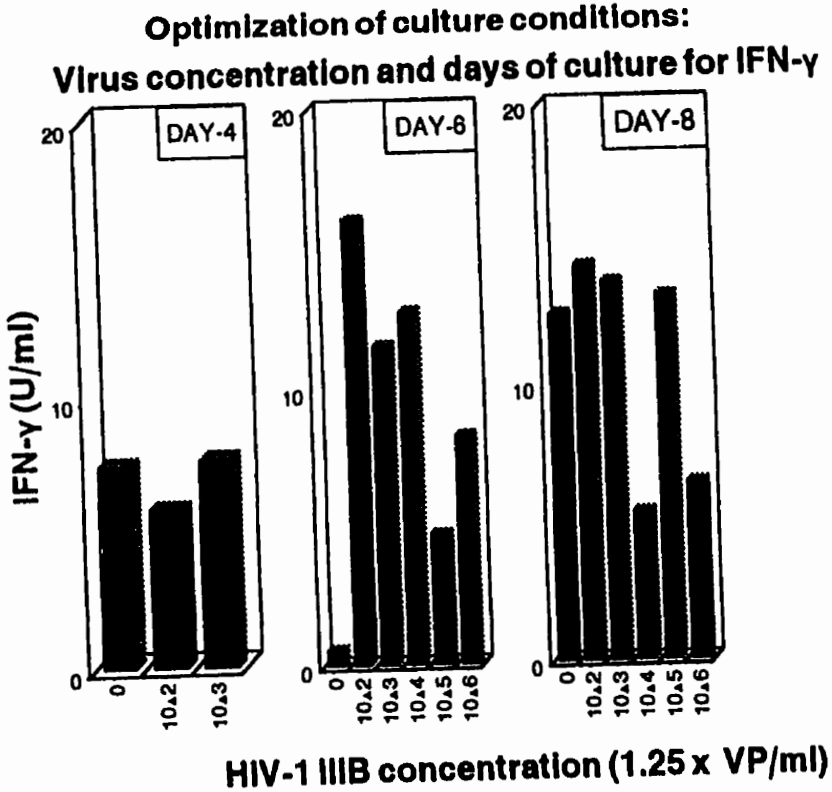

Figure 3. Optimization of culture conditions for measuring HIV driven IFN-γ. PBMCs (1.5 x 10^6/ml) from HIV resistant women were cultured with inactivated HIV IIIB at various concentrations as shown on X-axis. Each panel shows IFN-γ levels (as indicated on Y-axis) produced in the culture on indicated days of supernatant harvest. Results from one representative subject are shown.

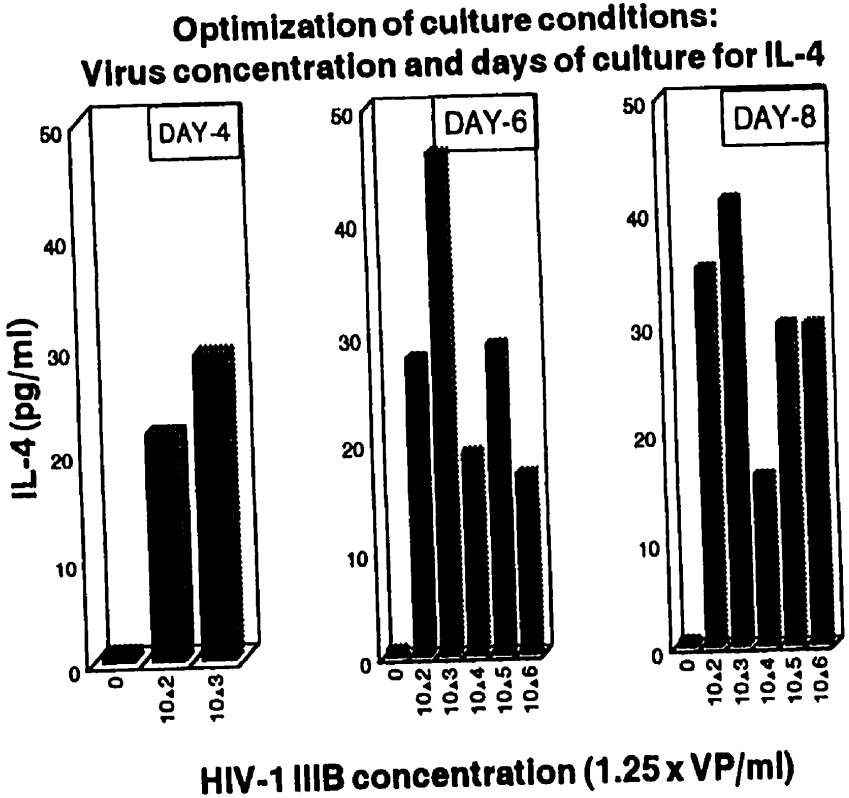

Figure 4. Optimization of culture conditions for measuring HIV driven IL-4. PBMCs (1.5×10^6/ml) from HIV positive women were cultured with inactivated HIV IIIB at various concentrations as shown on X-axis. Each panel shows IL-4 levels (as indicated on Y-axis) produced in the culture on indicated days of supernatant harvest. Results from one representative subject are shown.

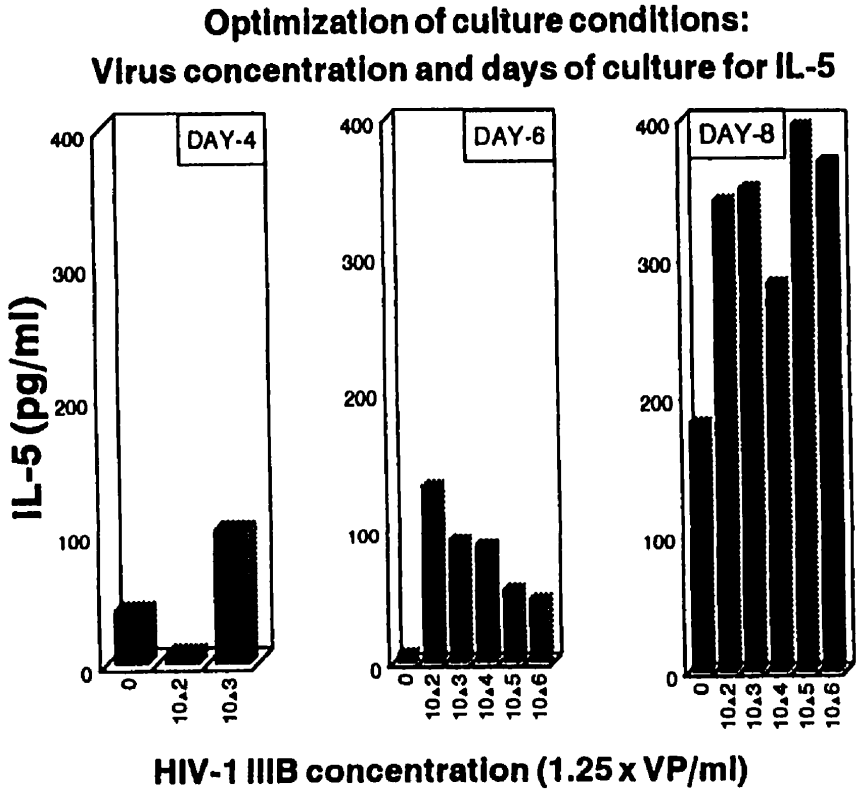

Figure 5. Optimization of culture conditions for measuring HIV driven IL-5. PBMCs (1.5 x 10^6/ml) from HIV resistant women were cultured with inactivated HIV IIIB at various concentrations as shown on X-axis. Each panel shows IL-5 levels (as indicated on Y-axis) produced in the culture on indicated days of supernatant harvest. Results from one representative subject are shown.

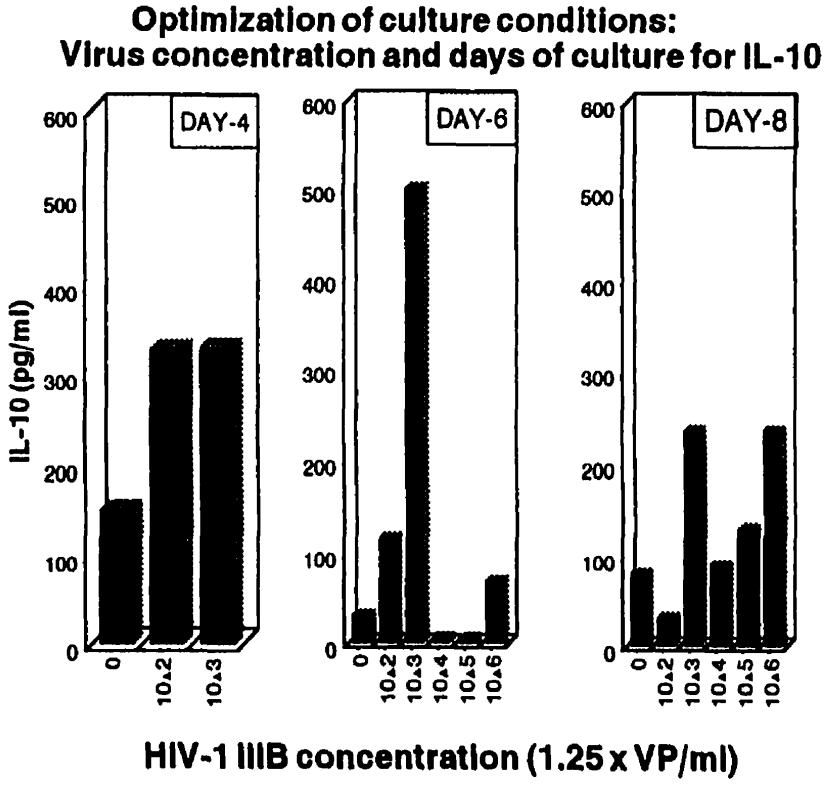

Figure 6. Optimization of culture conditions for measuring HIV driven IL-10. PBMCs (1.5 x 10^6/ml) from HIV resistant women were cultured with inactivated HIV IIIB at various concentrations as shown on X-axis. Each panel shows IL-10 levels (as indicated on Y-axis) produced in the culture on indicated days of supernatant harvest. Results from one representative subject are shown.

1.2.1.5 IL-13: PBMC isolated from two resistant women were cultured with the virus as described in the methods. Peak virus driven IL-10 response was observed with a concentration of 1.25×10^2 and 10^3 VP/ml on day 6 of the culture. Results from one representative subject of the two studied are shown (Figure 7).

1.2.2 Effect of psoralene on cytokine production

Impact of psoralene on Ag driven cytokine response was analyzed as described in the Methods Section. Presence of psoralene at the concentration used did not have a significant impact on Ag driven cytokine response. Figure 8 shows the results from a representative experiment.

1.2.3 Determination of the specificity of virus induced cytokine responses

To determine that the cytokine responses are recall HIV-1 specific responses, cytokine responses of low risk Kenyan women following virus stimulation were examined as described in Methods. These subjects did not generate cytokine responses to HIV-1. This confirmed that virus driven cytokine responses among resistant women and susceptible women are HIV-1 specific and so, low risk subjects were not used as controls for comparision of HIV-1 specific responses.

1.2.4 Optimization of culture conditions for assaying SK Ag driven cytokine responses

Experiments using PBMC from two low risk women were performed to optimize the SK Ag concentration and days of culture. The peak cytokine responses were observed with 5000 U/ml of SK Ag and 6 days of culture. Results from one representative subject is shown for the indicated cytokines (Figures 9 and 10).

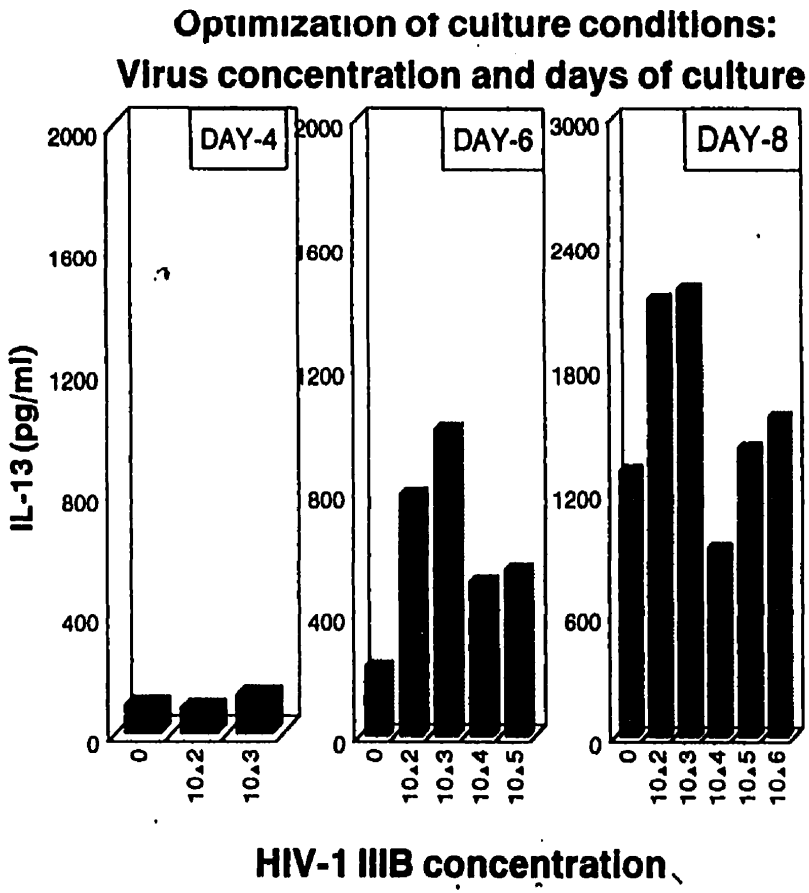

Figure 7. Optimization of culture conditions for measuring HIV-1 driven IL-13. PBMCs (1.5×10^6/ml) from HIV-1 resistant women were cultured with inactivated HIV-1 at various concentrations as shown on X-axis. Each panel shows IL-13 levels (as indicated on Y-axis) produced in the culture on indicated days of supernatant harvest. Results from one representative subject are shown.

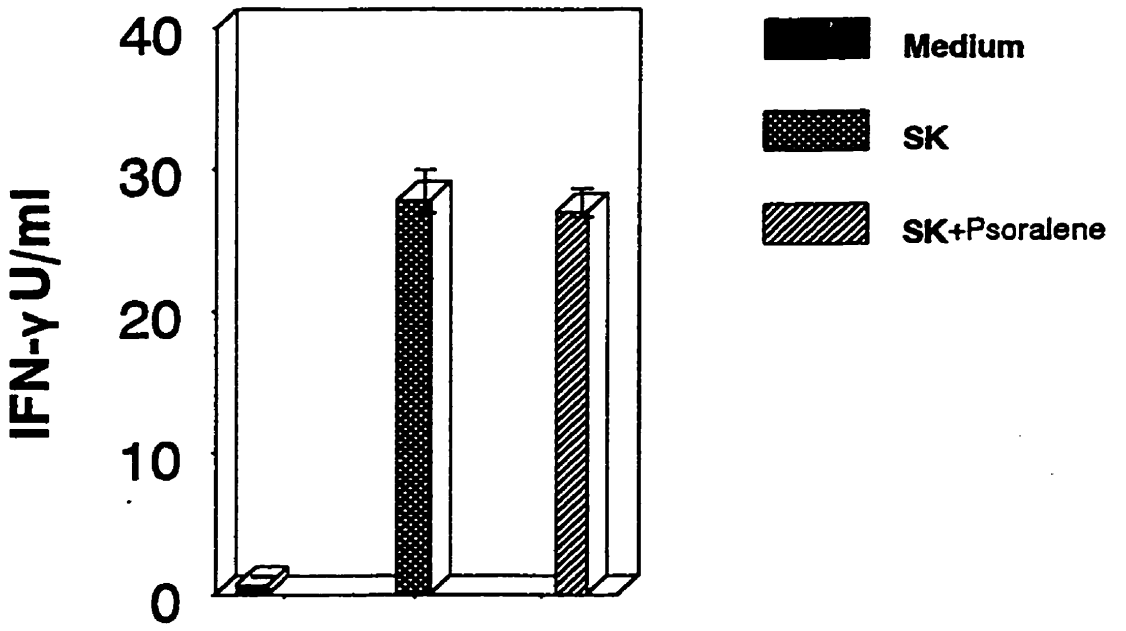

Figure 8. Effect of psoralene on antigen driven IFN-γ synthesis. PBMCs (1.5×10^6/ml) from a healthy subject were cultured in the absence or presence of streptokinase Ag (5000 U/ml) or streptokinase plus psoralene (at 1 pg/ml) as indicated on X-axis. Y-axis shows IFN-γ levels produced in the culture.

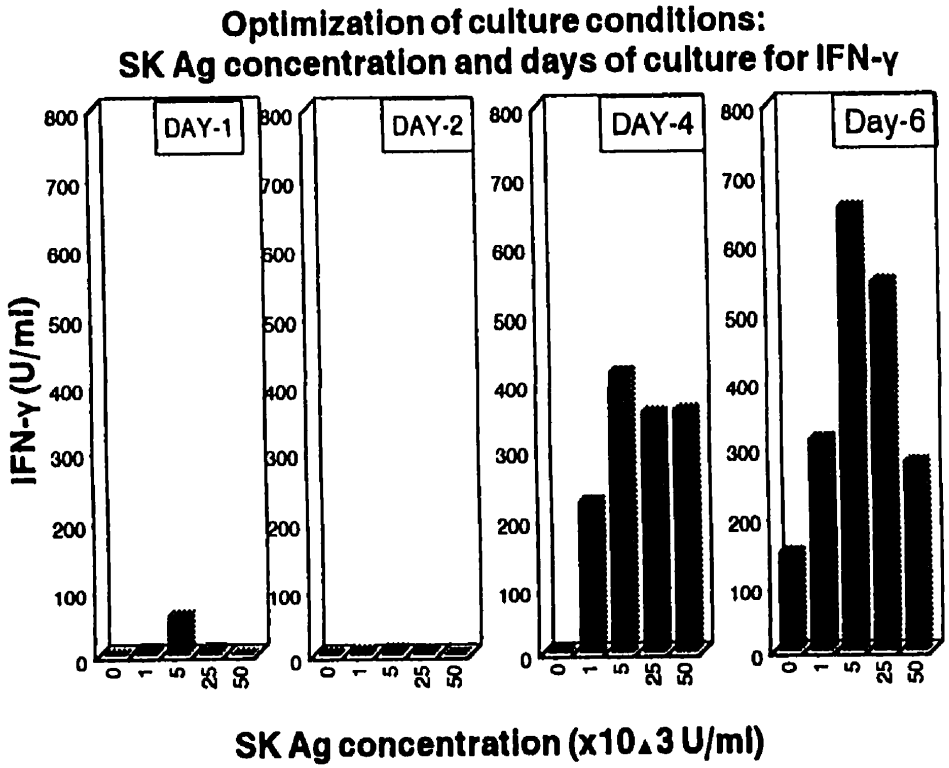

Figure 9. Optimization of culture conditions for measuring SK Ag driven IFN-γ. PBMCs from Low risk women were cultured with or without SK Ag at various concentrations as indicated on X-axis. Each panel shows IFN-γ production (on Y-axis) on indicated days. Results from one representative subject are shown.

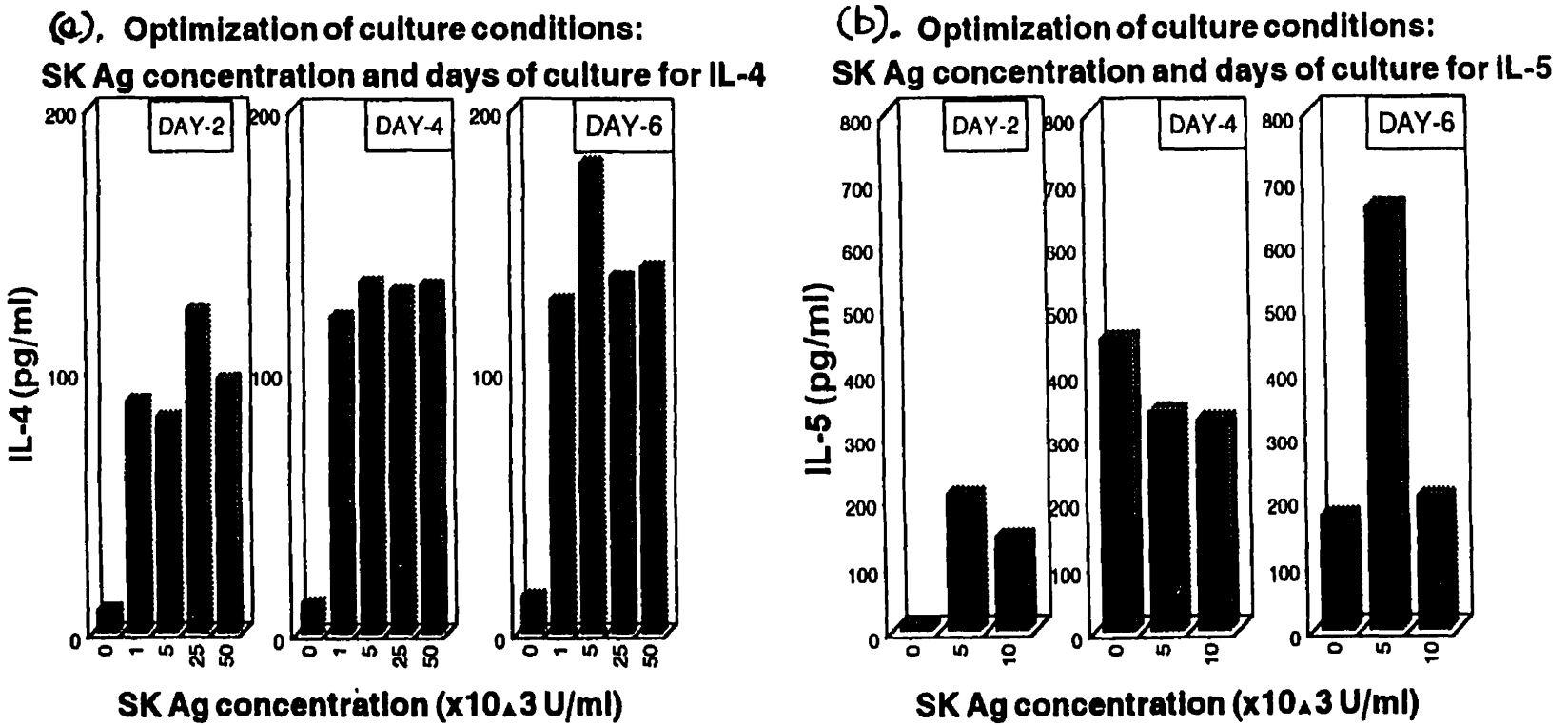

Figure 10 (a,b). Optimization of culture conditions for measuring SK Ag driven IL-4 and IL-5 production. PBMCs from Low risk women were cultured with or without SK Ag at various concentrations as indicated on X-axis. IL-4 (left panel) and IL-5 (right panel) production is shown (on Y-axis) on indicated days. Results from one representative subject are shown.

1.2.5 Optimization of culture conditions for evaluating PPD driven cytokine responses

The optimization experiments were done as described in Methods. Peak cytokine responses were observed at a concentration of 5 TU/ml. Day 6 was found to be the optimal culture period. Results from one representative subject of the two studied is shown for indicated cytokines (Figures 11 and 12).

2. ANALYSIS OF TYPE-1 AND TYPE-2 CYTOKINE RESPONSES OF HIV-1 RESISTANT KENYAN WOMEN

2.1 Analysis of Type-1 and Type-2 Cytokine Responses Among HIV-1 Resistant and Susceptible Kenyan Women

2.1.1 HIV-1 $_{IIIB}$ driven type-1 and type-2 cytokine responses among HIV-1 resistant and susceptible Kenyan women

In order to dissect the differential role of type-1 and type-2 cytokine responses underlying resistance or susceptibility to HIV-1 infection, HIV-1 driven recall cytokine responses were examined in two groups of Kenyan sex workers - HIV-1 resistant versus susceptible women. Thus, PBMC from resistant women (n=14) and susceptible women (n=11) were cultured using pre-optimized culture conditions. Culture supernatants were assayed for IFN-γ (a prototypic type-1 cytokine), IL-4, IL-5 and IL-13 (type-2 cytokines), and IL-10 (neither a type-1 nor type-2 cytokine) by ELISA as described in Methods.

2.1.1.1 HIV-1 resistant but not susceptible women exhibit markedly enhanced virus driven IFN-γ recall responses. Results of the

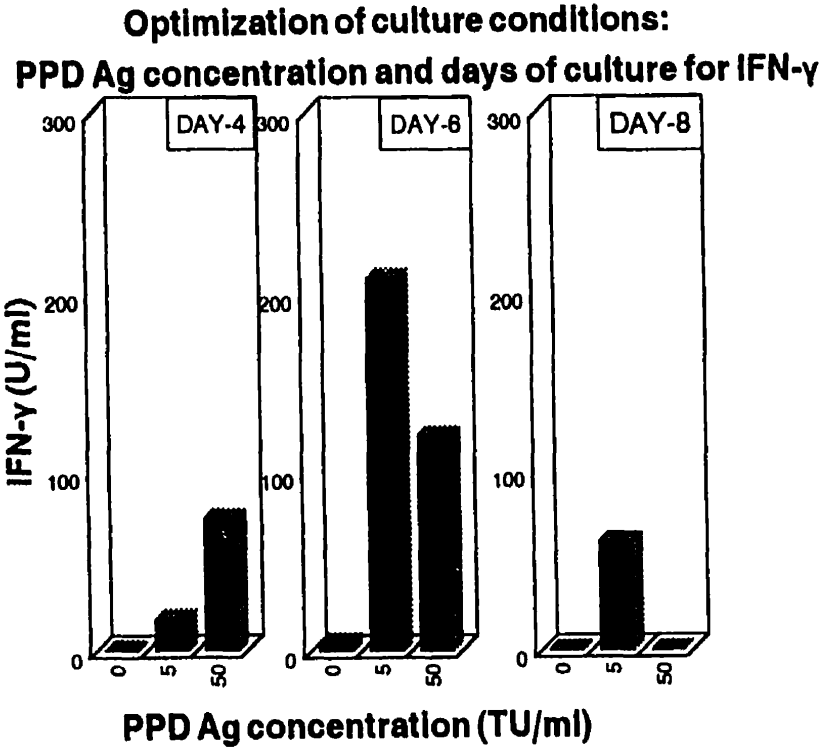

Figure 11. Optimization of culture conditions for measuring PPD Ag driven IFN-γ. PBMCs from Low risk women were cultured with or without PPD Ag at various concentrations as indicated on X-axis. Each panel shows IFN-γ production (on Y-axis) on indicated days. Results from one representative subject are shown.

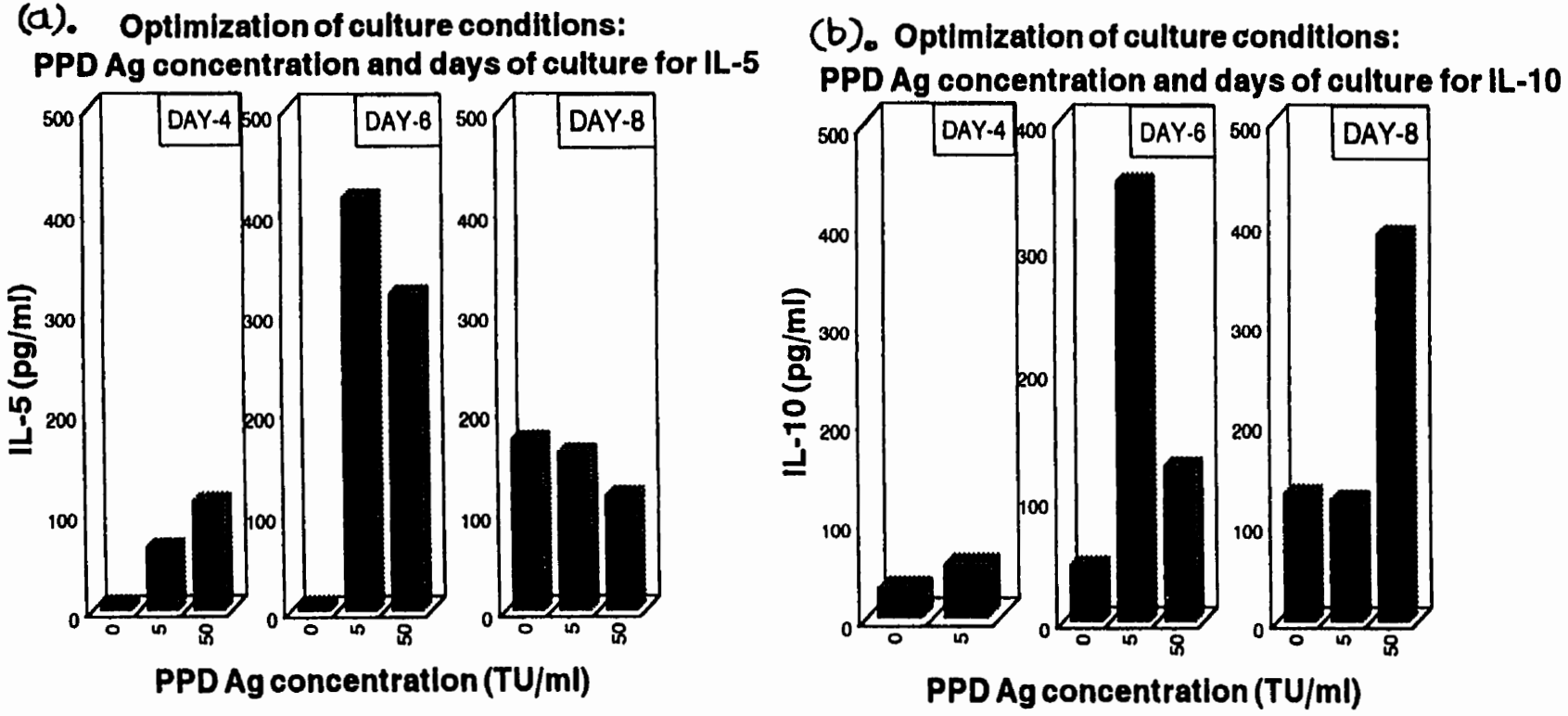

Figure 12 (a,b). Optimization of culture conditions for measuring PPD Ag driven IL-5 and IL-10 production. PBMCs from Low risk women were cultured with or without PPD Ag at various concentrations as indicated on X-axis. IL-5 (left panel) and IL-10 (right panel) production is shown (on Y-axis) on indicated days. Results from one representative subject are shown.

analysis of HIV-1-driven IFN-γ responses among resistant women and susceptible women are shown in Figure 13. Of the 14 resistant women examined, 12 (86%) exhibited detectable IFN-γ recall response while only 2 (18 %) of the 11 susceptible women showed detectable IFN-γ response to HIV-1 (Fisher's p=0.002). Subsequently, the intensity of recall cytokine response was analyzed. As evident in Figure 13, the intensity of the virus driven IFN-γ production was significantly higher (Mann-Whitney p=0.004) among the resistant women (median 1.1 U/ml, range 0.31-16 U/ml) relative to the susceptible women (median 0.31 U/ml, range 0.31-16.6 U/ml).

***2*.1.1.2 HIV-1 resistant but not susceptible women exhibit markedly reduced virus driven recall IL-4 responses.** Results of the analysis of HIV-1 driven recall IL-4 responses among resistant and susceptible women are shown in Figure 14. Two (14%) of the 14 resistant women examined exhibited detectable IL-4 recall response. In contrast, 10 (91%) of the 11 susceptible women (Fisher's p=0.0002) showed detectable IL-4 response to HIV-1 (Figure 14). Furthermore, intensity of the virus driven IL-4 production was significantly higher (Mann-Whitney p=0.005) among the susceptible women (median 9.3 pg/ml, range 0.45-77.2 pg/ml) relative to the resistant women (median 0.45 pg/ml, range 0.45-10.6 pg/ml).

2.1.1.3 HIV-1 resistant women exhibit marginally enhanced virus driven IL-5 responses relative to susceptible women. Results of the analysis of HIV-1 driven recall IL-5 responses among resistant and susceptible women are shown in Figure 15. Twelve (86%) of the 14 resistant

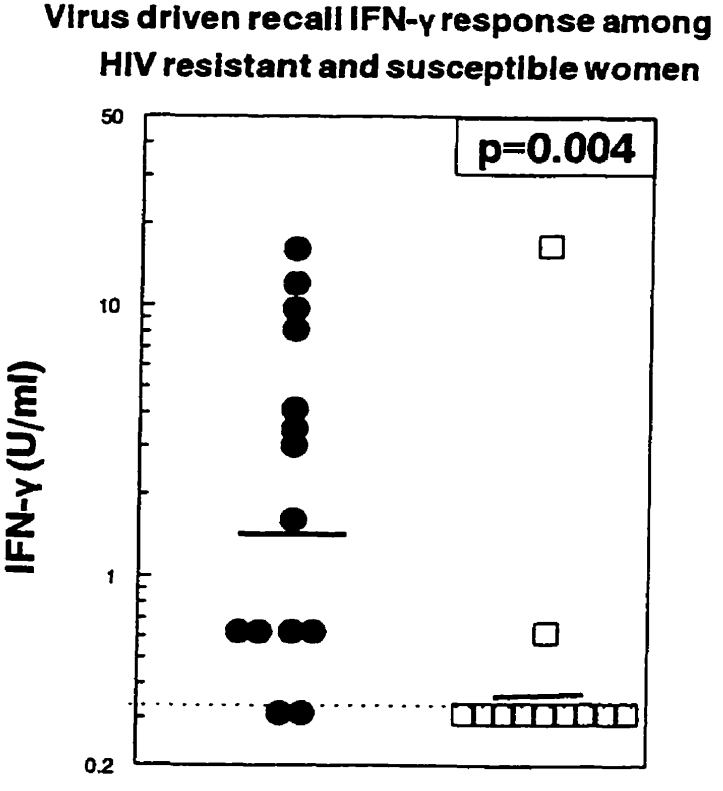

Figure 13. HIV resistant but not susceptible women exhibit markedly enhanced virus driven IFN-γ recall responses. PBMC (1.5 X10^6/ml) were cultured in the presence of 1.25 x 10^2 VP/ml and 1.25 x 10^3 VP/ml of inactivated HIV_{IIIB}. Culture supernatants were harvested and assayed for IFN-γ production (U/ml) by ELISA as described. The data represents peak IFN-γ response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann Whitney U test.

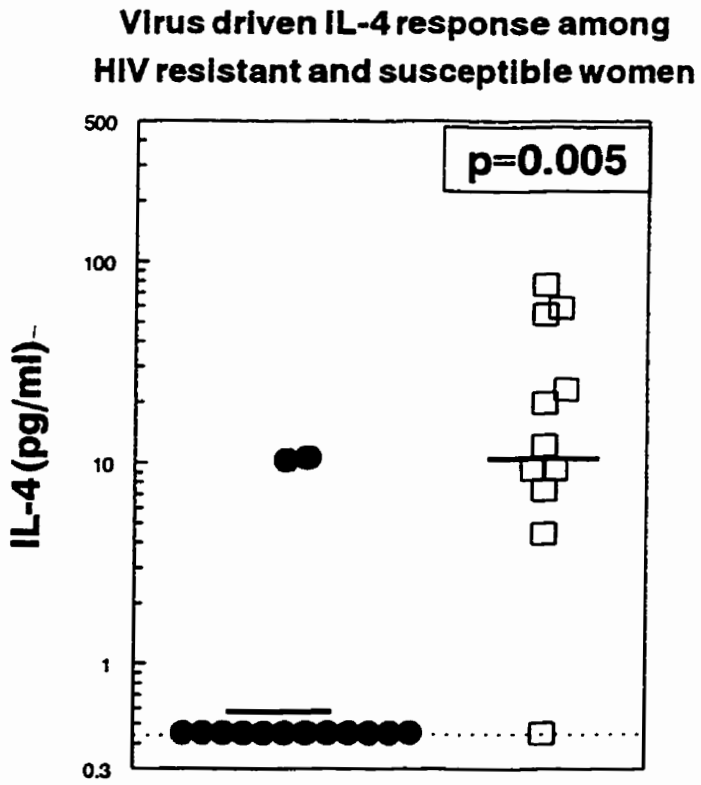

Figure 14. HIV resistant, but not resistant women exhibit markedly reduced virus driven recall IL-4 responses. PBMC (1.5 X10^6/ml) were cultured in the presence of 1.25 x 10^2 VP/ml and 1.25 x 10^3 VP/ml of inactivated HIV_{IIIB}. Culture supernatants were harvested and assayed for IL-4 production (U/ml) by ELISA as described. The data represents peak IFN-γ response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann Whitney U test.

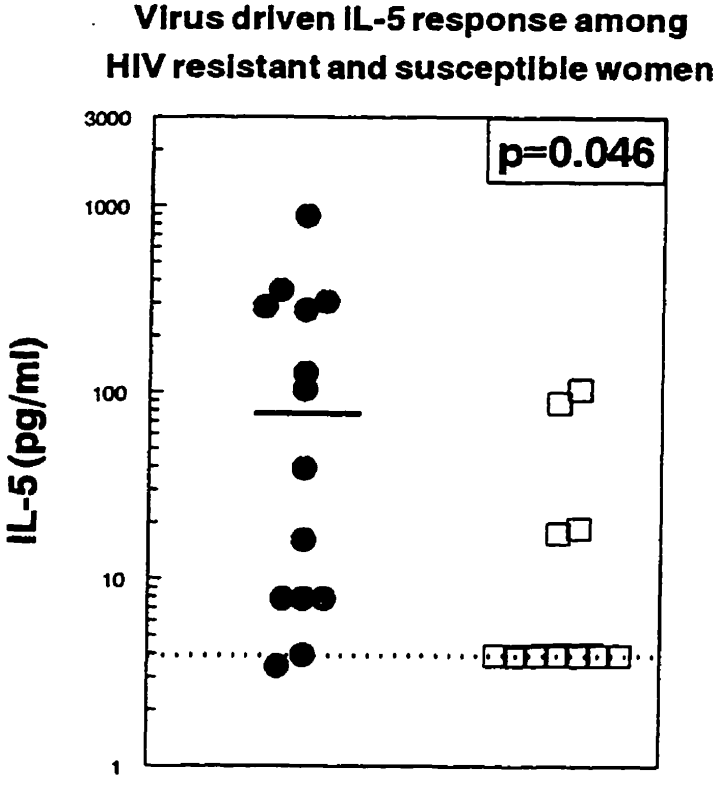

Figure 15. HIV-1 driven IL-5 response is marginally enhanced among resistant compared to susceptible women. PBMC (1.5 X10^6/ml) were cultured in the presence of 1.25 x 10^2 VP/ml and 1.25 x 10^3 VP/ml of inactivated HIV_{IIIB}. Culture supernatants were harvested and assayed for IL-5 production (U/ml) by ELISA as described. The data represents peak IL-5 response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann Whitney U test.

women examined exhibited detectable IL-5 recall response compared with 4 (36%) of the 11 HIV-1 susceptible women (Fisher's p=0.02). As evident in Figure 15, the intensity of the virus driven IL-5 production differed significantly (Mann-Whitney p=0.046) between the resistant women (median 71 pg/ml, range 3.9-877 pg/ml) and the susceptible women (median 3.9 pg/ml, range 3.9-104 pg/ml).

2.1.1.4 HIV-1 resistant and susceptible women exhibit comparable levels of virus driven IL-13 responses. Results of the analysis of HIV-1 driven recall IL-13 responses among resistant and susceptible women are shown in Figure 16. The frequency of virus driven IL-13 responders among resistant women (14/14,100%) was comparable to the susceptible women (8/11, 73%; Fisher's p=0.07). As shown in Figure 16, the intensity of the virus driven IL-13 production did not differ statistically between the resistant women (median 252 pg/ml; range 67-2176 pg/ml) and susceptible women (median 98 pg/ml; range 1-750 pg/ml; Mann-Whitney p=ns).

2.1.1.5 HIV-1 resistant and susceptible women do not differ in virus driven IL-10 responses. Results of the analysis of HIV-1-driven recall IL-10 responses among resistant and susceptible women are shown in Figure 17. The frequency of virus driven IL-10 responders among resistant (13/14, 93%) and susceptible women was similar (9/11, 82%; Fisher's p=0.57). The intensity of the virus driven IL-10 production did not differ statistically between the resistant women (median 321 pg/ml,

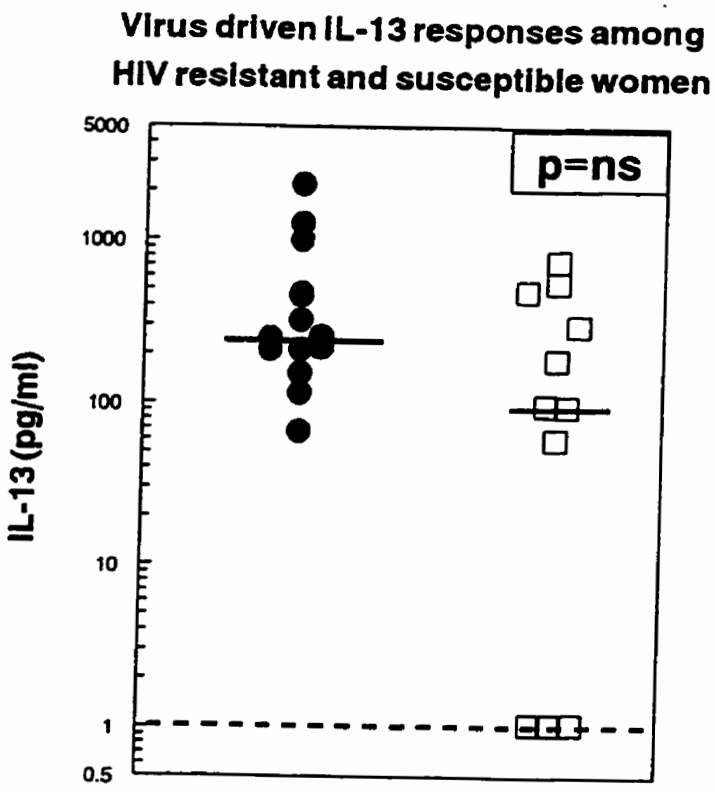

Figure 16. HIV resistant and susceptible Kenyan women exhibit comparable levels of virus driven recall IL-13 responses. PBMC (1.5 X10^6/ml) were cultured in the presence of 1.25 x 10^2 VP/ml and 1.25 x 10^3 VP/ml of inactivated HIV_{MB}. Culture supernatants were harvested and assayed for IL-13 production (U/ml) by ELISA as described. The data represent peak IL-13 response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann Whitney U test.

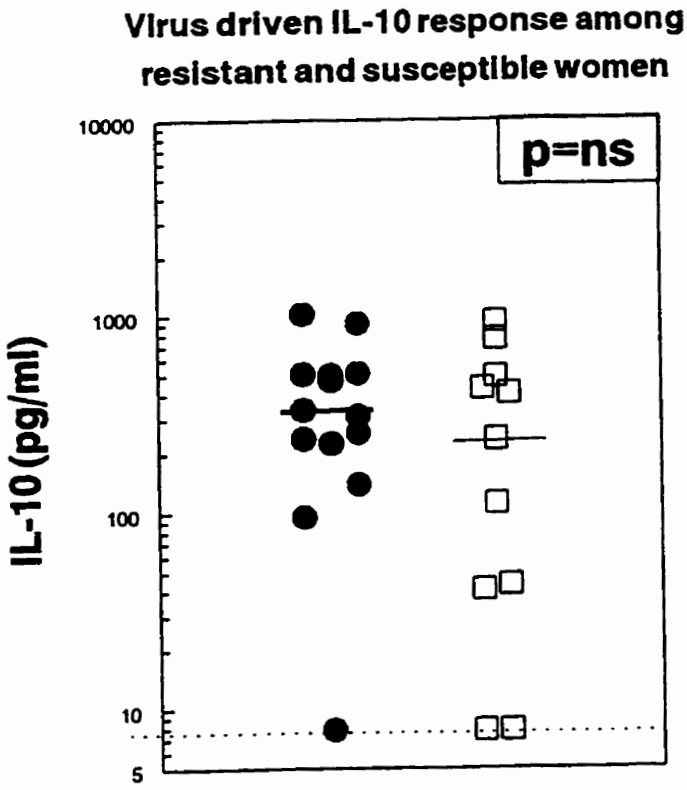

Figure 17. HIV-1 resistant and susceptible Kenyan women do not exhibit detectable differences in virus driven recall IL-10 responses. PBMC (1.5 X10^6/ml) were cultured in the presence of 1.25 x 10^2 VP/ml and 1.25 x 10^3 VP/ml of inactivated HIV-1_{IIIB}. Culture supernatants were harvested and assayed for IL-10 production (U/ml) by ELISA as described. The data represents peak IL-10 response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann-Whitney U test.

range 7.8-1017 pg/ml) and susceptible women (median 237 pg/ml, range 7.8-935 pg/ml; Mann-Whitney p=ns) (Figure 17).

2.1.2 Polyclonally (PHA) driven cytokine responses among HIV-1 resistant and susceptible Kenyan women

Preliminary experiments were performed to determine the optimal concentration and day of harvest as described in Methods. It was found that 1% PHA concentration and two days of culture were optimal for cytokine responses. All subsequent experiments were performed using these optimized conditions.

2.1.2.1 PHA driven IFN-γ response is marginally enhanced among resistant compared to susceptible women: The results of the analysis of PHA driven IFN-γ (type-1 cytokine) responses of resistant and susceptible women are shown in Figure 18. The frequency of resistant and susceptible women exhibiting detectable IFN-γ synthesis to PHA was 100% (14/14) and 54 % (6/11), respectively (Fisher's $p<0.009$). When analyzed for intensity of response, resistant women exhibited marginally (Mann-Whitney $p=0.047$) enhanced levels of PHA driven IFN-γ synthesis (median 33.4 U/ml, range 5.3-319 U/ml) compared to susceptible women (median, 3.7 U/ml; range 0.31-94 U/ml).

2.1.2.2 HIV-1 resistant and susceptible women do not differ in PHA driven Type-2 (IL-4, IL-5, IL-10, IL-13) cytokine responses. The results of the analysis of PHA driven IL-4 responses of resistant and susceptible Kenyan women is shown in Figure 19. The frequency of resistant and susceptible women exhibiting detectable IL-4 synthesis to PHA was 50%

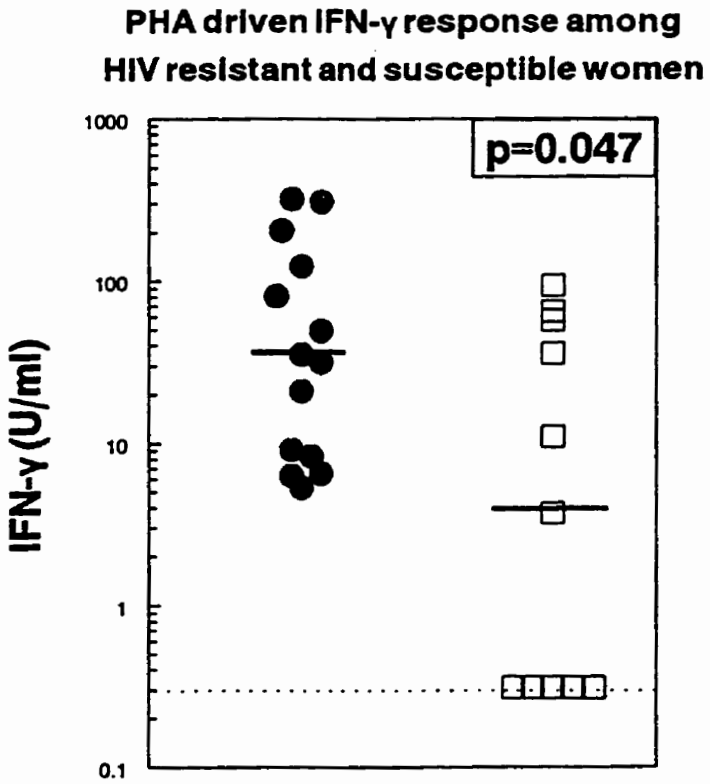

Figure 18. ***PHA driven IFN-γ response is marginally enhanced among resistant compared to susceptible women.*** PBMC (1.5 X10^6/ml) were cultured in the presence PHA (1%). Culture supernatants were harvested and assayed for IFN-γ production (U/ml) by ELISA as described. The data represent peak IFN-γ response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann Whitney U test.

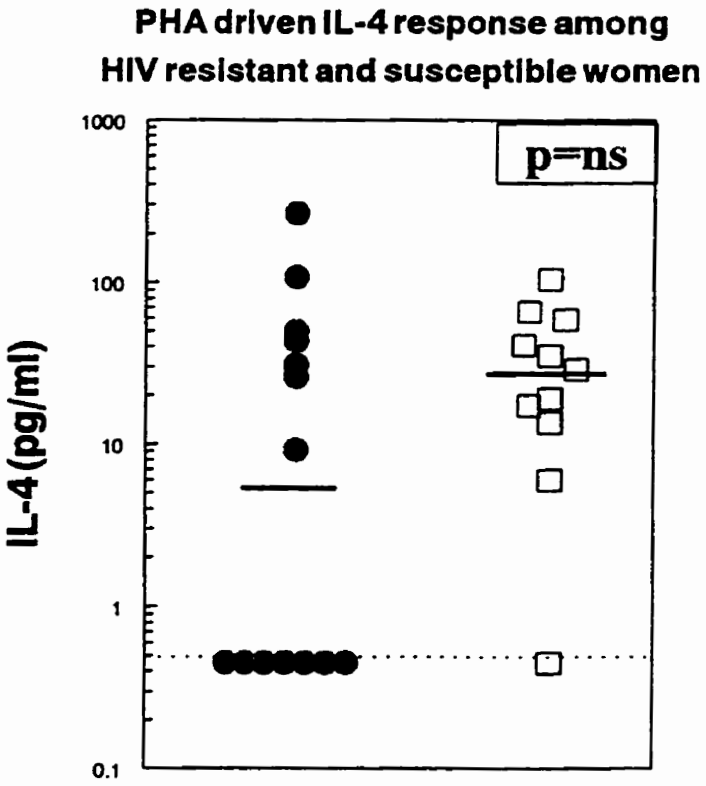

Figure 19. ***HIV resistant and susceptible women do not differ in PHA driven IL-4 responses.*** PBMC (1.5 X10^6/ml) were cultured in the presence PHA (1%). Culture supernatants were harvested and assayed for IL-4 production (pg/ml) by ELISA as described. The data represent peak IL-4 response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann Whitney U test.

(7/14) and 91% (10/11), respectively (Fisher's $p<0.05$). When analyzed for the intensity of response, resistant women exhibited comparable levels of PHA driven IL-4 synthesis (median 4.8 pg/ml, range 0.45-264 pg/ml) to that of susceptible women (median 28.6 pg/ml, range 0.45-105 pg/ml; Mann-Whitney $p=0.16$).

The results of the analysis of PHA driven IL-5 responses of resistant and susceptible women are shown in Figure 20. The frequency of responders among resistant women (11/14, 79%) and susceptible women (7/11, 64%) was comparable (Fisher's $p=0.66$). Furthermore the intensity of response in resistant women (median 122 pg/ml, range 3.9-1461 pg/ml) was comparable (Mann-Whitney $p=0.297$) to that in susceptible women (median 52.3 pg/ml, range 3.9-408 pg/ml).

The results of the analysis of PHA driven IL-13 responses of resistant and susceptible Kenyan women are shown in Figure 21. The frequency of resistant and susceptible women exhibiting detectable IL-13 synthesis to PHA was not different (14/14 and 11/11, respectively). When analyzed for the intensity of response, resistant women exhibited comparable levels (Mann-Whitney p=ns) of PHA-driven IL-13 synthesis (median 832 pg/ml, range 214-9522) to that of susceptible women (median 472 pg/ml, range 33-4147 pg/ml).

The results of the analysis of PHA driven IL-10 responses of resistant and susceptible Kenyan women are shown in Figure 22. The frequency of resistant and susceptible women exhibiting detectable IL-10 synthesis to

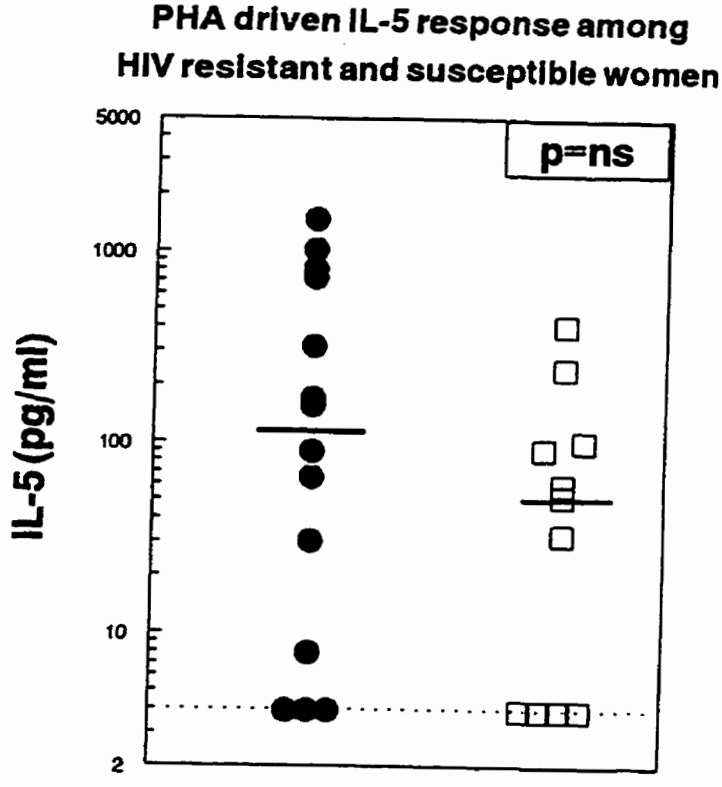

Figure 20. HIV resistant and susceptible women do not differ in PHA driven IL-5 responses. PBMC (1.5 X10^6/ml) were cultured in the presence PHA (1%). Culture supernatants were harvested and assayed for IL-5 production (pg/ml) by ELISA as described. The data represent peak IL-5 response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann Whitney U test.

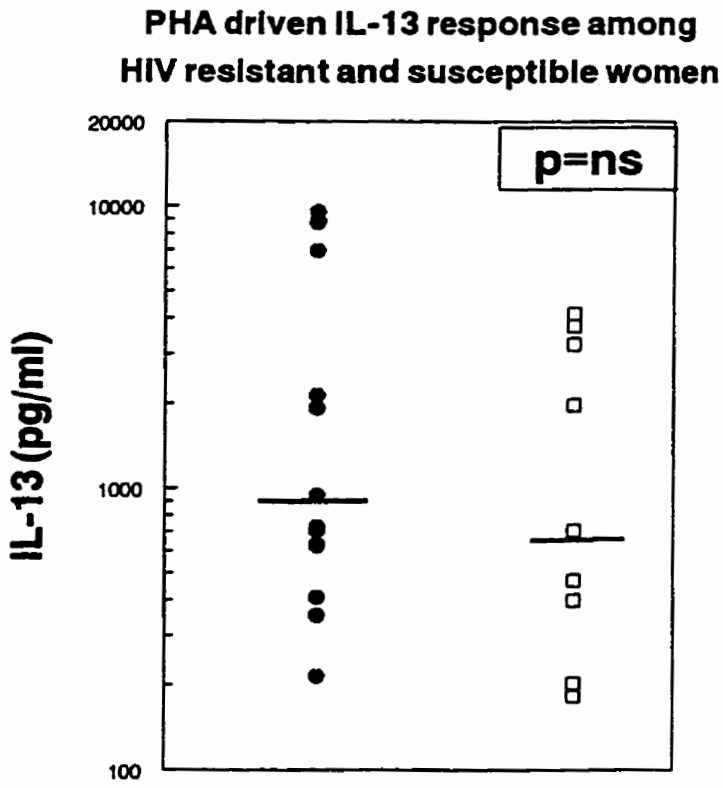

Figure 21. HIV-1 resistant and susceptible women do not differ in PHA driven IL-13 responses. PBMC (1.5 X10^6/ml) were cultured in the presence PHA (1%). Culture supernatants were harvested and assayed for IL-13 production (pg/ml) by ELISA as described. The data represents peak IL-13 response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values . Statistical significance was determined by Mann-Whitney U test.

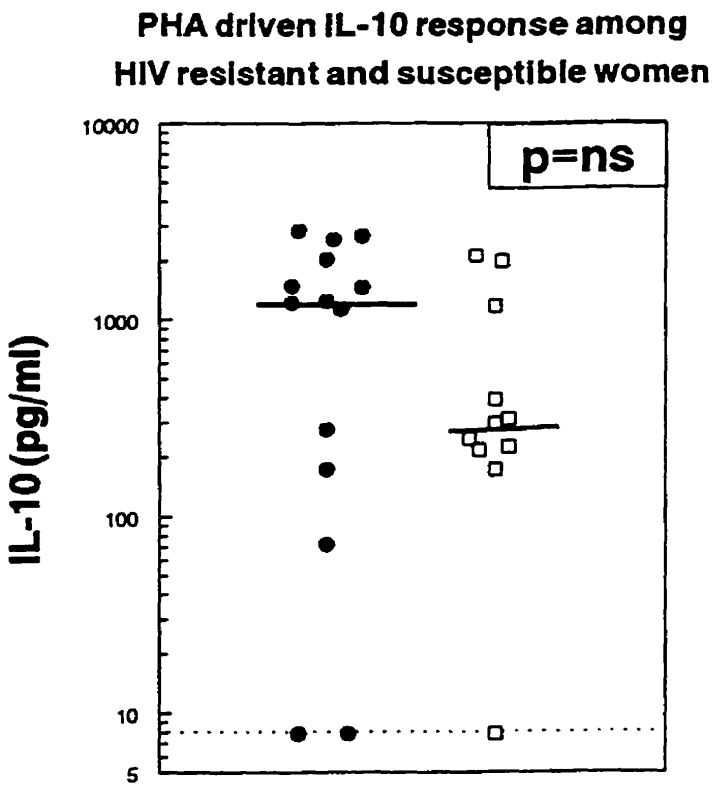

Figure 22. HIV-1 resistant and susceptible women do not differ in PHA driven IL-10 responses. PBMC (1.5 X10^6/ml) were cultured in the presence PHA (1%). Culture supernatants were harvested and assayed for IL-10 production (pg/ml) by ELISA as described. The data represent peak IL-10 response of resistant (●) and susceptible (□) women. Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann-Whitney U test.

PHA was 86% (12/14) and 91% (10/11), respectively (Fisher's p=ns). When analyzed for the intensity of response, resistant women exhibited comparable levels (Mann-Whitney p=0.56) of PHA-driven IL-10 synthesis (median 1228 pg/ml, range 7.8-2824 pg/ml) to that of susceptible women (median 297 pg/ml, range 7.8-2119 pg/ml).

Together, these data provide evidence that resistance or susceptibility to HIV-1 infection among Kenyan women is associated with enhanced PHA driven type-1 (IFN-γ) and similar type-2 (IL-4, IL-5 IL-10, IL-13) cytokine synthesis.

2.1.3 Analysis of type-2:type-1 cytokine balance among resistant and susceptible Kenyan women

2.1.3.1 HIV-1 driven type-2:type-1 ratio in resistant versus susceptible women. For each subject, the ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-13:IFN-γ, and IL-10:IFN-γ were determined using the data for each cytokine obtained following HIV-1 mediated activation. The results of this analysis are shown in Figure 23. The ratio of IL-4:IFN-γ was significantly lower (Mann-Whitney p=0.0002) among resistant women (median 0.503, range 0.028-33.2) relative to the susceptible women (median 30.09, range 0.74-249). In marked contrast, the two groups did not differ in other type-2:type-1 ratios examined: IL-13:IFN-γ (resistant women: median 0.199, range 0.022-0.803; susceptible women: median 0.60, range 0.0032-2.42; Mann-Whitney p=ns); IL-5:IFN-γ (resistant women: median 28.95, range 0.326-1414.5; susceptible women: median 12.58, range 1.13-335.5; Mann-Whitney p=ns) and IL-10:IFN-γ (resistant women: median 175.7, range 0.332-819.4;

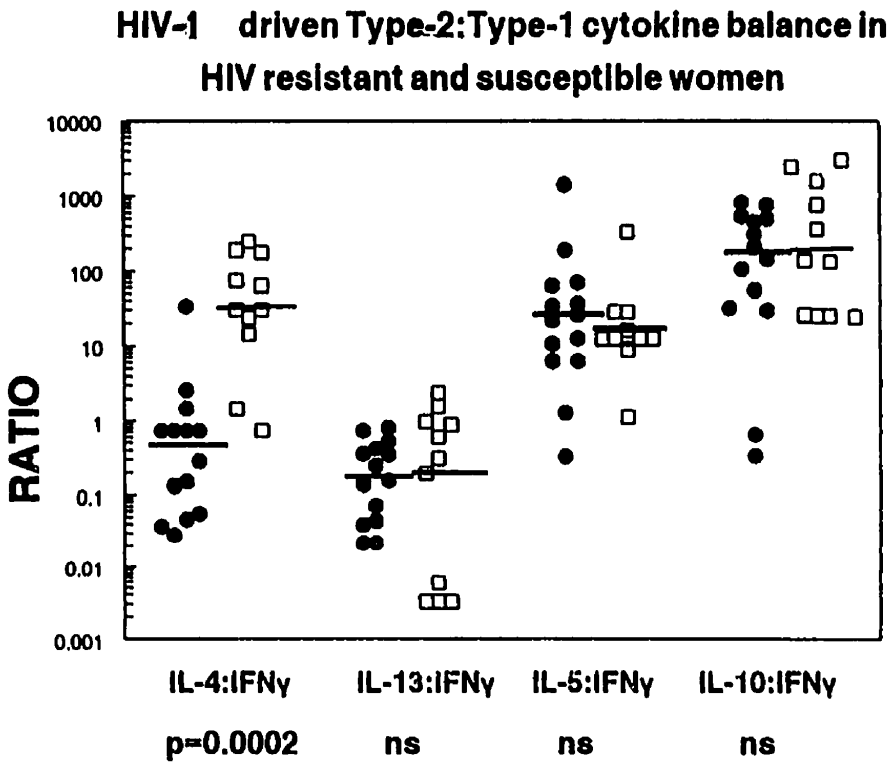

Figure 23. Selective imbalance in HIV-1 driven IL-4:IFN-γ, but not in other Type-2:Type-1 responses among HIV-1 resistant compared to susceptible women. PBMC (1.5 X 10^6/ml) from resistant (●) and susceptible women (□) were each cultured with two different concentrations (1.25 x 10^2 and 1.25 x 10^3/ml) of inactivated HIV-1 . Culture supernatants were assayed for IFN-γ, IL-4, IL-5, IL-10 and IL-13 protein levels as described and the peak production of each cytokine was determined. The ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-10: IFN-γ, and IL-13 :IFN-γ, calculated for each subject are represented on the Y-axis. Each symbol represents one subject. The horizontal bars represent the median values. Statistical significance was evaluated by Mann-Whitney U test.

susceptible women: median 138.7, range 24.3-3016; Mann-Whitney p=ns). Together, these data provide evidence for selective imbalance in virus driven IL-4:IFN-γ but not IL-13:IFN-γ, IL-5:IFN-γ, or IL-10:IFN-γ among resistant relative to the susceptible women.

2.1.3.2 PHA driven type-2:type-1 cytokine ratio in resistant versus susceptible women. For each subject, the ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-13:IFN-γ, and IL-10:IFN-γ were determined using the data for each cytokine obtained following PHA mediated activation. The results of this analysis are shown in Figure 24. The relative balance of IL-4:IFN-γ was significantly different (Mann-Whitney p=0.006) between the resistant (median 0.146, range 0.001-4.83) and susceptible groups (median 1.229, range ,0.17-337.7). In contrast, the two groups did not differ markedly in other type-2:type-1 ratios examined: IL-13:IFN-γ (resistant women: median 0.0482, range 0.0033-0.834; susceptible women: median 0.106, range 0.012-10.42; Mann-Whitney p=ns); IL-5:IFN-γ (resistant women: median 1.43, range 0.326-123.23; susceptible women: median 4.34, range 0.92-787; Mann-Whitney p=ns) and IL-10:IFN-γ (resistant women: median 15.52, range 1.24-393.2; susceptible women: median 12.43, range 0.71-6836; Mann-Whitney p=ns).

These data provide evidence for a selective imbalance in IL-4:IFN-γ among HIV-1 resistant women compared to susceptible women following

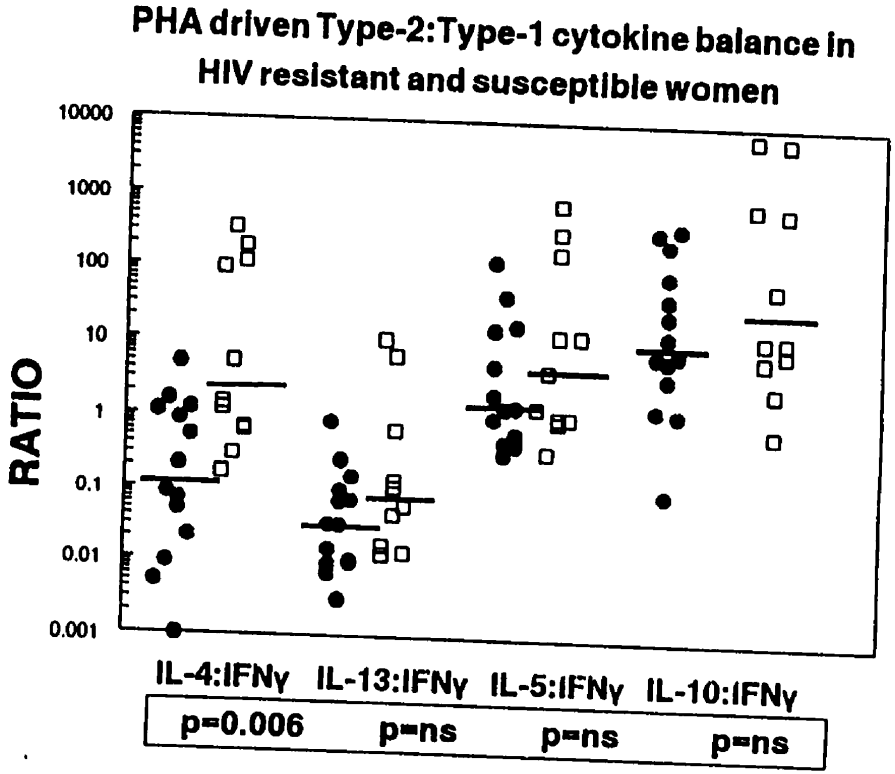

Figure 24. Selective imbalance of PHA driven IL-4:IFN-γ, but not in other Type-2:Type-1 ratios among HIV-1 resistant compared to susceptible Kenyan women. PBMC (1.5 X 10^6/ml) from resistant (●) and susceptible women (□) were cultured in the presence of 1 % PHA. Culture supernatants were assayed for IFN-γ, IL-4, IL-5, IL-10 and IL-13 protein levels as described. The ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-10: IFN-γ, and IL-13 :IFN-γ calculated for each subject are represented on Y-axis. Each symbol represents one subject. The horizontal bars represent the median values. Statistical significance was evaluated by Mann-Whitney U test.

PHA mediated activation. Other type-2:type-1 cytokine ratios examined were similar in the two groups.

2.2 Analysis of Recall Ag (SK and PPD) Driven Cytokine Responses Among Resistant and Low Risk Kenyan Women

2.2.1 Analysis of recall Ag (SK and PPD) driven cytokine responses among resistant and low risk women

2.2.1.1 HIV-1 resistant women do not exhibit enhanced type-1 cytokine (IFN-γ) response to recall Ags (SK and PPD) compared to low risk women. To examine whether resistant women have a generalized ability to produce increased levels of IFN-γ on recall Ag (SK, PPD) Ag mediated stimulation, their cytokine responses were compared with the low risk women. PBMC from resistant women and low risk women were stimulated with pre-optimized concentrations of SK and PPD. IFN-γ production was determined by ELISA. The results from the analysis using SK Ag are shown in Figure 25a. The frequency of subjects exhibiting detectable IFN-γ responses to SK among resistant women and low risk women was not different (12/14 vs 9/11; Fisher's p=ns). The intensity of SK Ag driven IFN-γ production was similar between the two groups (resistant women: median 6.54, range 0.62-147; low risk women: median 44; range 0.31-317; Mann-Whitney p=ns).

The results from the analysis using PPD Ag are shown in Figure 25b. The frequency of subjects exhibiting detectable IFN-γ responses to PPD among resistant women was 9 (75%) and among low risk women was 12

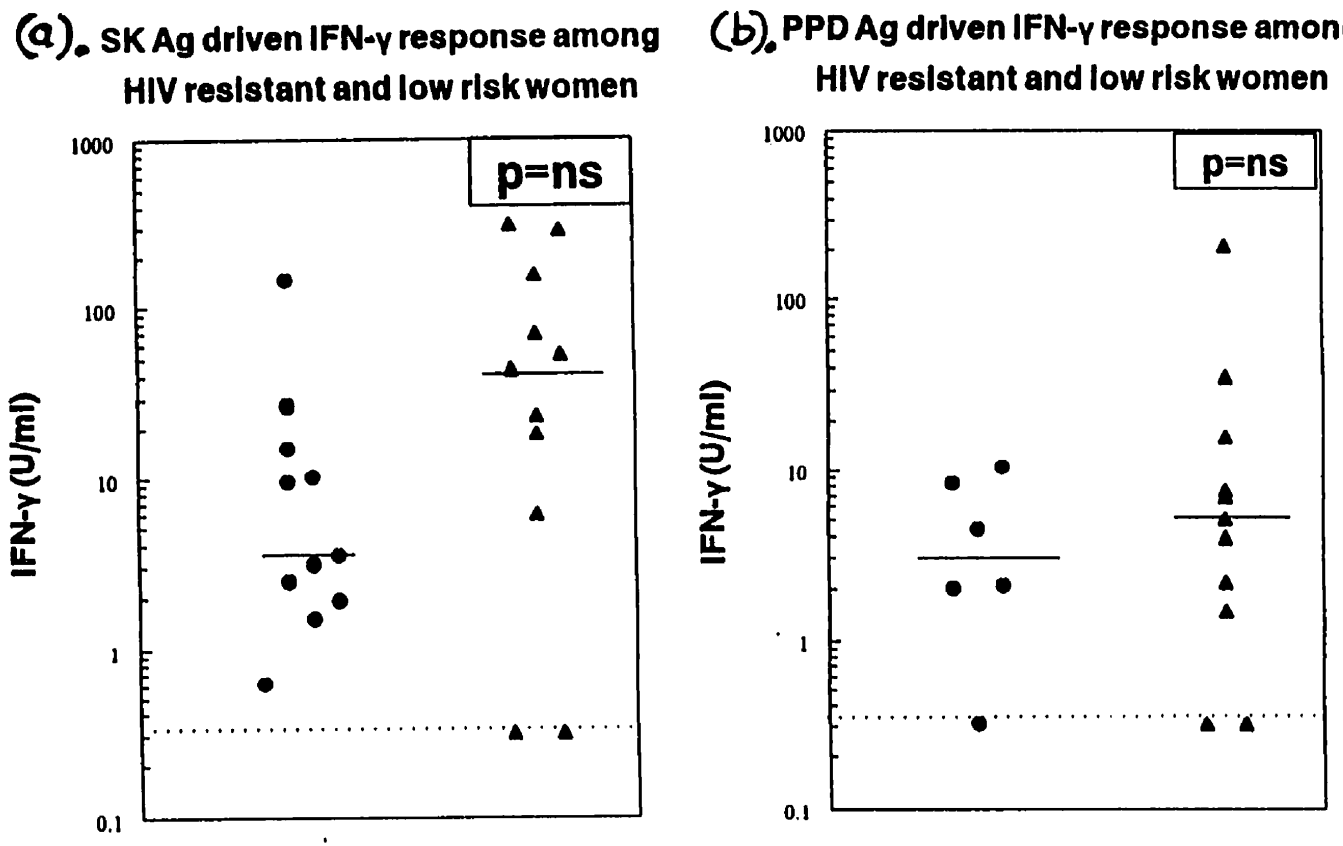

Figure 25(a,b). HIV-1 resistant women do not exhibit enhanced IFN-γ responses to SK (left panel) and PPD Ags (right panel) compared to low risk women. PBMC (1.5 X10^6/ml) were cultured with 5000 U/ml of SK or 5 TU/ml of PPD as described. Culture supernatants were harvested and assayed for IFN-γ production (U/ml) by ELISA as described. The data represent IFN-γ response of resistant (●) and presumably unexposed low risk, HIV-1 negative Kenyan women (Low risk women) (▲) women. Each symbol represents one subject. Horizontal bars represent median values and broken line indicates sensitivity of the assay. Statistical significance was determined by Mann-Whitney U test.

(86%) (Fisher's p=ns). The intensity of PPD Ag driven IFN-γ production was also similar between the two groups (resistant women: median 3.3, range 0.31-10.4; low risk women: median 5.1, range 0.31-209; Mann-Whitney p=ns). Together, the above data argue that HIV-1 resistant women do not exhibit a general tendency of enhanced recall IFN-γ responses on Ag mediated activation relative to the low risk women.

2.2.1.2 HIV-1 resistant women exhibit significantly diminished recall Ag driven IL-4 responses relative to low risk women. The results from the analysis of IL-4 response to SK Ag among resistant women and low risk women are shown in Figure 26a. The frequency of subjects exhibiting detectable IL-4 responses to SK among resistant women was 3/14 (21%) and among low risk women was 10/11 (71%) (Fisher's $p<0.05$). The intensity of SK Ag driven IL-4 production was also significantly reduced among resistant women relative to the low risk women (resistant women: median 0.45, range 0.45-16.8 pg/ml; low risk women: median 6.19, range 0.45-71 pg/ml; Mann-Whitney p=0.016).

The results from the analysis of IL-4 response using PPD Ag are shown in Figure 26b. The frequency of subjects exhibiting detectable recall IL-4 response to PPD among resistant women was 2/12 (17%) and among low risk women was 11/14 (79%) (Fisher's $p<0.02$). The intensity of PPD Ag driven IL-4 production was significantly reduced among the resistant women compared to the low risk women (resistant women: median 0.45, range 0.45-

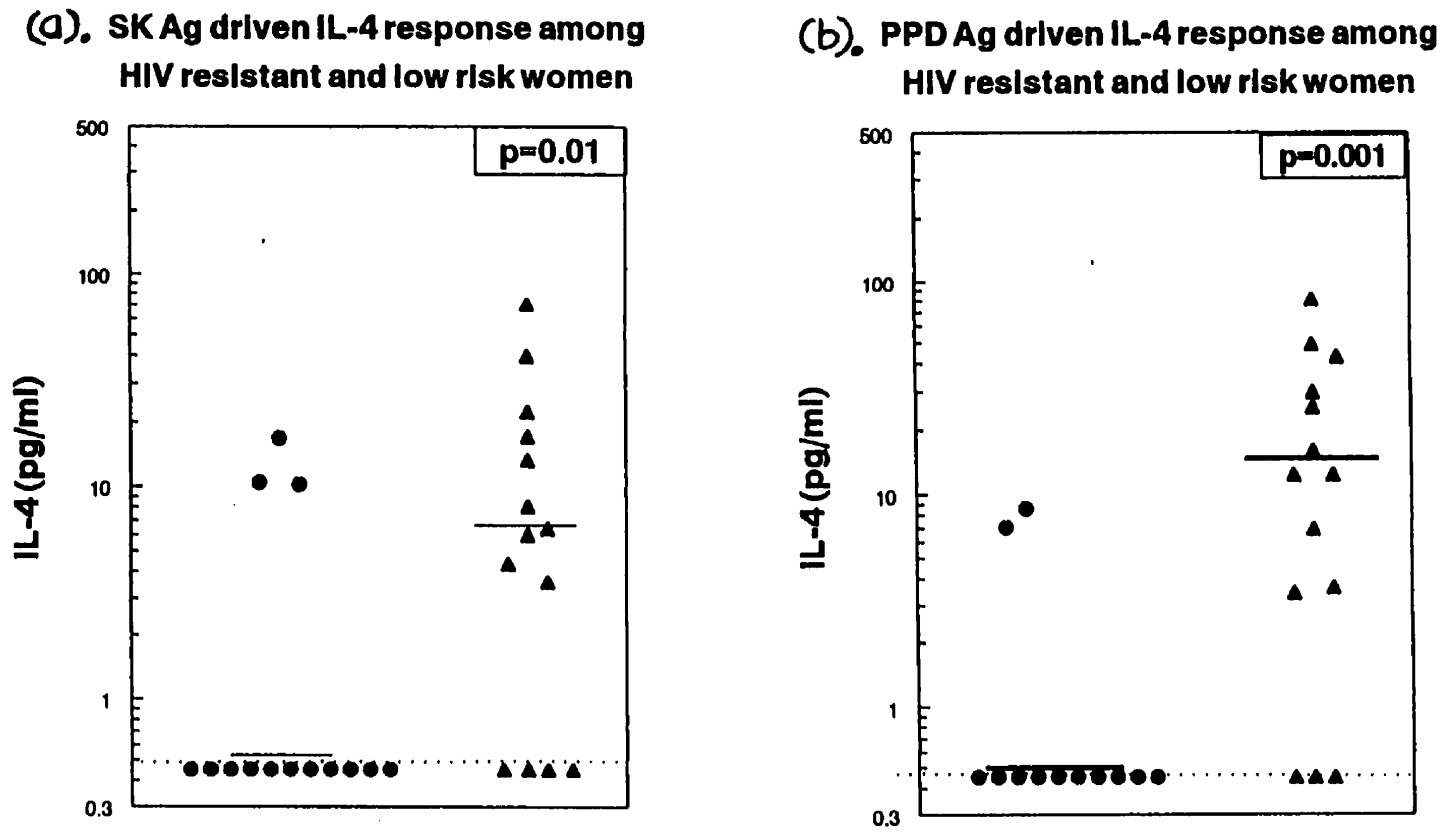

Figure 26(a,b) . HIV-1 resistant women exhibit significantly diminished SK (left panel) and PPD Ag (right panel) driven IL-4 responses compared to HIV-1 negative Low risk Kenyan women. PBMC (1.5 X10^6/ml) were cultured with 5000 U/ml of SK or 5 TU/ml PPD as described. Culture supernatants were harvested and assayed for IL-4 production (pg/ml) by ELISA as described. The data represent IL-4 responses of resistant (●) and presumably unexposed Low risk, HIV-1 negative Kenyan women (Low risk women) (▲). Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates the sensitivity of the assay. Statistical significance was determined by Mann-Whitney U test.

8.55 pg/ml; low risk women: median12.33, range 0.45-81; Mann-Whitney p=0.001).

The above data provide evidence that HIV-1 resistant women exhibit dramatically decreased frequency as well as intensity of recall IL-4 responses to the two common recall Ags used relative to low risk women.

2.2.1.3 HIV-1 resistant women exhibit marginally diminished recall Ag driven IL-13 response compared to low risk women. The results from the analysis of IL-13 response to SK Ag among resistant and low risk Kenyan women are shown in Figure 27a. The frequency of subjects exhibiting detectable IL-13 responses to SK among resistant women was not different (12/12) than among low risk women (11/11). The intensity of SK Ag driven IL-13 production was marginally (Mann-Whitney p=0.04) decreased in the resistant women (median 311, range 108-1596 pg/ml) relative to the low risk women (median 1128, range 11-7735 pg/ml).

The results from the analysis of IL-13 response using PPD Ag are shown in Figure 27b. The frequency of subjects exhibiting detectable recall IL-13 response to PPD among resistant women (6/6) and low risk women (11/11) was identical. However, the intensity of PPD Ag driven IL-13 production was significantly reduced (Mann-Whitney p=0.02) among resistant women relative to the low risk women (resistant women: median 368, range 82-445 pg/ml; low risk women: median 1182, range 1-8140).

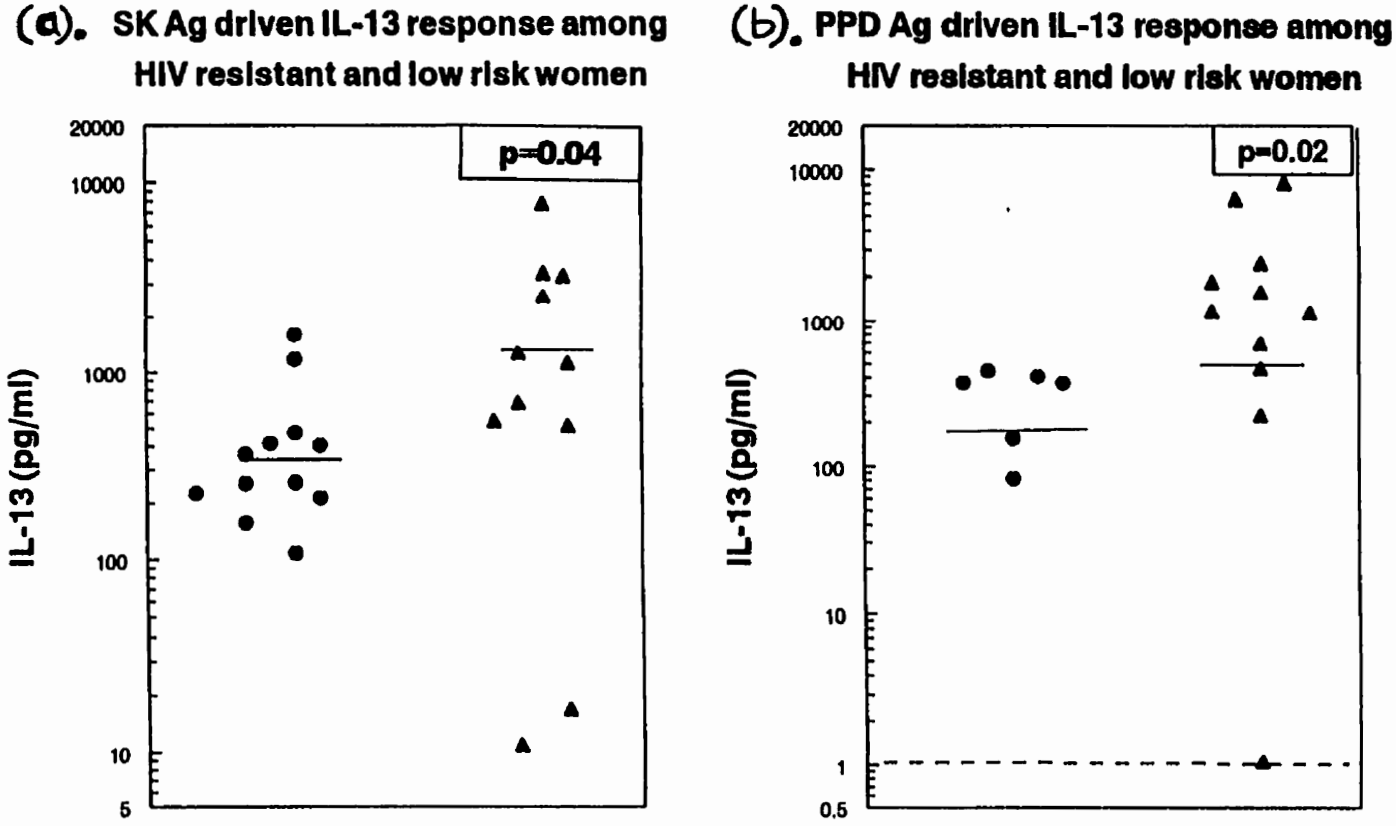

Figure 27(a,b). SK (left panel) and PPD (right panel) Ag driven recall IL-13 responses among HIV-1 resistant low risk Kenyan women. PBMC (1.5 X10^6/ml) were cultured with 5000 units/ml of streptokinase or 5 U/ml PPD as described. Culture supernatants were harvested and assayed for IL-13 production (pg/ml) by ELISA as described. The data represent IL-13 responses of resistant (●) and presumably unexposed low risk HIV-1 negative Kenyan women (Low risk women) (▲). Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test.

The above data suggest that HIV-1 resistant women exhibit similar frequency but marginally reduced intensity of recall IL-13 response to the two common recall Ags used, relative to low risk women.

2.2.1.4 Recall Ag driven IL-5 response among HIV-1 resistant and low risk women. The results from the analysis of IL-5 response to SK Ag among resistant and low risk Kenyan women are shown in Figure 28a. Frequency of subjects exhibiting detectable IL-5 responses to SK among resistant women was 6/12 (50%) and among low risk women was 9/11 (82%). The intensity of SK Ag driven IL-5 production was not significantly different (Mann-Whitney p=ns) between the two groups (resistant women: median 17.9, range 3.9-376; low risk women: median 175, range 3.9-966).

The results from the analysis of IL-5 response using PPD Ag are shown in Figure 28b. Frequency of subjects exhibiting detectable recall IL-5 response to PPD among resistant women was 1/6 (17%) and among low risk women was 7/11 (64%) (Fisher's $p<0.05$). The intensity of PPD Ag driven IL-5 production was also reduced among resistant women (resistant women: median 3.9, range 3.9-33 pg/ml; low risk women: median 193, range 3.9-1901 pg/ml; Mann-Whitney p=0.03).

2.2.1.5 Recall Ag driven IL-10 responses among resistant women and low risk women. The results from the analysis of IL-10 response to SK Ag among resistant and low risk Kenyan women are shown in Figure 29a. The frequency of subjects exhibiting detectable IL-10 responses to SK among resistant women was 11/12 (92%) and among low risk women was 8/11

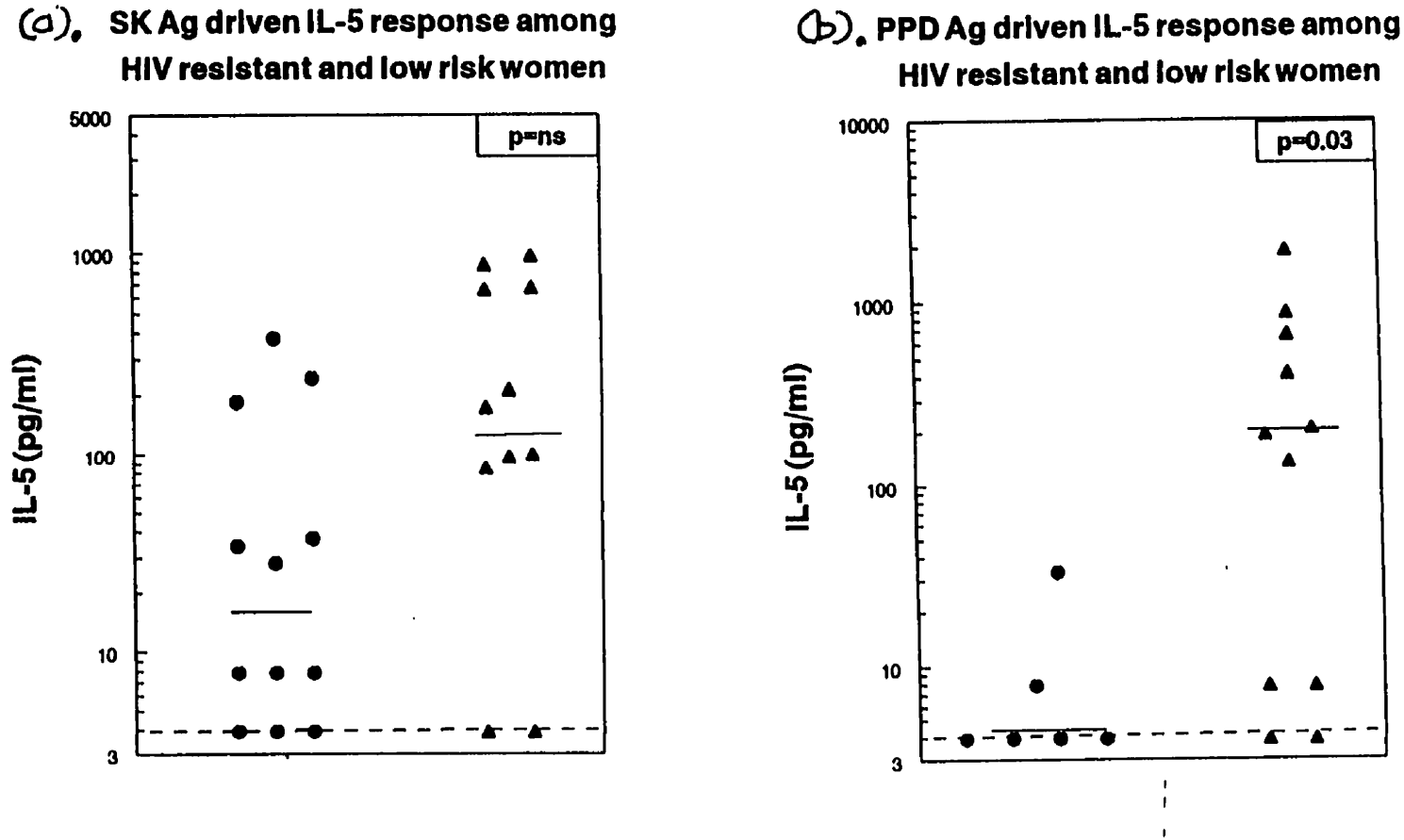

Figure 28a,b. SK (left panel) and PPD (right panel) Ag driven recall IL-5 responses among HIV resistant and low risk Kenyan women. PBMC (1.5 X10^6/ml) were cultured with 5000 units/ml of streptokinase or 5 U/ml PPD as described. Culture supernatants were harvested and assayed for IL-5 production (pg/ml) by ELISA as described. The data represent IL-5 responses of resistant (●) and presumably unexposed low risk HIV negative Kenyan women (low risk women) (▲). Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann Whitney U test.

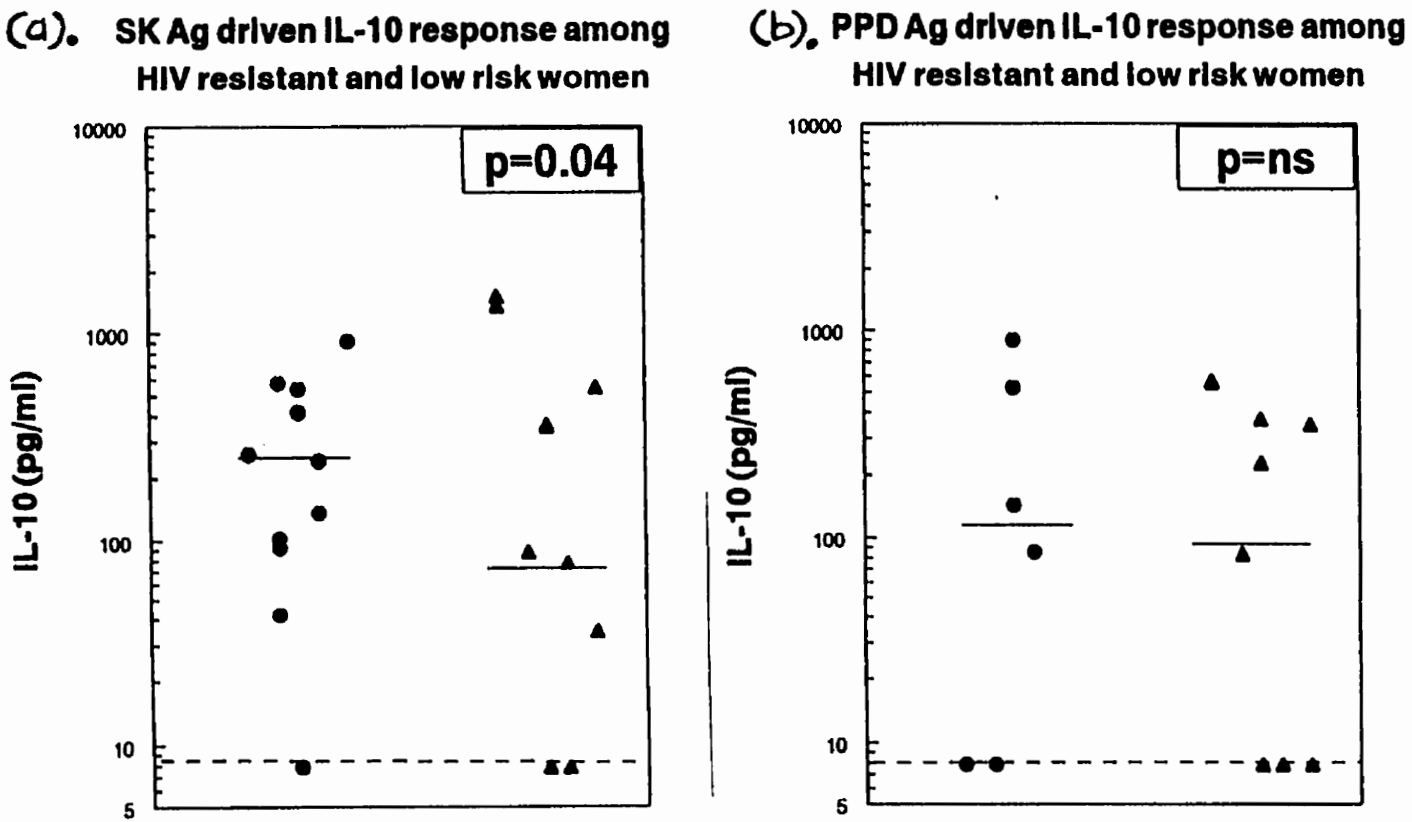

Figure 29a,b. SK (left panel) and PPD (right panel) Ag driven recall IL-10 responses among HIV-1 resistant and low risk Kenyan women. PBMC (1.5 X10^6/ml) were cultured with 5000 units/ml of streptokinase or 5 U/ml PPD as described. Culture supernatants were harvested and assayed for IL-10 production (pg/ml) by ELISA as described. The data represent IL-10 responses of resistant (●) and HIV-1 negative low risk women (▲). Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test.

(73%). The intensity of SK Ag driven IL-10 production was slightly higher (Mann-Whitney p=0.03) among resistant women (resistant women: median 250, range 7.8-910 pg/ml; low risk women: median 53, range 7.8-317 pg/ml).

The results from the analysis of IL-10 response using PPD Ag are shown in Figure 29b. The frequency of subjects exhibiting detectable recall IL-10 response to PPD among resistant women was 67% (4/6) and among low risk women was 55% (6/11) (Fisher's p=ns). The intensity of PPD Ag driven IL-10 production was similar between the two groups (resistant women: median 115, range 7.8-893 pg/ml; low risk women: median 83, range 7.8-566 pg/ml; Mann-Whitney p=ns).

2.2.2 Analysis of polyclonally driven cytokine responses among resistant and low risk women

2.2.2.1 Analysis of PHA driven cytokine responses

2.2.2.1.1 Type-1 (IFN-γ) cytokine response to PHA mediated activation is similar among resistant women and low risk women. The results of the analysis of PHA driven IFN-γ (type-1 cytokine) responses of resistant women and low risk women are shown in Figure 30. The frequency of resistant and low risk women exhibiting detectable IFN-γ synthesis to PHA was identical (14/14 and 11/11, respectively). When analyzed for the intensity of response, both groups exhibited comparable levels of PHA driven IFN-γ synthesis (resistant women: median 33.4 U/ml, range 5.3-319 U/ml;

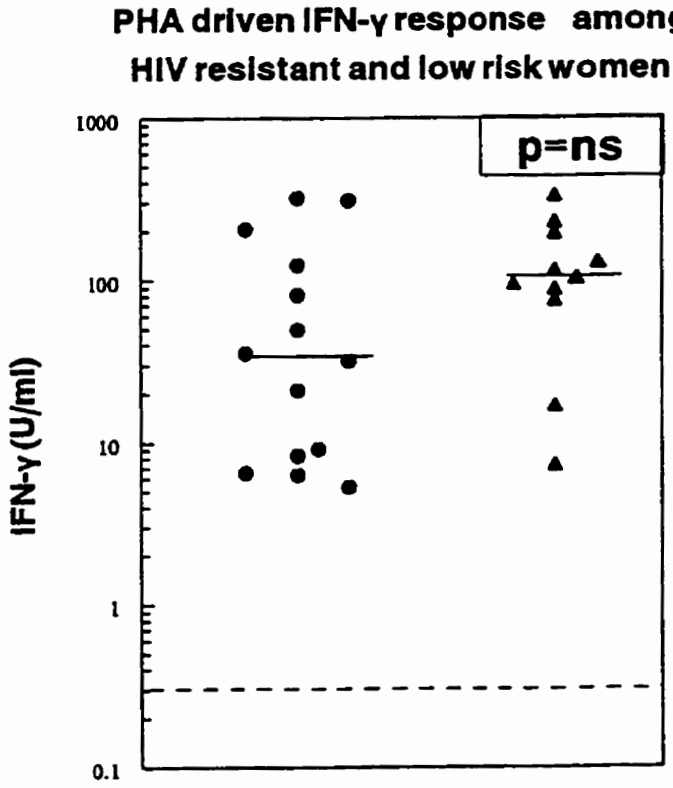

Figure 30. Type-1 (IFN-γ) cytokine response to PHA mediated activation is similar in resistant and low risk women. PBMC (1.5 X10^6/ml) were cultured in the presence of 1% PHA . Culture supernatants were harvested and assayed for IFN-γ production (U/ml) by ELISA as described. The data represent PHA driven IFN-γ response of resistant (●) and low risk women (▲). Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test.

low risk women: median 103 U/ml, range 7.3- 332 U/ml; Mann-Whitney p=ns).

2.2.2.1.2 PHA driven type-2 cytokine responses among resistant women and low risk women. The results of the analysis of PHA driven IL-4 responses of resistant women and low risk women are shown in Figure 31a. The frequency of resistant and low risk women exhibiting detectable IL-4 synthesis to PHA was 50% (7/14) and 100% (11/11), respectively. When analyzed for the intensity of response, resistant women exhibited diminished (Mann-Whitney p=0.006) levels of PHA driven IL-4 synthesis (median 4.8 pg/ml, range 0.45-264 pg/ml) relative to that of low risk women (median 54.4 pg/ml, range 12.9-375 pg/ml).

The results of the analysis of PHA driven IL-5 responses of resistant and low risk women is shown in Figure 31b. The frequency of resistant women and low risk women exhibiting detectable IL-5 synthesis to PHA was 10/14 (71%) and 8/11 (73%), respectively (Fisher's p=ns). When analyzed for the intensity of response, resistant women exhibited similar (Mann-Whitney p=ns) levels of PHA-driven IL-5 synthesis (median 122 pg/ml, range 3.9-1461 pg/ml) to that of low risk women (median 443 pg/ml, range 7.8-5225 pg/ml).

The results of the analysis of PHA driven IL-13 responses of resistant and low risk women are shown in Figure 32a. The frequency of resistant and low risk women exhibiting detectable IL-13 synthesis

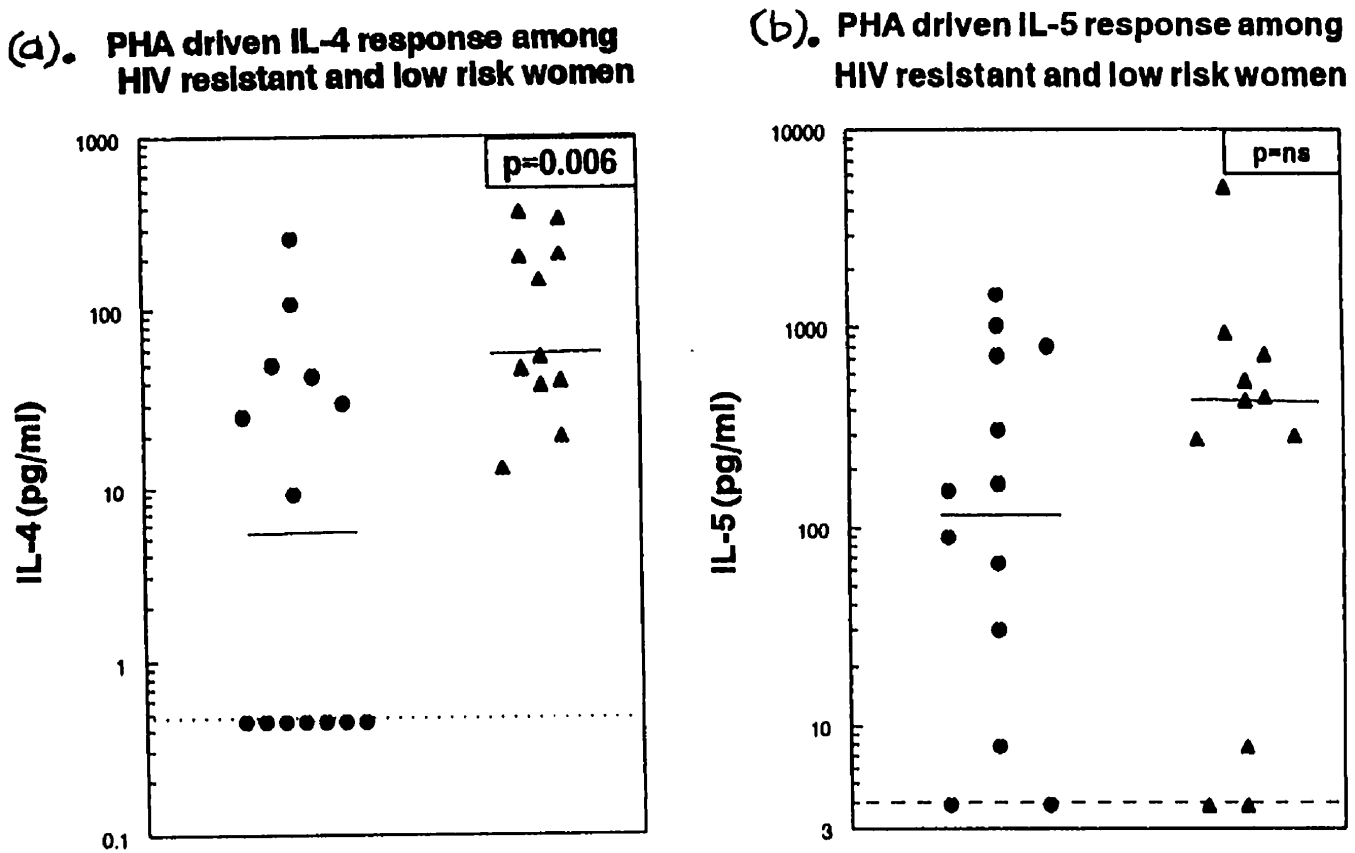

Figure 31(a,b). HIV-1 resistant women exhibit diminished levels of PHA-driven IL-4 (left panel) but similar levels of IL-5 (right panel) synthesis relative to low risk women: PBMC (1.5 X10^6/ml) were cultured in the presence of 1% PHA. Culture supernatants were harvested and assayed for IL-4, IL-5 production (pg/ml) by ELISA as described. The data represent IL-4 and IL-5 responses of resistant (●) and low risk women (▲). Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann-Whitney U test.

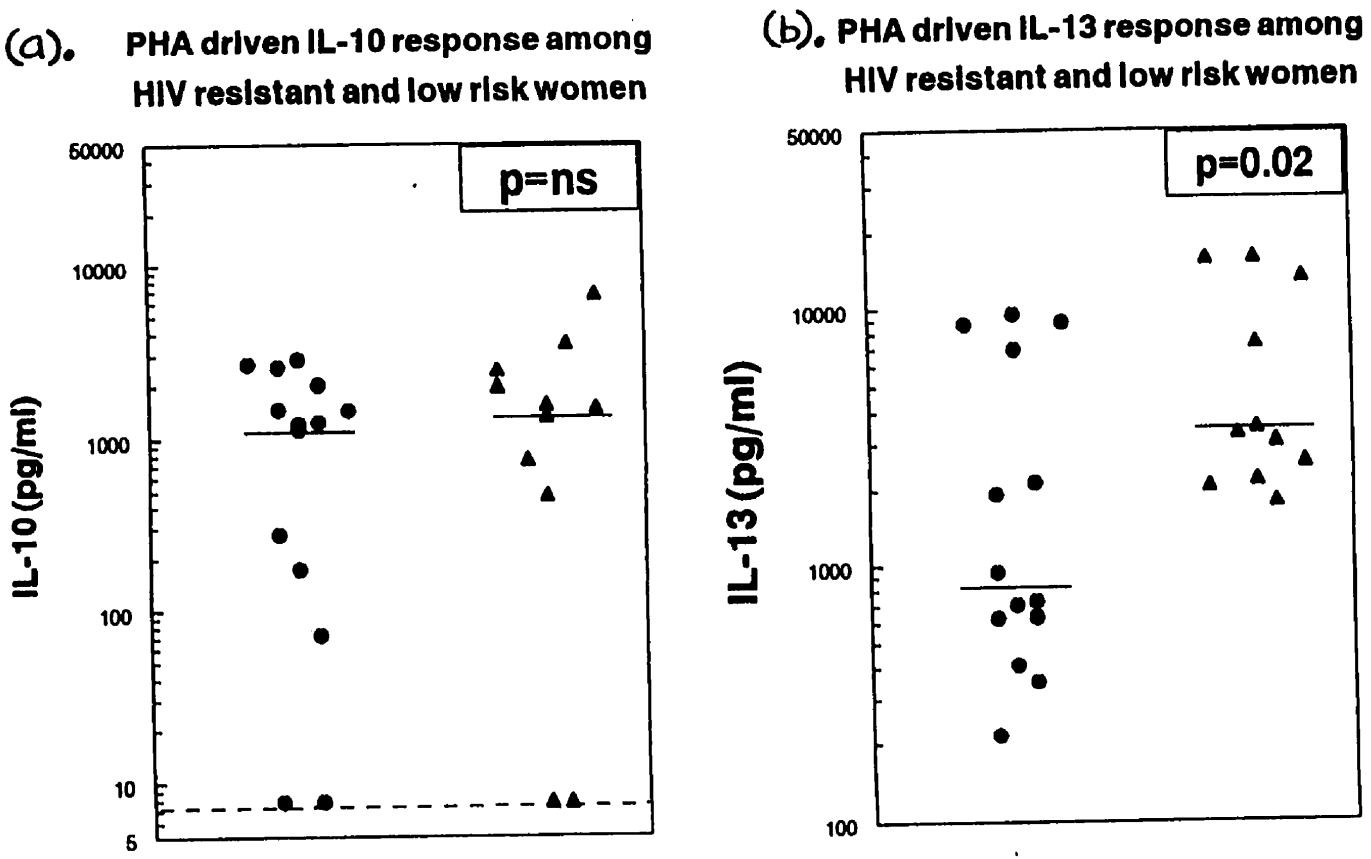

Figure 32a,b. HIV-1 resistant women exhibit modestly diminished levels of PHA-driven IL-13 (left panel) but similar levels of IL-10 (right panel) synthesis compared to low risk HIV-1 negative women: PBMC (1.5 X10^6/ml) were cultured in the presence of 1% PHA. Culture supernatants were harvested and assayed for IL-10, IL-13 production (pg/ml) by ELISA as described. The data represent IL-10 and IL-13 responses of resistant (●) and low risk women (▲). Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test.

to PHA was identical (14/14 and 11/11, respectively; Fisher's p=ns). When analyzed for the intensity of response, resistant women exhibited significantly (Mann-Whitney p=0.02) diminished levels of PHA driven IL-13 synthesis (median 832 pg/ml, range 214-9522) to that of low risk women (median 3315 pg/ml, range 1803-16000 pg/ml)..

The results of the analysis of PHA driven IL-10 responses of resistant and low risk women are shown in Figure 32b. The frequency of resistant and low risk women exhibiting detectable IL-10 synthesis to PHA was 12/14 (86%) and 10/11 (91%), respectively (Fisher's p=ns). When analyzed for the intensity of response, resistant women exhibited similar (Mann-Whitney p=ns) levels of PHA driven IL-10 synthesis (median 1228 pg/ml, range 7.8-2824 pg/ml) to that of low risk women (median 1485 pg/ml, range 7.81-6682 pg/ml).

These data provide evidence that the resistant women differ from low risk women in reduced IL-4 and IL-13 synthesis to PHA but not in other type-2 or type-1 cytokines.

2.2.2.2 Analysis of anti-CD3 mAb driven cytokine responses

2.2.2.2.1. Soluble anti-CD3 mAb driven type-1 (IFN-γ) cytokine response is similar in resistant women and low risk women. The results of the analysis of anti-CD3 mAb driven IFN-γ (type-1 cytokine) responses of resistant women and low risk women are shown in Figure 33. The frequency of resistant and low risk women

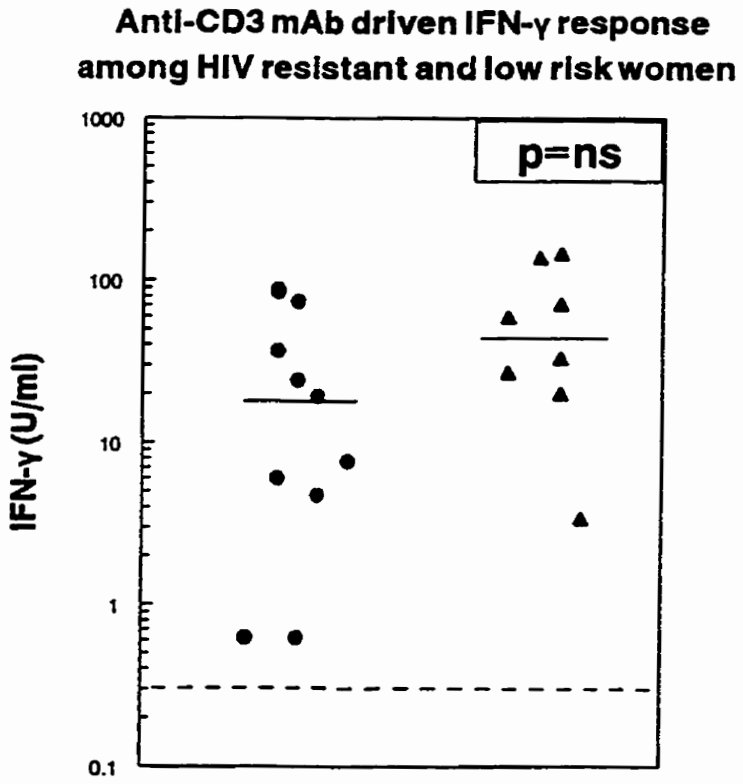

Figure 33. HIV-1 Resistant women exhibit similar frequency and intensity of soluble anti-CD3 mAb driven IFN-γ synthesis relative to low risk women. PBMC (1.5 X10^6/ml) were cultured in the presence of 100 ng/ml soluble anti-CD3 mAb. Culture supernatants were harvested and assayed for IFN-γ production (U/ml) by ELISA as described. The data represent soluble anti-CD3 driven IFN-γ response of resistant (●) and low risk women (▲). Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test.

exhibiting detectable IFN-γ synthesis to anti-CD3 mAb was 9/11 (82%) and 8/8 (100%), respectively (Fisher's p=ns). When analyzed for the intensity of response, both groups exhibited comparable levels of anti-CD3 mAb-driven IFN-γ synthesis (resistant women: median 19.2 U/ml, range 0.62-87.1 U/ml; low risk women: median 46 U/ml, range 3.4-145 U/ml; Mann-Whitney p=ns).

2.2.2.2.2. Anti-CD3 mAb driven type-2 cytokine response among resistant women and low risk women. The results of the analysis of anti-CD3 mAb driven IL-4 responses of resistant women and low risk women are shown in Figure 34a. The frequency of resistant and low risk women exhibiting detectable IL-4 synthesis to anti-CD3 mAb was 9/11 (82%) and 8/8 (100%), respectively. When analyzed for the intensity of response, resistant women exhibited similar (Mann-Whitney p=ns) levels of IL-4 synthesis (median 16.8 pg/ml, range 0.45-70.7 pg/ml) relative to that of low risk women (median 36.5 pg/ml, range 0.45-53 pg/ml).

The results of the analysis of anti-CD3 mAb driven IL-10 responses of resistant and low risk women are shown in Figure 35b. The frequency of resistant and low risk women exhibiting detectable IL-10 synthesis to anti-CD3 mAb was 9/11 (82%) and 5/8 (63%), respectively (Fisher's p=ns). When analyzed for the intensity of response, resistant women exhibited comparable (Mann-Whitney p=ns) levels of anti-CD3 mAb-driven IL-10

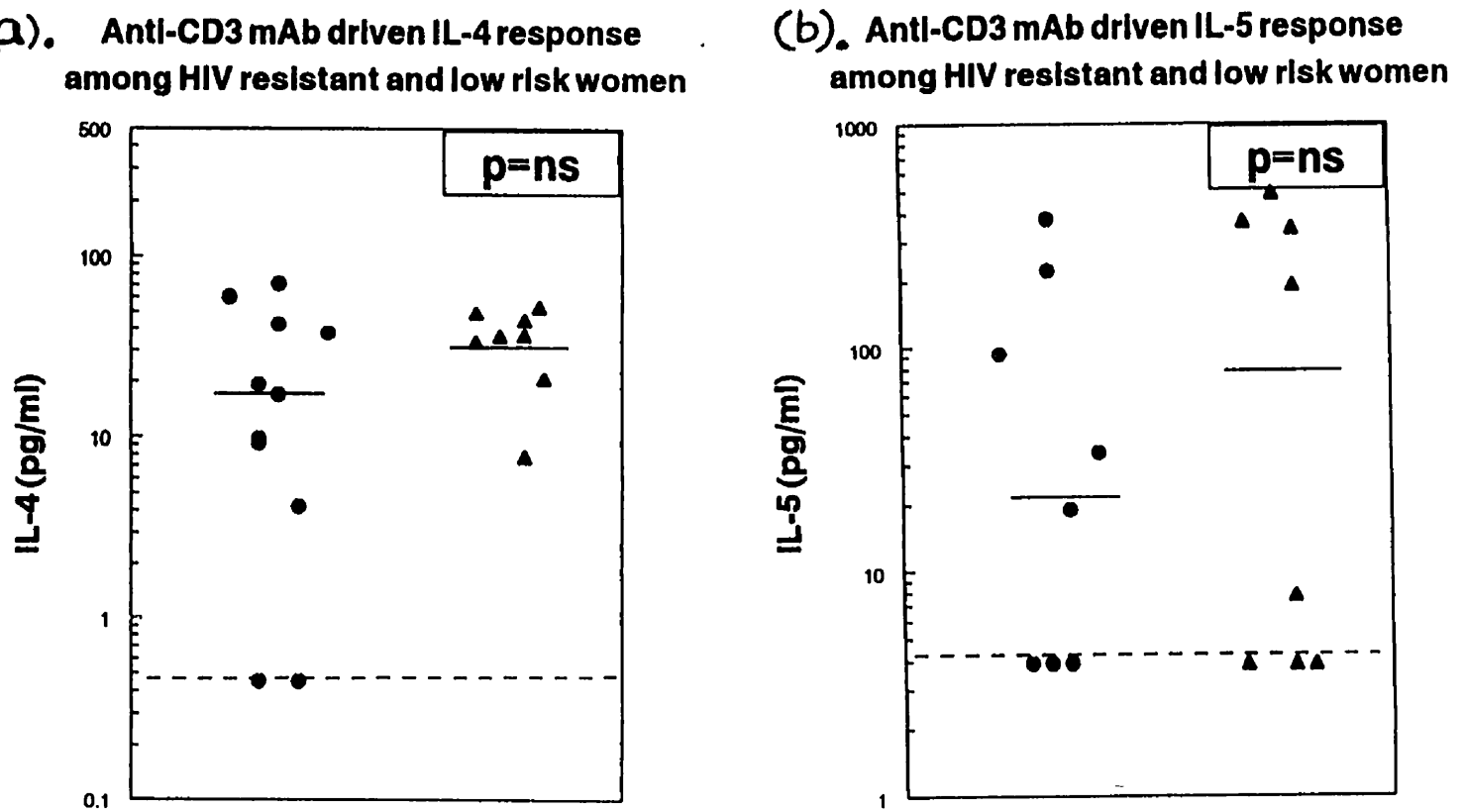

Figure 34. HIV-1 resistant women do not differ from low risk women in frequency or intensity of soluble anti-CD3 mAb driven IL-4 (left panel) and IL-5 (right panel) synthesis. PBMC (1.5 X10^6/ml) were cultured in the presence of 100 ng/ml soluble anti-CD3 antibody. Culture supernatants were harvested and assayed for IL-4 and IL-5 production (pg/ml) by ELISA as described. The data represent: soluble anti-CD3 mAb driven IL-4 responses of resistant (●) and low risk women (▲). Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test.

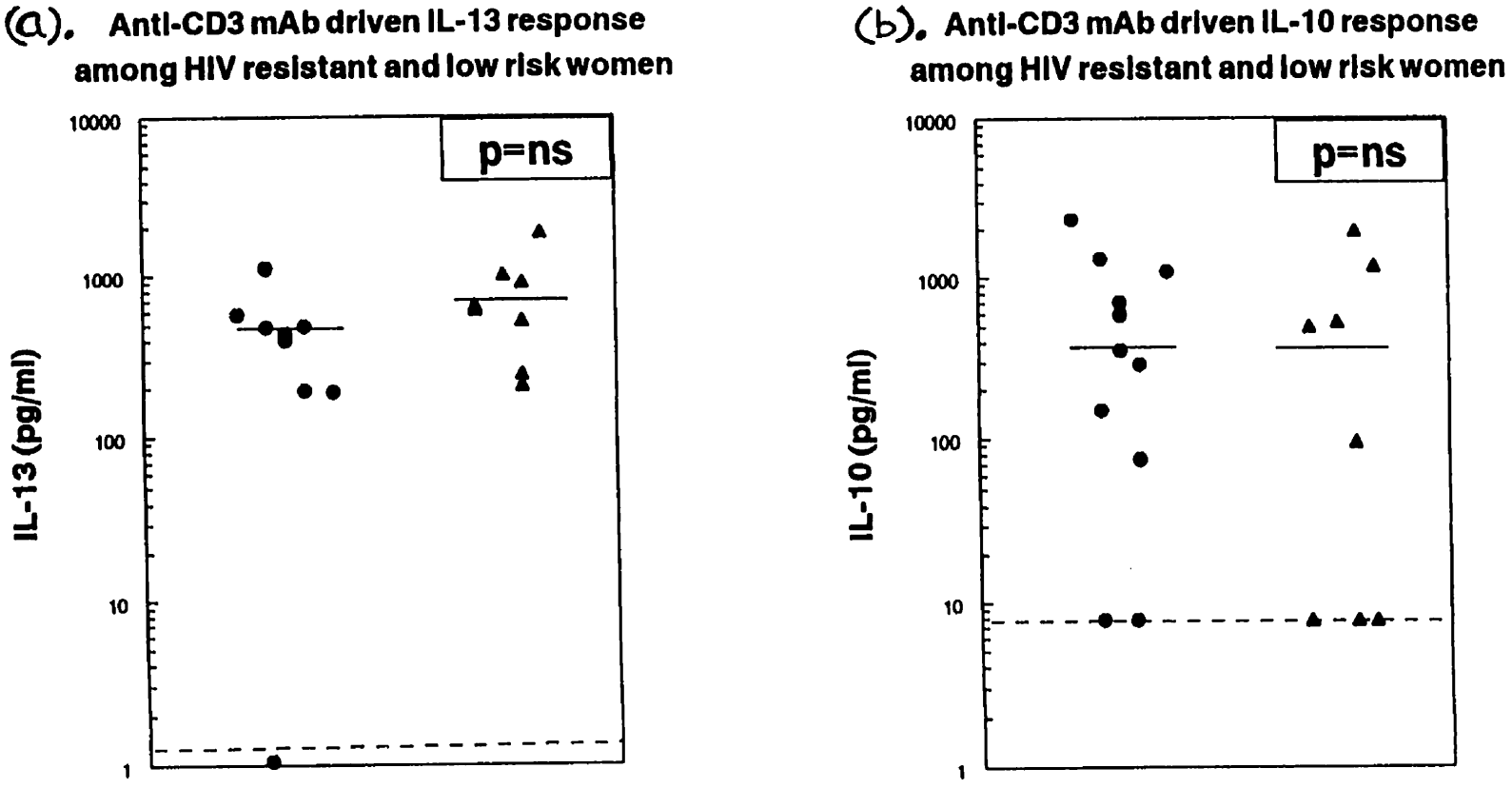

Figure 35a,b. HIV-1 resistant women exhibit similar frequency and intensity of soluble anti-CD3 mAb driven IL-13 (left panel) and IL-10 (right panel) synthesis relative to low risk women. PBMC (1.5 x 10^6/ml) were cultured in the presence of 100 ng/ml soluble anti-CD3 mAb. Culture supernatants were harvested and assayed for IL-10 and IL-13 production (pg/ml) by ELISA as described. The data represent. IL-10 responses of resistant (●) and low risk women (▲). Each symbol represents one subject. Horizontal bars represent median values and the broken line indicates assay sensitivity. Statistical significance was determined by Mann-Whitney U test.

synthesis (median 358 pg/ml, range 7.8-2325 pg/ml) to that of low risk women (median 298 pg/ml, range 7.8-1960 pg/ml).

These data provide evidence that resistant women do not differ from low risk women in type-2 cytokine responses to anti-CD3 mAb mediated activation.

2.2.3 Analysis of type-2:type-1 cytokine balance among resistant and low risk women

2.2.3.1 Analysis of recall Ag (SK, PPD) driven type-2:type-1 cytokine balance among resistant and low risk women. For each subject in both groups of Kenyan women, the ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-13:IFN-γ, and IL-10:IFN-γ were determined using the data for each cytokine obtained following SK and PPD Ag mediated activation.

The results of the analysis of SK Ag driven cytokine response are shown in Figure 36. The relative balance of IL-4:IFN-γ was not significantly different (Mann-Whitney p=ns) between resistant (median 0.136, range, 0.003-6.8) and low risk women (median 0.169, range, 0.003-1.45). Also, the two groups did not differ markedly in other type-2:type-1 ratios examined: IL-13:IFN-γ (resistant women: median 0.045, range, 0.002-0.769; low risk women: median 0.021, range 0.011-1.77; Mann-Whitney p=ns); IL-5:IFN-γ (resistant women: median 2.28, range 0.026-54.8; low risk women: median 4.54, range 0.61-40.3; Mann-Whitney p=ns) and IL-10:IFN-γ (resistant women: median 34.65, range 0.63-418; low risk women: median 1.78, range 0.11-4358; Mann-Whitney p=ns).

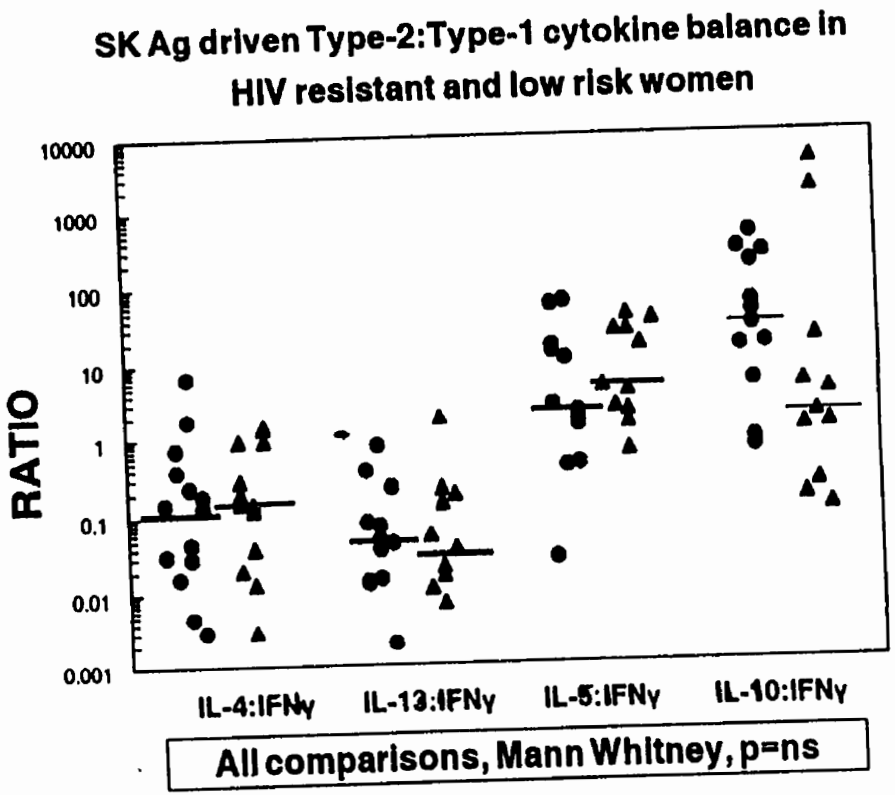

Figure 36. HIV-1 resistant women and low risk women do not differ significantly in SK Ag driven Type-2:Type-1 cytokine balance. PBMC (1.5 X 10^6/ml) from resistant women (●) and low risk women (▲) were each cultured with 5000 U/ml of streptokinase . Culture supernatants were assayed for IFN-γ, IL-4, IL-5, IL-10 and IL-13 protein levels as described. The ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-10: IFN-γ, and IL-13 :IFN-γ calculated for each subject are represented on Y-axis. Each symbol represents one subject. The horizontal bars represent the median values. Statistical significance was evaluated by Mann-Whitney U test.

The results of the analysis of PPD Ag driven response are shown in Figure 37. The relative balance of IL-4:IFN-γ was not significantly different (Mann-Whitney p= ns) between resistant (median 0.127, range 0.004-22.5) and the low risk women group (median 1.45, range0.016-13.6). Also, the two groups did not differ markedly in other type-2:type-1 ratios examined: IL-13:IFN-γ (resistant women: median 0.124, range 0.035-1.2; low risk women: median0.471, range 0.001-2.24; Mann-Whitney p=ns); IL-5:IFN-γ (resistant women: median 1.41, range 0.38-12.6; low risk women: median 25.2, range 0.49-279; Mann-Whitney p=ns) and IL-10:IFN-γ (resistant women: median 21.23, range 0.75-250; low risk women: median 2.27; range 0.49-1790; Mann-Whitney p=ns).

This data show that there is no detectable imbalance in IL-4:IFN-γ, IL-13:IFN-γ, IL-5:IFN-γ, or IL-10:IFN-γ among resistant relative to the low risk women following either SK or PPD Ag mediated activation.

2.2.3.2 Analysis of polyclonally driven Type-2:Type-1 cytokine balance among resistant women and low risk women. For each subject, the ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-13:IFN-γ, and IL-10:IFN-γ were determined using the data for each cytokine, obtained following PHA and anti-CD3 mAb mediated activation. The results of the analysis using PHA is shown in Figure 38. The relative balance of IL-4:IFN-γ was not significantly different (Mann-Whitney, p=ns) between resistant (median 0.146, range 0.001-4.83) and low risk women (median 0.647, range 0.39-7.42;). Also, the

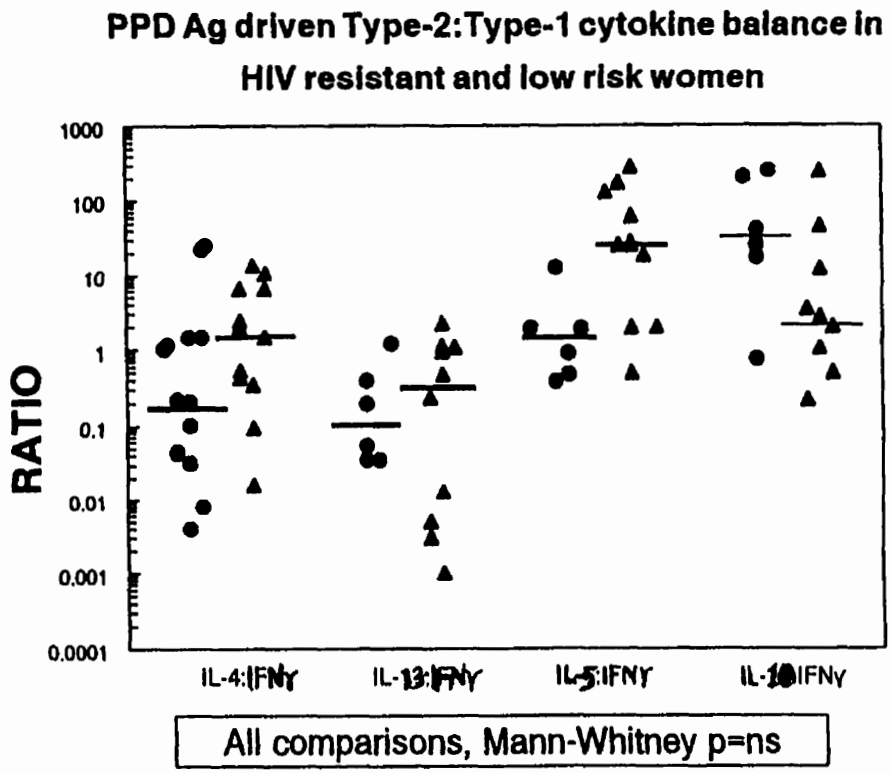

Figure 37. HIV-1 resistant women and low risk women do not differ significantly in PPD driven Type-2:Type-1 cytokine balance. PBMC (1.5 X 10^6/ml) from resistant women (●) and low risk women (▲) were each cultured with 5 tuberculin units/ml of Pure protein derivative (PPD) . Culture supernatants were assayed for IFN-γ, IL-4, IL-5, IL-10 and IL-13 protein levels as described. The ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-10: IFN-γ, and IL-13 :IFN-γ calculated for each subject are represented on Y-axis. Each symbol represents one subject. The horizontal bars represent the median values. Statistical significance was evaluated by Mann-Whitney U test.

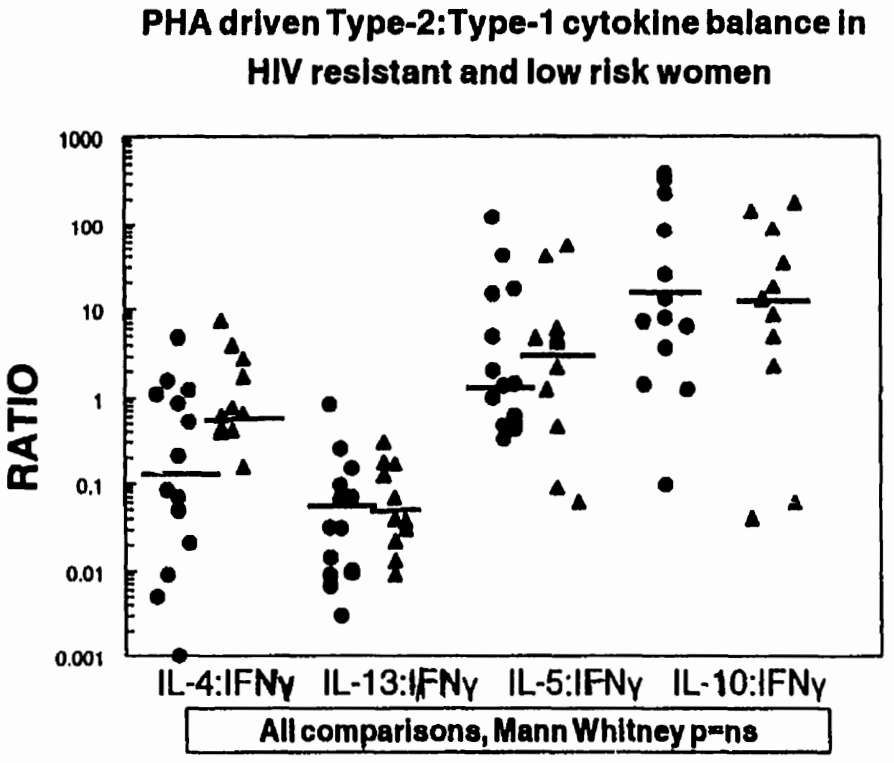

Figure 38. Lack of detectable difference in PHA driven Type-2:Type-1 cytokine responses between resistant and the low risk Kenyan women. PBMC (1.5 X 10^6/ml) from resistant women (●) and low risk HIV-1 negative Kenyan women (▲) were each cultured with 1% PHA . Culture supernatants were assayed for IFN-γ, IL-4, IL-5, IL-10 and IL-13 protein levels as described and the peak production of each cytokine was determined from both these concentrations . The ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-10: IFN-γ, and IL-13 :IFN-γ calculated for each subject are represented on Y-axis. Each symbol represents one subject. The horizontal bars represent the median values. Statistical significance was evaluated by Mann-Whitney U test.

two groups did not differ markedly in other type-2:type-1 ratios examined: IL-13:IFN-γ (resistant women: median0.048,; range 0.003-0.834; low risk women: median 0.037,; range 0.009-0.175; Mann-Whitney p=ns); IL-5:IFN-γ (resistant women: median 1.43, range 0.33-123; low risk women: median 4.30, range 0.061-55; Mann-Whitney p=ns) and IL-10:IFN-γ (resistant women: median 15.52, range 0.096-393; low risk women: median 15.30, range 0.04-182; Mann-Whitney p=ns).

The results of the analysis using anti-CD3 mAb mediated activation are shown in Figure 39. The relative balance of IL-4:IFN-γ was not significantly different (Mann-Whitney p=ns) between resistant (median 0.228, range 0.012-114) and low risk women (median 0.963, range 0.11-6.1). Also, the two groups did not differ markedly in other type-2:type-1 ratios examined: IL-13:IFN-γ (resistant women: median 0.018, range 0.00002-0.103; low risk women: median 0.008, range 0.0029-0.55; Mann-Whitney p=ns); IL-5:IFN-γ (resistant women: median0.989, range 0.044-150; low risk women: median 2.35, range 0.11-6.3; Mann-Whitney p=ns) and IL-10:IFN-γ (resistant women: median 14.85, range 0.21-3750; low risk women: median 2.27, range 0.11-345; Mann-Whitney p=ns).

These data provide evidence for lack of detectable difference in IL-4:IFN-γ, IL-13:IFN-γ, IL-5:IFN-γ, or IL-10:IFN-γ ratios between resistant women and low risk women using either PHA or anti-CD3 mAb mediated activation.

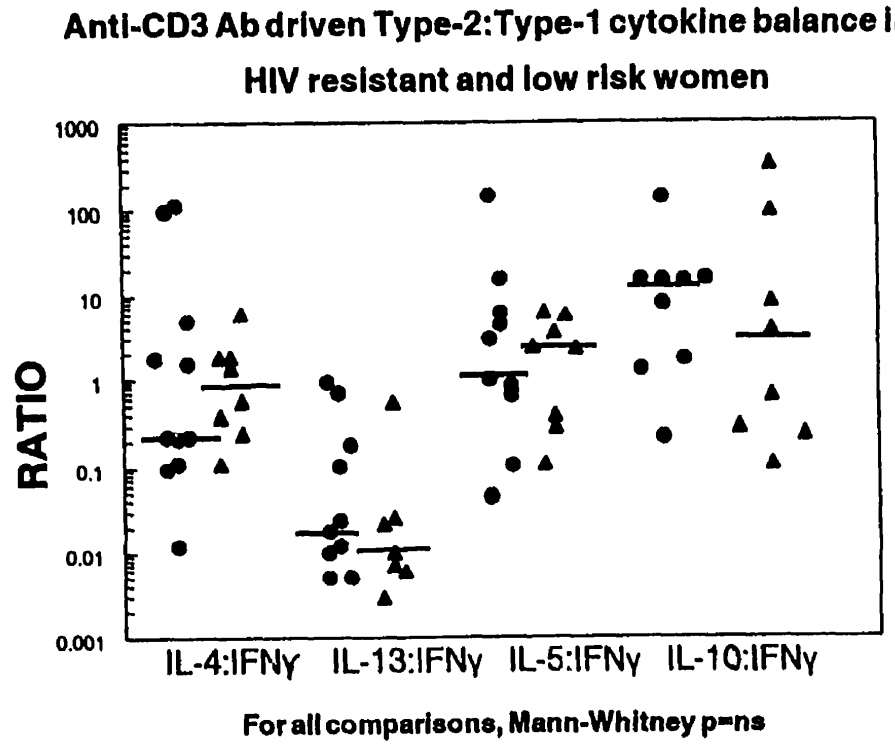

Figure 39. HIV Resistant women and low risk women do not differ significantly in anti-CD3 mAb driven Type-2:Type-1 cytokine balance. PBMC (1.5 X 10^6/ml) from resistant women (●) and low risk HIV negative Kenyan women (▲) were cultured with soluble anti-CD3 mAb (100 ng/ml). Culture supernatants were assayed for IFN-γ, IL-4, IL-5, IL-10 and IL-13 protein levels as described and the peak production of each cytokine was determined from both these concentrations. The ratios of IL-4:IFN-γ, IL-5:IFN-γ, IL-10: IFN-γ, and IL-13 :IFN-γ calculated for each subject is represented on Y-axis. Each symbol represents one subject. The horizontal bars represent the median values. Statistical significance was evaluated by Mann Whitney U test.

3. CHARACTERIZATION OF IL-12 AND IP-10 RESPONSIVENESS OF HIV-1 RESISTANT KENYA WOMEN

The following experiments were performed to characterize the IL-12 responsiveness of HIV-1 resistant Kenyan women. IL-12 responsiveness was defined as the ability to respond to exogenous recombinant (rh IL-12) *in vitro* by producing IFN-γ. In these experiments, rh IL-12 responsiveness of deliberately unstimulated cells (referred to as constitutive IL-12 responsiveness) and on Ag mediated cellular activation were examined.

3.1 Characterization of IL-12 Responsiveness of HIV Resistant Kenyan Women

3.1.1 Analysis of IL-12 responsiveness among HIV-1 resistant and susceptible Kenyan women

3.1.1.1 HIV-1 resistant women exhibit significantly higher constitutive IL-12 responsiveness relative to susceptible women. Analysis of the ability to respond to rIL-12 was carried out in both groups of resistant and susceptible women by comparing IFN-γ production by their PBMC in medium alone, and in the presence of two different concentrations of rIL-12. This analysis showed that both resistant and susceptible women responded significantly to rIL-12 (with 25 pg/ml IL-12: median 92 U/ml, range 5.4-355 U/ml in resistant women vs 35 U/ml: range 1.9-66.4 U/ml in susceptible women; Mann-Whitney $p=0.002$; with 100 pg/ml IL-12 median 179 U/ml, range 10.1-1065 U/ml vs 51 U/ml range 9-81 U/ml; Mann-Whitney $p<0.0001$; in both groups examined independently).

Subsequently, constitutive IL-12 responsiveness was compared between the two groups of women. Results of this analysis are shown in

Figure 40(a,b). As shown, resistant women exhibit significantly (Mann-Whitney p=0.01) higher constitutive IL-12 responsiveness (fFor 25 pg/ml of IL-12: median 92, range 2.50-355 U/ml) compared to susceptible women (median 35, range 1.9-66.4 U/ml). Subsequently, IL-12 responsiveness using a 4-fold higher concentration of IL-12 (100 pg/ml) was examined. As shown, whereas the frequency of responsiveness was identical in the two groups (100% each), the intensity of response was significantly higher (Mann-Whitney p=0.008) among resistant women (median 179, range 10.1-1065 U/ml) compared to susceptible women (median 51, range 9-81 U/ml).

3.1.1.2 HIV-1 resistant women also exhibit significantly higher IL-12 responsiveness relative to susceptible women, on HIV-1 mediated activation. The HIV-1 driven IFN-γ response among resistant women and susceptible women is shown in Figure 41(a,b,c). As evident, resistant women exhibited significantly higher frequency, as well as intensity, of virus driven IFN-γ response relative to susceptible women (resistant women: median 15.1, range 0.31-285 U/ml; susceptible women: median 0.7m range 0.31-8 U/ml; Mann-Whitney p=0.009). The capacity of resistant women and susceptible women to exhibit IL-12 responsiveness on HIV-1 mediated activation was first analyzed within the two groups by comparing their virus driven IFN-γ production in the absence and presence of exogenous IL-12. Both groups exhibited significant IL-12 responsiveness. Following virus mediated activation, resistant women exhibited significantly higher (Mann-Whitney p=0.02) IL-12 responsiveness (median 51, range 3.30-845 U/ml) of IFN-γ

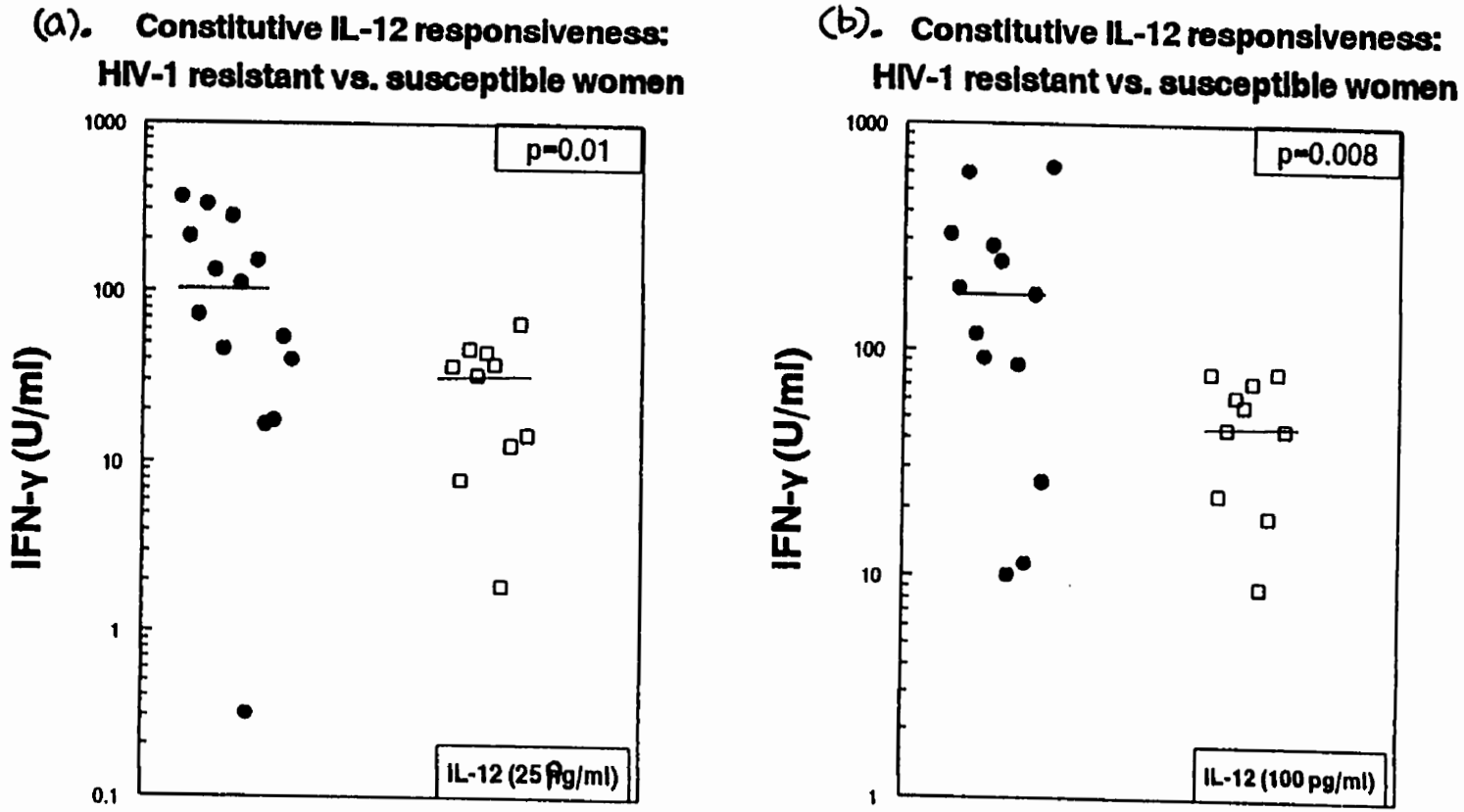

Figure 40a,b: ***HIV resistant women exhibit significantly higher constitutive IL-12 responsiveness relative to susceptible women.*** **(a).** PBMC (1.5 x 10^6/ml) from HIV resistant women (●) and susceptible women (□) were cultured with 25 pg/ml (left panel) and 100 pg/ml (right panel) of rIL-12. Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

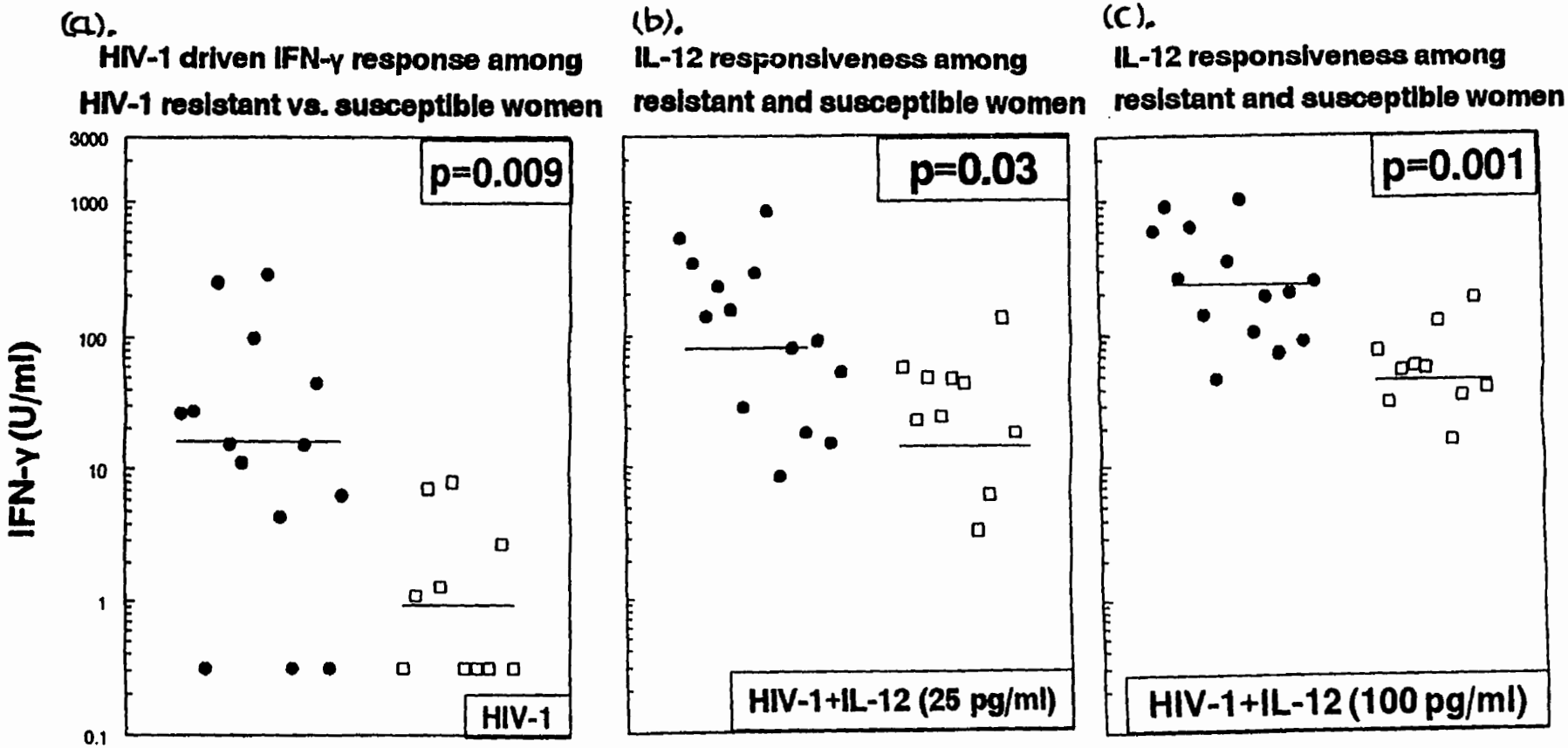

Figure 41a,b,c : HIV-1 resistant women exhibit significantly higher IL-12 responsiveness relative to Susceptible women, on HIV-1 mediated activation. PBMC (1.5 x 10^6/ml) from HIV-1 resistant (●) and susceptible (□) women were cultured in the presence of inactivated HIV-1 $_{IIIB}$ (at two different concentrations 1.25 x 10^2 and 1.25 x 10^3 V.P/ml) alone (left panel) and HIV-1 plus rhIL-12 at 25 (middle panel) and 100pg/ml (right panel). Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

synthesis relative to susceptible women (median 33.50, range 3.3-129 U/ml). Subsequently, IL-12 responsiveness using a 4-fold higher concentration of IL-12 (100 pg/ml) was examined. As shown in Figure 41, the response was significantly higher among resistant women (median 113, range 16-1000 U/ml) compared to susceptible women (median 55, range 16-181 U/ml; Mann-Whitney p=0.001).

Taken together, the above results demonstrate that resistance versus susceptibility to HIV-1 infection is associated with differential responsiveness to IL-12 (both constitutive and on virus stimulation) among these groups of Kenyan women.

3.1.1.3 Reconstitution of IFN-γ production in vitro among susceptible women using recombinant human IL-12. Susceptible women exhibited significantly reduced IFN-γ responses on HIV-1 mediated activation (Figure 41a). To examine whether exogenous IL-12 could restore the reduced levels of IFN-γ in susceptible women *in vitro*, rh IL-12 was added to the virus activated PBMC of susceptible women at two different concentrations (25 and 100 pg/ml). These concentrations are in the range of IL-12 (p70) synthesis typically observed following bacterial Ag (SAC) mediated stimulation of fresh PBMC (data not shown). IFN-γ levels in the culture supernatants were compared with virus driven IFN-γ responses of resistant women elicited in the absence of exogenous IL-12. Results of this analysis are shown in Figure 42. The addition of as little as 25 pg/ml rIL-12 could reconstitute the IFN-γ responses of susceptible women to a level

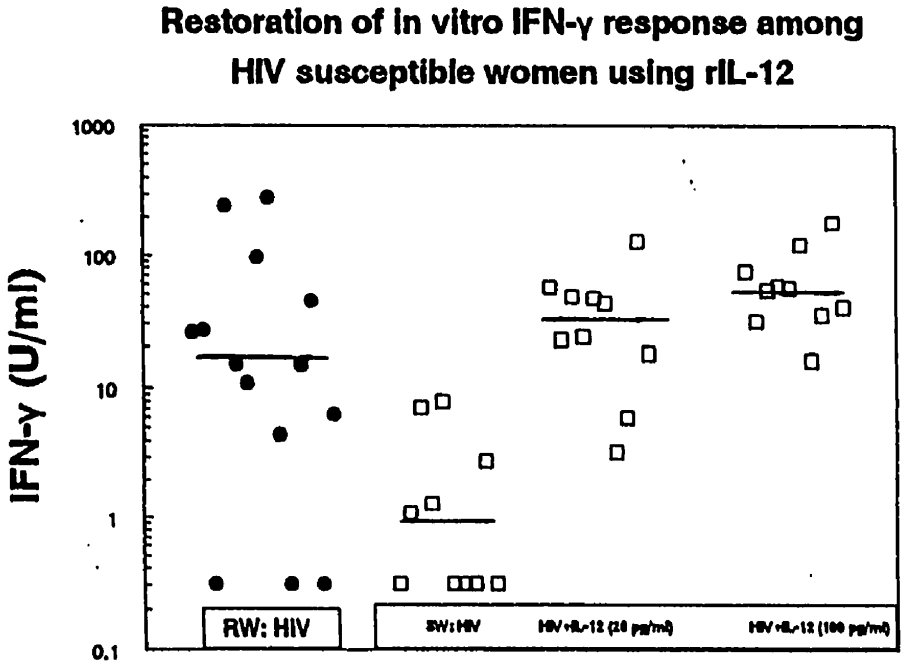

Figure 42: Reconstitution of IFN-γ production *in vitro* among susceptible women using exogenous rIL-12.PBMC (1.5 x 10^6/ml) from HIV-1 resistant (●) and susceptible (□) women were cultured in the presence of inactivated HIV-1 $_{IIIB}$ (at two different concentrations 1.25 x 10^2 and 1.25 x 10^3 V.P/ml) alone and for susceptible women, HIV-1 plus rhIL-12 at 25 and 100pg/ml. Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject. The significance was assessed by Mann-Whitney U test. {resistant women vs. susceptible women : HIV-1 alone: p=0.009; resistant women (HIV-1) vs. susceptible women (HIV-1+IL-12, 25 pg/ml), p=ns; resistant women (HIV-1) vs. susceptible women (HIV-1 +IL-12, 100 pg/ml), p=0.04}.

essentially identical to that of resistant women (susceptible women with IL-12: median 33.5, range 3.3-129 vs resistant women without IL-12: median 15.1, range 0.31-285 U/ml; Mann-Whitney p=ns). Furthermore, addition of the higher concentration of IL-12 to the cultures (100 pg/ml) enhanced responses of susceptible women to levels much higher than those of resistant women observed in the absence of IL-12 (susceptible women with 100 pg/ml of IL-12: median 55, range 16-181 vs resistant women without IL-12: median 15.1, range 0.31-285; Mann-Whitney p=0.04).

Thus, these data demonstrate that exogenous IL-12 restored IFN-γ responses in HIV-1 susceptible women to levels comparable with HIV-1 resistant women. These results also suggest a potential therapeutic value of IL-12 in boosting IFN-γ responses in HIV-1 infected subjects.

3.1.2 Analysis of IL-12 responsiveness among HIV-1 resistant and low risk Kenyan women

3.1.2.1 Constitutive IL-12 responsiveness is similar among HIV-1 resistant and low risk women. The above results, demonstrating a higher responsiveness to IL-12 among resistant women, prompted us to examine their relative responsiveness in comparison to HIV-1 negative, low risk Kenyan women.

For this, the ability to respond to IL-12 was assessed for resistant women and low risk HIV-1 negative women by comparing IFN-γ production by PBMC in medium alone and in the presence of two different concentrations of IL-12. This analysis showed that both resistant women and

low risk HIV-1 negative women can respond to IL-12. In medium alone for resistant women, the median response was 5.3 U/ml and with 25 pg/ml IL-12, the median was 92 U/ml (range 5.4-355; Mann-Whitney p=0.002). In low risk women, in medium alone, the median response was 8.60 U/ml and with 25 pg/ml IL-12, the median was 56 U/ml (range 3.4-1039; Mann-Whitney p=0.01). With a 4-fold higher concentration (100 pg/ml), IL-12 responsiveness becomes stronger (for resistant women: median 179 U/ml, range 10.1-1065 in the presence of IL-12 vs median 5.25 U/ml, range 0.31-199 in the absence of IL-12; Mann-Whitney $p<0.0001$ and for low risk women, median 156 U/ml, range 24-1298 U/ml in the presence of IL-12 vs median 8.60, range 0.31-46.1 in the absence of IL-12; Mann-Whitney $p<0.001$).

Subsequently, IL-12 responsiveness, measured as the capacity of fresh PBMC to mount IFN-γ responses, was compared between the two groups of women. Results of these analysis are shown in Figure 43(a,b). As shown, IL-12 responsiveness was similar between the two groups of women (median 92, range 2.5- 355 U/ml for resistant women vs median 56, range 3.4-1039 U/ml for low risk women; Mann-Whitney p= ns). Analysis of IL-12 responsiveness with a higher dose of 100 pg/ml for low risk women provided similar findings (median 179, range 10.1-1065 U/ml for resistant women vs median 156, range 24-1298 U/ml for low risk women; Mann-Whitney p=ns).

3.1.2.2 Resistant women do not differ from low risk women in IL-12 responsiveness on Ag (SK and PPD) mediated activation. These

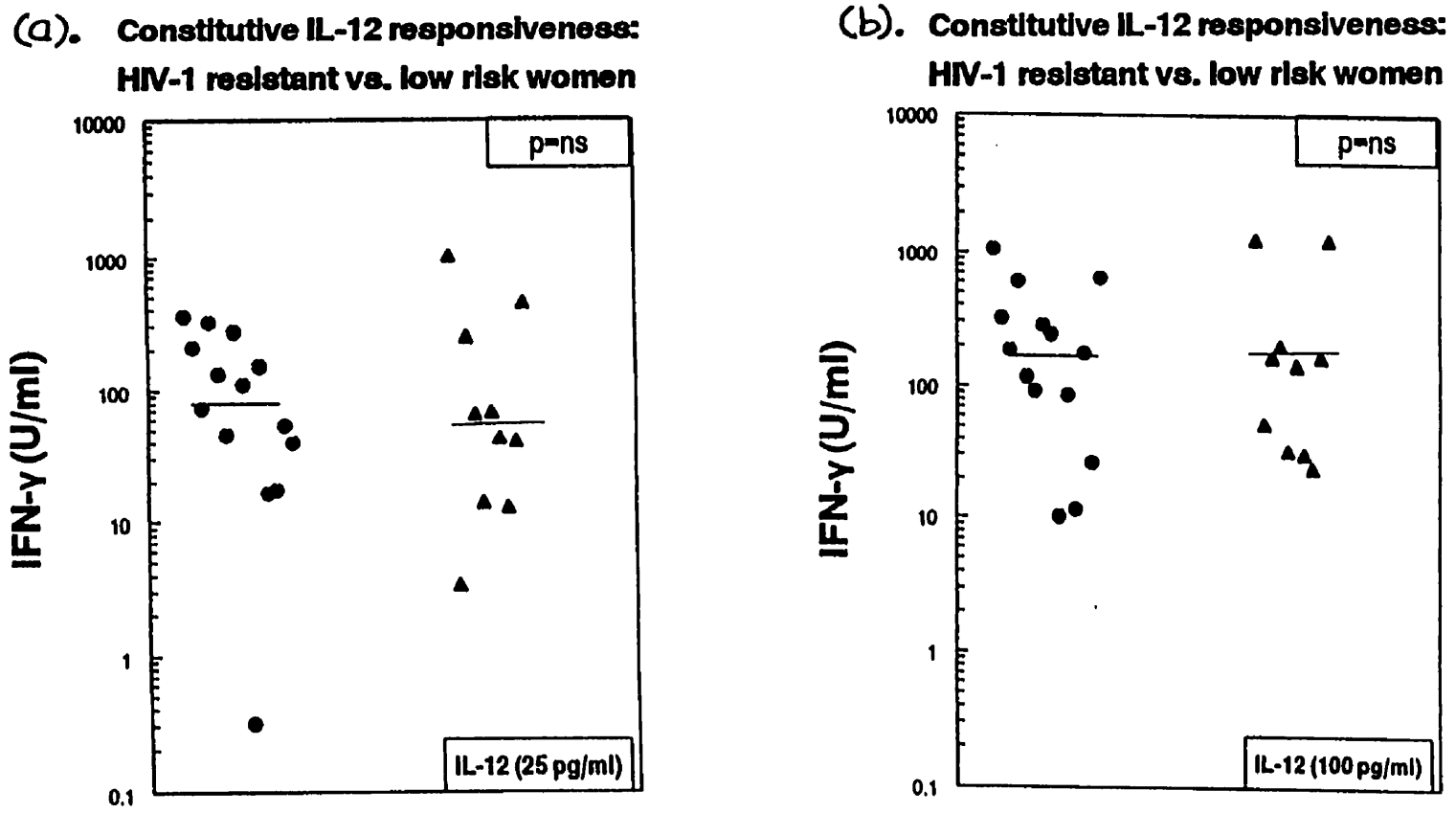

Figure 43a,b: ***Constitutive IL-12 responsiveness is identical among HIV resistant and low risk women.*** PBMC (1.5 x 10^6/ml) from HIV resistant women (●) and low risk women (▲) were cultured with 25 (left panel) and 100 pg/ml (right panel) of rhIL-12. Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

experiments were performed to address the hypothesis that the resistant women may have an increased responsiveness to IL-12 on Ag mediated activation compared with low risk women. For this, recall Ags SK and PPD were used for cellular activation. The capacity of resistant women and low risk women to exhibit IL-12 responsiveness on SK mediated activation was analyzed first within the two groups by comparing the SK driven IFN-γ production in the absence and the presence of exogenous IL-12. Following SK mediated activation, in the presence of 25 pg/ml IL-12, both resistant and low risk women responded significantly (resistant women with SK alone: median 61.5, range 0.31-608 vs SK+IL-12: median 271, range 21.3-1621 U/ml; Mann-Whitney p=0.01; low risk women with SK alone: median 36.5, range 0.31-257 U/ml vs SK+IL-12: median 172, range 28-1120 U/ml; Mann-Whitney p=0.01). Examination of responsiveness to 100 pg/ml of rIL-12 within both groups again demonstrated the same trend (resistant women with SK alone: median 61.5, range 0.31-608 U/ml vs SK+IL-12: median 437.5, range 25-1280 U/ml; Mann-Whitney p=0.001; low risk women with SK alone: median 36.5, range 0.31-257 U/ml vs SK+IL-12: median 341.5, range 77-1280, Mann-Whitney p=0.001).

Subsequently, IL-12 responsiveness was compared between the two groups. Results of the analysis of IL-12 responsiveness on SK Ag mediated activation are shown in Figure 44(a,b,c). The intensity of IL-12 responsiveness was similar in both groups (resistant women: median 220, range 21.3-1621 U/ml vs median 172, range 28-1120 U/m in low risk

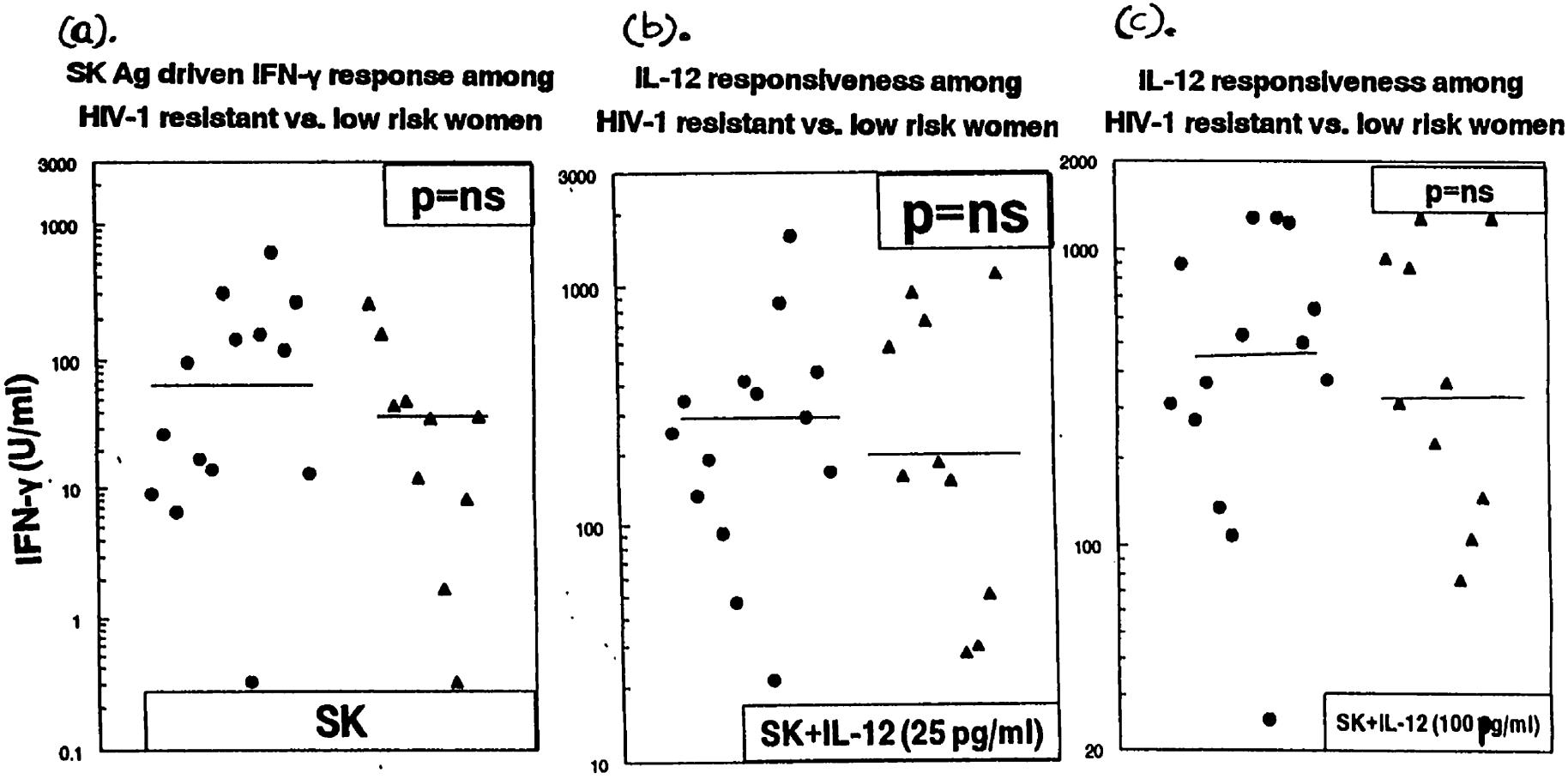

Figure 44a,b,c: Resistant women do not differ from low risk women in IL-12 responsiveness on SK mediated activation. PBMC (1.5×10^6/ml) from HIV-1 resistant (●) and low risk (▲) women were cultured in the presence of 5000 U/ml of streptokinase alone (left panel), SK plus rhIL-12 at 25 (middle panel) and 100pg/ml (rigth panel). Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

women; Mann-Whitney p=ns). With 100 pg/ml of IL-12, similar results were obtained (median 373, range 25-1280 U/ml in resistant women vs median 342, range 77-1280 U/ml in low risk women; Mann-Whitney p=ns).

The capacity of resistant women and low risk women to exhibit IL-12 responsiveness on PPD mediated activation was analyzed first within the two groups by comparing the PPD driven IFN-γ production in the absence and presence of exogenous IL-12 (Figure 44d-f). It was observed that following PPD mediated activation in the presence of 25 pg/ml, IL-12 resistant and low risk women responded and produced significantly higher levels of IFN-γ (resistant women with PPD alone: median 55.5, range 0.31-191 U/ml vs PPD+IL-12: median 214, range 55-706 U/ml; Mann-Whitney p<0.0001; low risk women with PPD alone: median 16.5, range 0.31-168 U/ml vs PPD+IL-12: median 280, range 14.5-899 U/ml; Mann-Whitney p=0.004). Examination of responsiveness to 100 pg/ml of rIL-12 within both groups again demonstrated the same trend (resistant women with PPD alone, median 55.5, range 0.31-199 U/ml vs PPD+IL-12: median 413, range 99-1230 U/ml; Mann-Whitney p<0.0001; low risk women with PPD alone: median16.5, range 0.31-168 U/ml vs PPD+IL-12: median 269, range 55-1280 U/ml; Mann-Whitney p=0.001).

Subsequently, IL-12 responsiveness was compared between the two groups. The results from the analysis of IL-12 responsiveness on PPD Ag mediated activation are shown in Figure 44e,f. The intensity of IL-12 responsiveness was similar in both groups (resistant women: median 214,

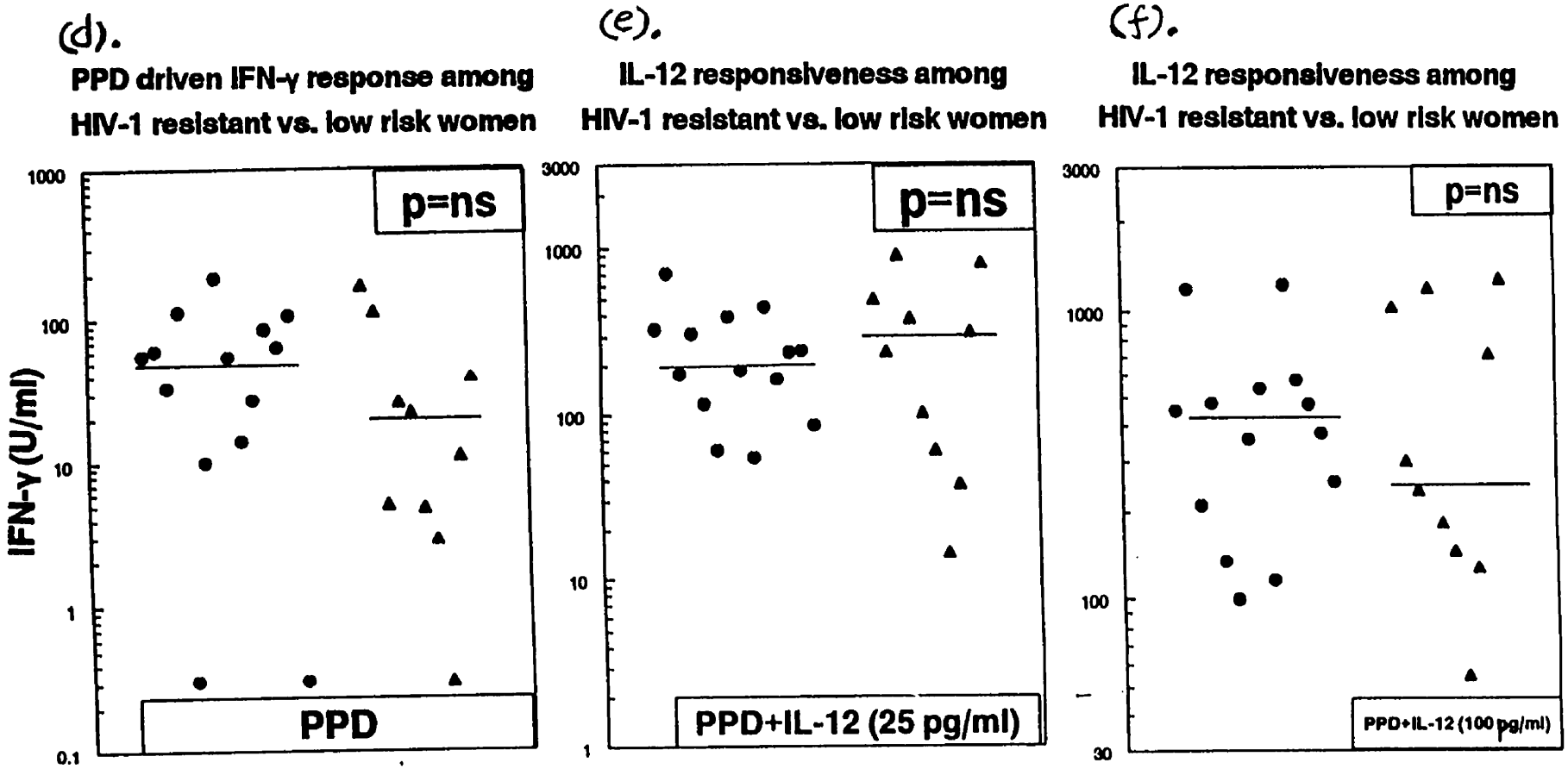

Figure 44d,e,f: Resistant women do not differ from low risk women in IL-12 responsiveness on PPD mediated activation PBMC (1.5×10^6/ml) from HIV-1 resistant (●) and low risk (▲) women were cultured in the presence of 5 T.U./ml of PPD alone (left panel), PPD plus rhIL-12 at 25 (middle panel) and 100pg/ml (rigth panel). Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

range 55-706 U/ml vs low risk women: median 280, range 15-899 U/ml; Mann-Whitney p=ns). With 100 pg/ml of IL-12, similar results were obtained (resistant women: median 413, range 99-1230 U/ml vs low risk women: median 269, range 55-1280 U/ml; Mann-Whitney p=ns).

These data provide evidence that HIV-1 resistant women do not differ from low risk women in either constitutive IL-12 responsiveness or IL-12 responsiveness with SK and PPD Ag mediated activation.

3.2 Characterization of IP-10 Responsiveness of HIV-1 Resistant Kenyan women

The following experiments were performed to characterize the IP-10 responsiveness of HIV-1 resistant Kenyan women. IP-10 responsiveness was defined as the ability to respond to recombinant (r) human IP-10 in culture by producing IFN-γ. In these experiments, IP-10 responsiveness of deliberately unstimulated cells (referred to as constitutive IP-10 responsiveness) and on Ag mediated cellular activation were examined.

3.2.1 Analysis of IP-10 responsiveness among HIV-1 resistant and susceptible women

3.2.1.1 HIV-1 resistant women do not differ from susceptible women in constitutive IP-10 responsiveness. The IP-10 responsiveness was compared between the two groups of women. Analysis of constitutive IP-10 responsiveness between resistant and susceptible women are shown in Figure 45a,b. Analysis of the intensity of responses did not reveal significant differences between the groups with either 0.1 ng/ml (resistant women: median 18.4, range 0.31-244U/ml; susceptible women: median 9.8, range 0.31-99 U/ml; Mann-Whitney p=ns) or 1 ng/ml of exogenous IP-10 (resistant

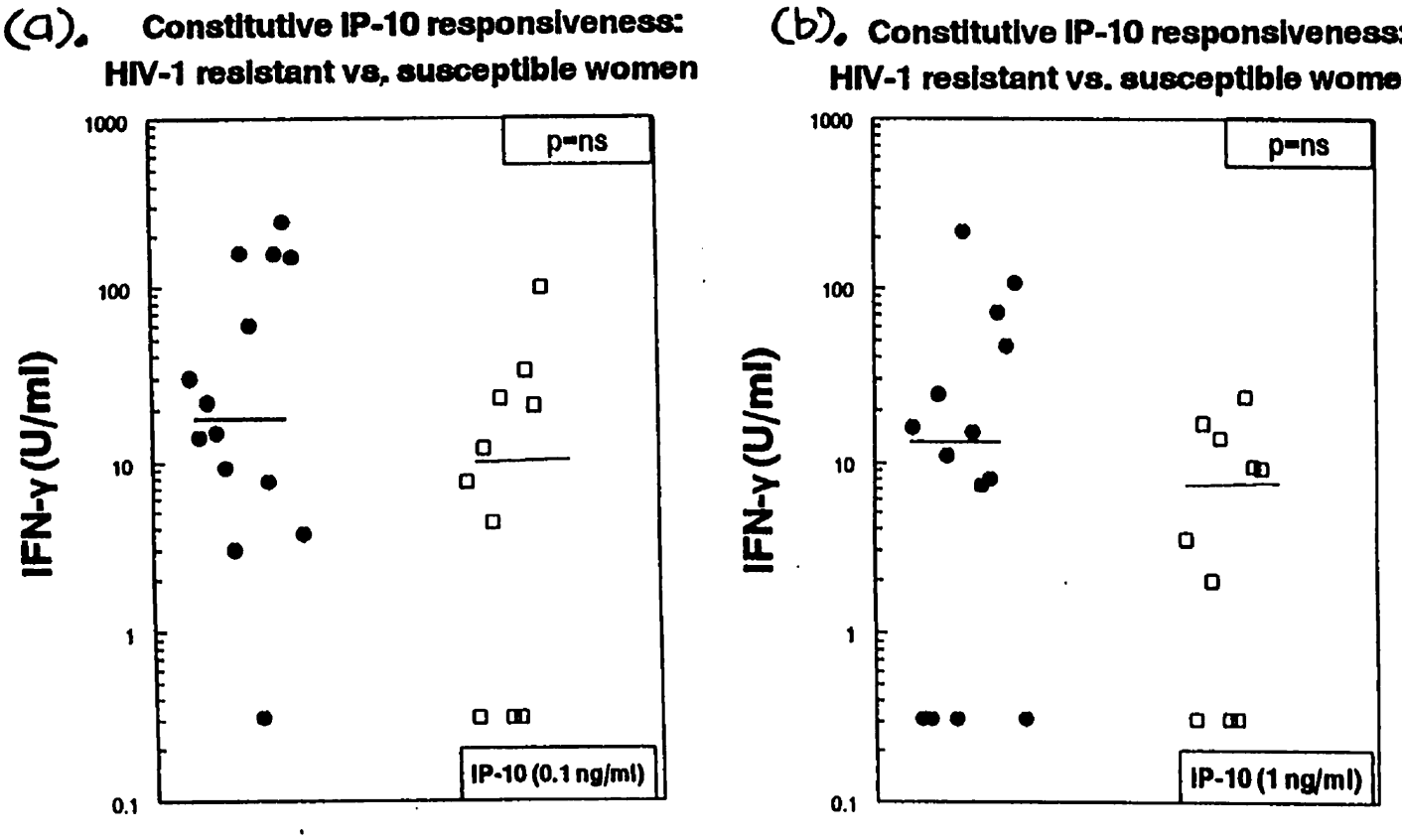

Figure 45a,b: ***HIV resistant women do not differ from susceptible women in constitutive IP-10 responsiveness.*** PBMC (1.5 x 10^6/ml) from HIV resistant women (●) and susceptible women (□) were cultured with 0.1 (left panel) and 1 ng/ml (right panel) of rhIP-10. Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

women: median 13, range 0.31-217 U/ml; susceptible women: median 6.4, range 0.31-24 U/ml; Mann-Whitney p=ns).

3.2.1.2 HIV-1 resistant women exhibit significantly higher IP-10 responsiveness relative to susceptible women, on HIV-1 mediated activation. The capacity of resistant women and susceptible women to exhibit IP-10 responsiveness on HIV-1 mediated activation was compared between the groups.

Results of the analysis of IP-10 responsiveness elicited, following HIV-1 mediated cellular activation, are shown in Figure 46a,b. The frequency of responders to 0.1 ng/ml IP-10 was similar in both groups of women (resistant women: 13/14; susceptible women: 9/10). Also, following virus mediated activation, resistant women exhibited a trend towards higher responsiveness to 0.1 ng/ml of IP-10 (median 40, range 0.31-259 U/ml) than that of susceptible women (median 12, range 0.31-205 U/ml; Mann-Whitney p=0.06). However, responsiveness to 1 ng/ml of IP-10 was significantly different between the two groups (resistant women: median 38, range 1.72-222 U/ml vs susceptible women: median 15, range 0.31-35 U/ml; Mann-Whitney p=0.03).

Taken together, the above results demonstrate that clinical resistance versus susceptibility to HIV-1 infection is associated with differential responsiveness to IP-10 on virus stimulation among these groups of Kenyan women.

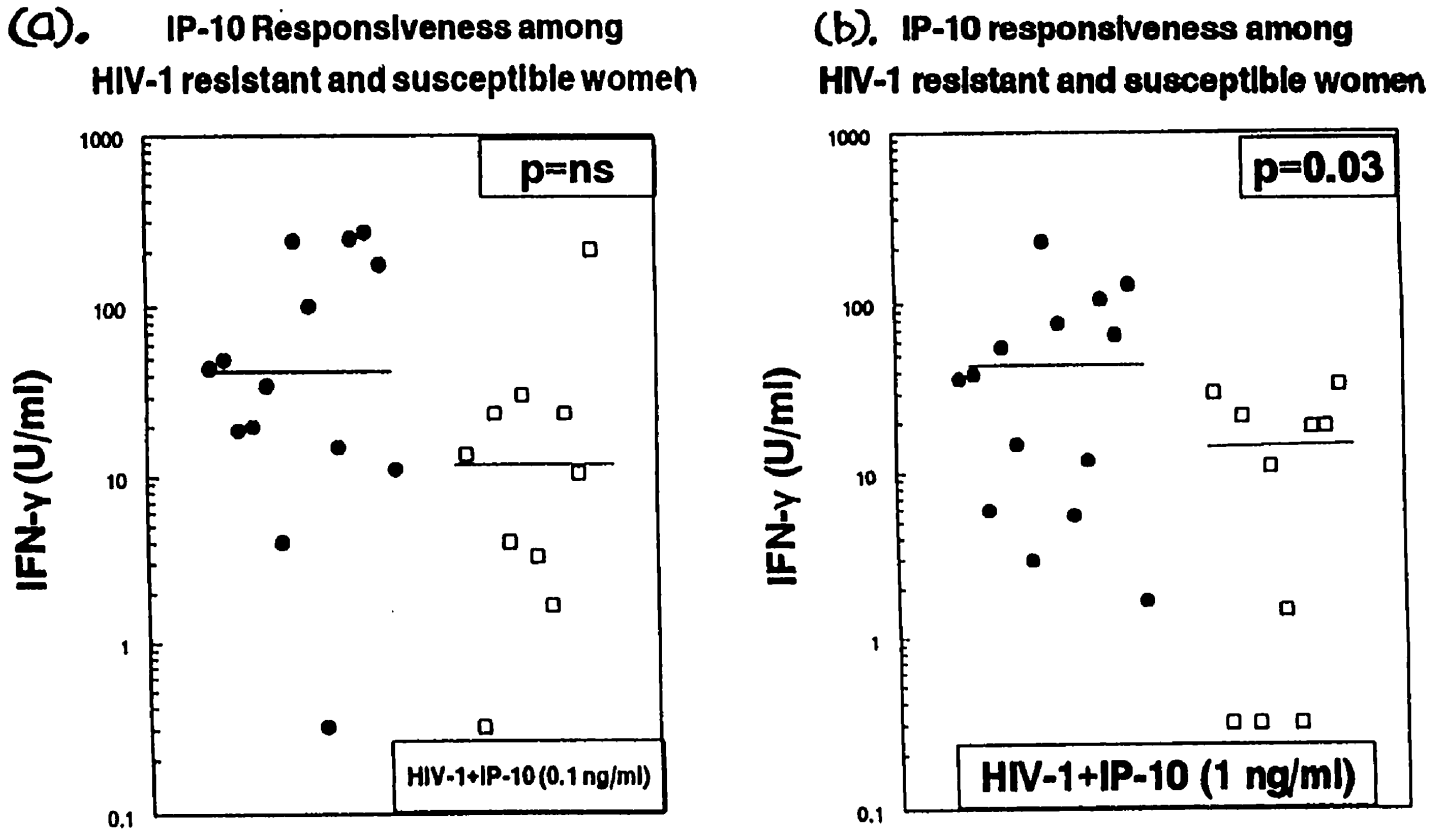

Figure 46a,b: HIV-1 resistant women exhibit higher IP-10 responsiveness relative to susceptible women on HIV-1 mediated activation. PBMC (1.5×10^6/ml) from HIV-1 resistant (●) and susceptible (□) women were cultured in the presence of inactivated HIV-1_{IIIB} (at two different concentrations 1.25×10^2 and 1.25×10^3 VP/ml) plus rhIP-10 at 0.1 (left panel) and 1ng/ml (rigth panel). Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

3.2.1.3 Reconstitution of IFN-γ production in vitro among susceptible women using recombinant human IP-10. Resistant women produced significantly higher levels of HIV-1 driven IFN-γ compared to susceptible women (Figure 47). Therefore, in view of potential therapeutic interest, whether exogenous rIP-10 can restore the decreased levels of IFN-γ responses in susceptible women, responsiveness was measured *in vitro* after the addition of rIP-10 at two different concentrations (0.1 and 1 ng/ml). IFN-γ levels production were compared with virus driven IFN-γ responses of resistant women without any exogenous IP-10. Results of this analysis are shown in Figure 47. Addition of as low as 0.1 ng/ml of rIP-10 could reconstitute IFN-γ responses of susceptible women to a level similar to those of resistant women with HIV-1 alone (susceptible women with HIV-1+IP-10: median 12, range 0.31-205 vs resistant women with HIV-1 alone: median 15.1, range 0.31-285 U/ml; Mann-Whitney p=ns). Furthermore, the addition of higher concentrations of IP-10 to the cultures (at 1 ng/ml) also had similar effects on virus driven IFN-γ responses of susceptible women (with HIV-1+IP-10: median 15, range 0.31-34 vs resistant women with HIV-1 alone: median 15.1, range 0.31-285 U/ml; Mann-Whitney p=ns).

Thus, these data demonstrate that exogenous IP-10 could restore the *in vitro* IFN-γ responses of susceptible women at par with those of resistant women. These results also indicate a potential therapeutic value of IP-10 in boosting IFN-γ responses of HIV-1 infected subjects.

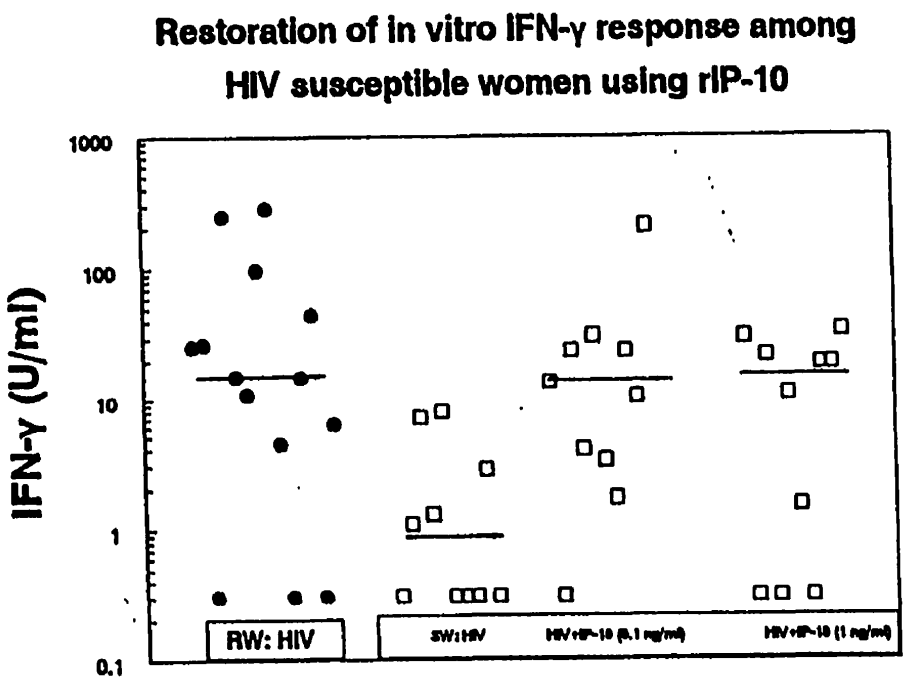

Figure 47. ***Reconstition of IFN-γ production in vitro among susceptible women using exogenous recombinant IP-10.*** PBMC (1.5×10^6/ml) from HIV resistant (●) and susceptible (□) women were cultured in the presence of inactivated HIV_{IIIB} (at two different concentrations 1.25×10^2 and 1.25×10^3 V.P/ml) alone and for susceptible women, HIV plus rhIP-10 at 0.1and 1ng/ml. Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject. The significance was assessed by Mann-Whitney U test {Resistant vs. susceptible, HIV alone: p=0.009; Resistant (HIV) vs. susceptible (HIV+IP-10, 0.1 ng/ml), p=ns; Resistant (HIV) vs. susceptible (HIV +IP-10, 1 ng/ml, p=ns}.

3.2.2 Analysis of IP-10 responsiveness among HIV-1 resistant and low risk Kenyan women.

3.2.2.1 Constitutive IP-10 responsiveness is similar among HIV-1 resistant and low risk women. The IP-10 responsiveness was compared between the two groups of women (resistant vs low risk). Results of these analysis are shown in Figure 48a,b. The intensity of responsiveness was similar between the two groups (median 18.4, range 0.31-244 U/ml in resistant women vs median 15.2, range 2.7-107 U/ml; Mann-Whitney p=ns). Analysis of IP-10 responsiveness to a higher dose of 1 ng/ml provided similar findings (median 13, range 0.31-217 U/ml in resistant women vs median 6.3, range 0.31-119 U/ml among low risk women; Mann-Whitney p=ns). These data suggest that constitutive IP-10 responsiveness is similar in both resistant women and low risk women.

3.2.2.2 Resistant women do not differ from low risk women in IP-10 responsiveness on Ag (SK and PPD) mediated activation. These experiments were performed to address the hypothesis that the resistant women may have an increased responsiveness to IP-10 on Ag mediated activation. For this, recall Ags SK and PPD were used for cellular activation. The IP-10 responsiveness was compared between the two groups. The results from the analysis of IP-10 responsiveness on SK Ag mediated activation are shown in Figure 49a,b. The intensity of IP-10 responsiveness was not significantly different between the groups with 0.1 ng/ml (resistant women: median 50, range 5-509 U/ml vs median 44, range 4.3-230 U/m in low risk

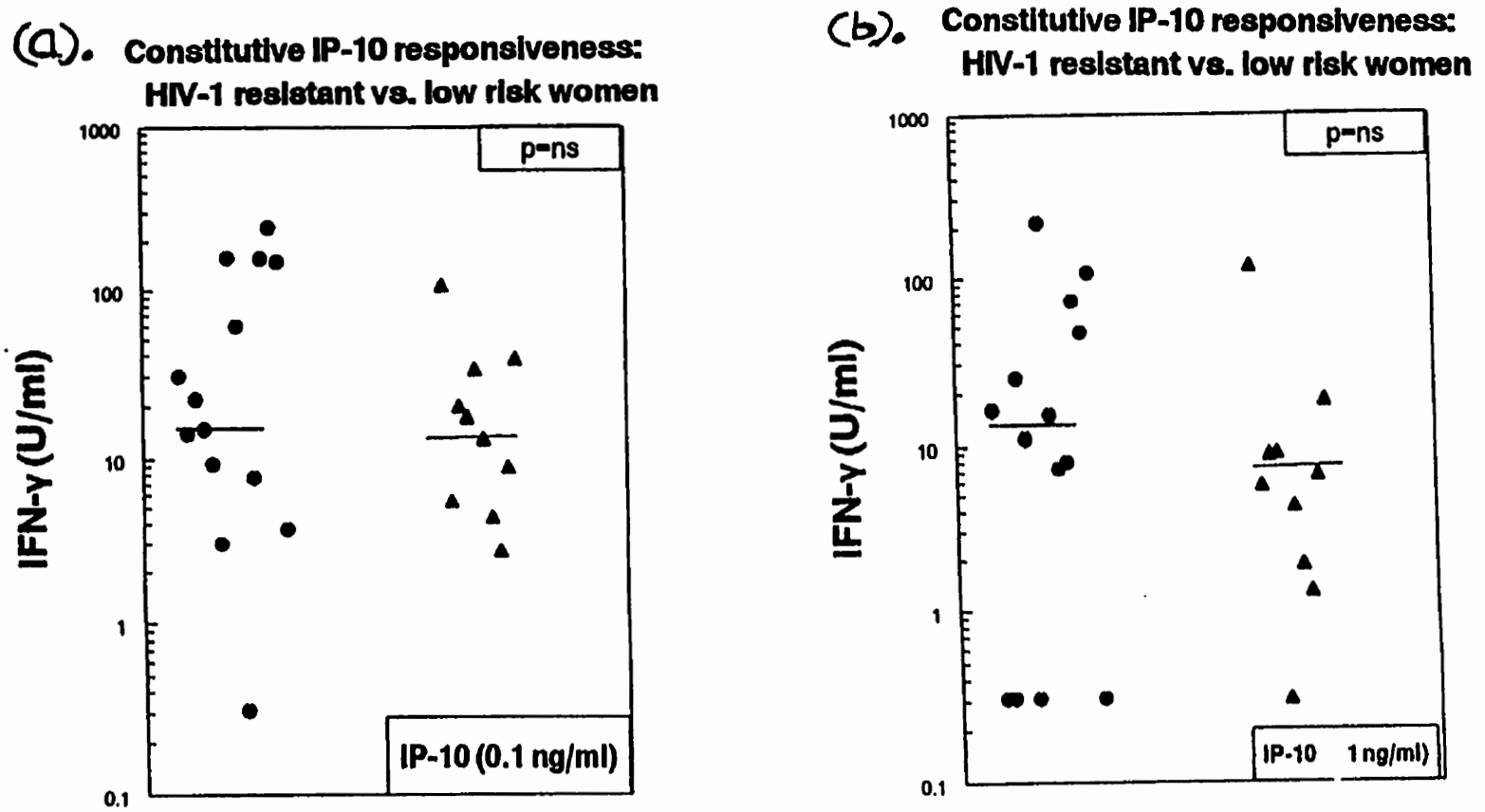

Figure 48a,b: ***Constitutive IP-10 responsiveness is identical among HIV resistant and low risk women.*** PBMC (1.5 x 10^6/ml) from HIV resistant women (●) and low risk women (▲) were cultured with 0.1 (left panel) and 1ng/ml (right panel) of rhIP-10. Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

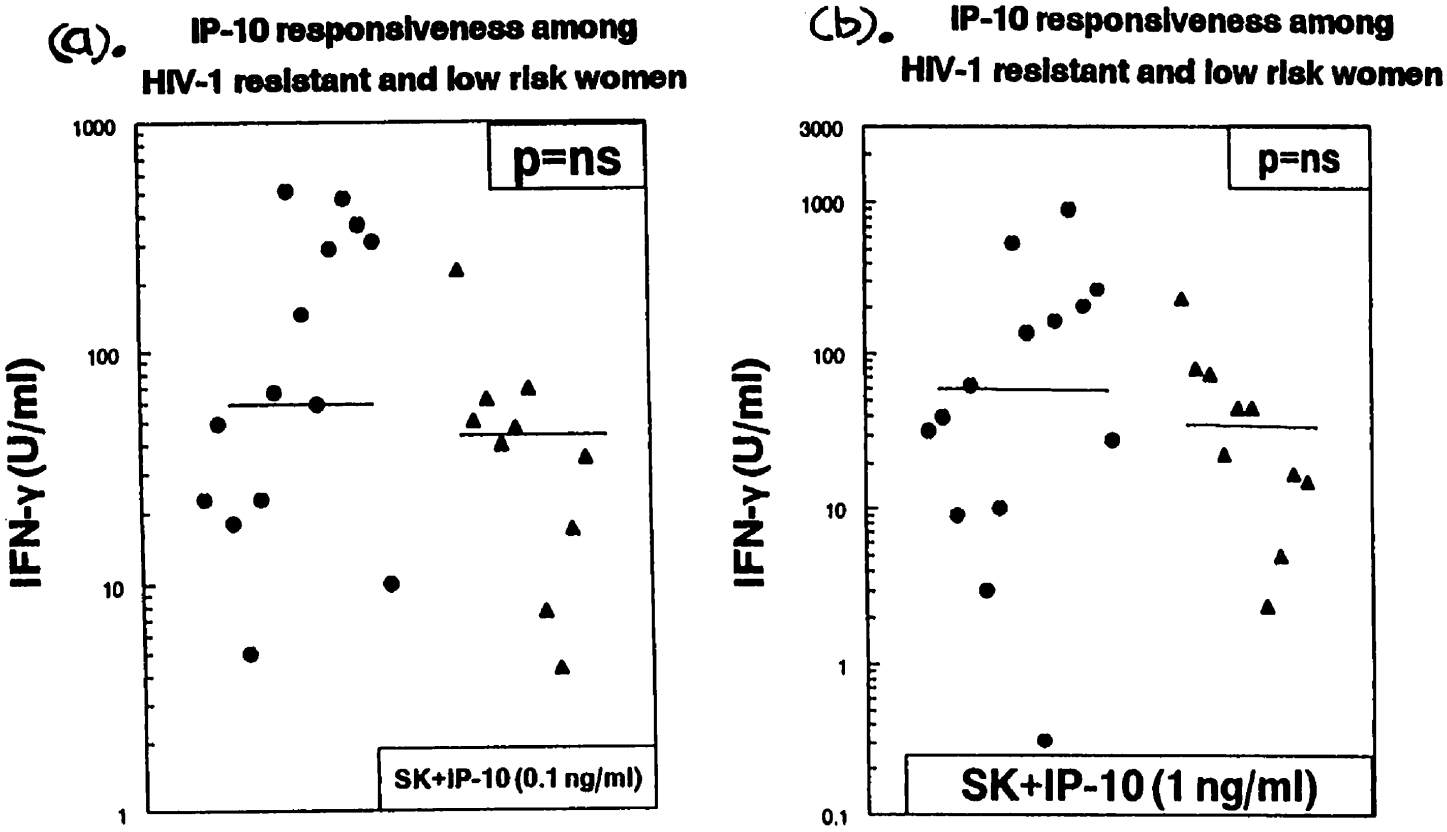

Figure 49a,b: ***Resistant women do not differ from low risk women in IP-10 responsiveness on Streptokinase (SK) mediated activation.*** PBMC (1.5 x 10^6/ml) from HIV-1 resistant (●) and low risk (▲) women were cultured in the presence of 5000 U/ml of streptokinase plus rhIP-10 at 0.1 (left panel) and 1ng/ml (rigth panel). Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

women; Mann-Whitney p=ns). With 1 ng/ml of IP-10, similar results were obtained (median 42, range 0.31-893 U/ml in resistant women vs median 34, range 2.4-229 U/ml in low risk women; Mann-Whitney p=ns). The results from the analysis of IP-10 responsiveness on PPD Ag mediated activation are shown in Figure 49c,d. The intensity of IP-10 (0.1 ng/ml) responsiveness was not significantly different between the groups (resistant women: median 79, range 11-300 U/ml vs median 29, range 4-158 U/m in low risk women; Mann-Whitney p=ns). With 1 ng/ml of IP-10, similar results were obtained (median 49, range 3.6-230 U/ml in resistant women vs median 10.7, range 2.9-204 U/ml in low risk women; Mann-Whitney p=ns). These data provide evidence that HIV-1 resistant women do not differ from low risk women in either constitutive IP-10 responsiveness or IP-10 responsiveness on SK and PPD Ag mediated activation.

4. CHARACTERIZATION OF PLASMA IgE PROFILE OF HIV-1 RESISTANT KENYAN WOMEN

Clinical resistance versus susceptibility to HIV-1 infection is associated with reciprocal synthesis of virus driven IFN-γ and IL-4 cytokines (Figures 13 and 14). It is now well established that IgE synthesis is reciprocally regulated by IFN-γ (negative regulator) and IL-4/IL-13 (positive regulators) cytokines (Romagnani, 1997; Bacharier et al, 1998). Therefore, it was of obvious interest to characterize IgE profile of these women, especially their systemic levels of HIV-1 specific IgE Abs. The hypothesis that the differential

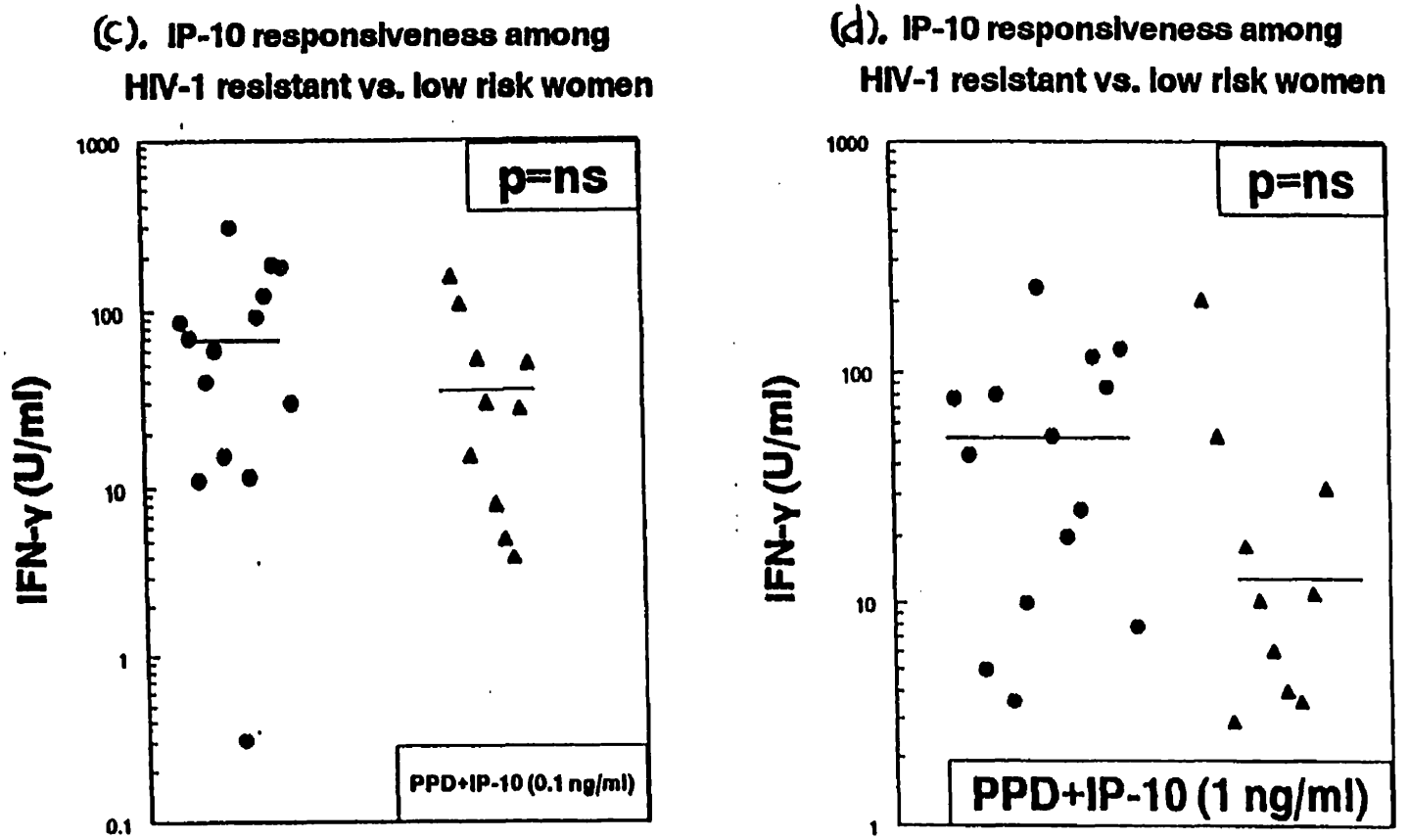

Figure 49c,d: Resistant women do not differ from low risk women in IP-10 responsiveness on PPD mediated activation. PBMC (1.5 x 10^6/ml) from HIV-1 resistant (●) and low risk (▲) women were cultured in the presence of 5T.U/ml of PPD plus rhIP-10 at 0.1 (left panel) and 1 ng/ml (rigth panel). Culture supernatants were harvested and assayed for IFN-γ protein by ELISA. The peak level of IFN-γ synthesis observed with each subject over a 6 day culture period is shown along the Y axis. Each symbol represents one subject and the horizontal bar indicates median value in each group. The significance was assessed by Mann-Whitney U test.

production of virus specific IgE Ab may be associated with resistance versus susceptibility to HIV-1 infection was addressed in this component of the study.

4.1 HIV-1 Resistant Kenyan Women Exhibit Marked Deficiency of HIV-1 Specific IgE Ab in the Plasma Relative to Susceptible Women

Results of the analysis of HIV-1 specific IgE Ab levels in the plasma of resistant women and susceptible women are shown in Figure 50. Most of the resistant women (24/27 examined, ~89%) did not exhibit detectable HIV-1-specific IgE Abs in the plasma. In contrast, the majority of the susceptible women (16/21 examined, ~77%) exhibited HIV-1-specific IgE Ab (Fisher's $p=0.01$). The frequency of HIV-1-specific IgE Ab among low risk women (2/18) was similar to that of the resistant women (3/27).

The magnitude of the HIV-1 specific IgE Ab levels was significantly higher among the susceptible women (OD: median 0.545, range 0.14-1.15) relative to the resistant women (OD: median 0.132, range 09-0.48; Mann-Whitney $p<0.00001$). Also, susceptible women differed significantly in their plasma levels of HIV-1-specific IgE from the low risk women (OD: median 0.163, range 0.09-0.36; Mann-Whitney $p<0.00001$).

From this data, it was concluded that resistance, but not susceptibility, to HIV-1 infection is associated with marked deficiency of HIV-1-specific IgE Ab in the plasma.

4.2 HIV-1 Resistant Kenyan Women Produce IgE Ab to Environmental Allergens Similar to Low Risk Women

Results of the analysis of HDM and cat allergen specific IgE Ab among three groups of women are shown in Figures 51 and 52, respectively. All women in each of the three groups exhibited detectable specific IgE Abs to both HDM and cat allergens. As evident in Figure 51, resistant women exhibited similar levels of HDM specific IgE Abs in the plasma

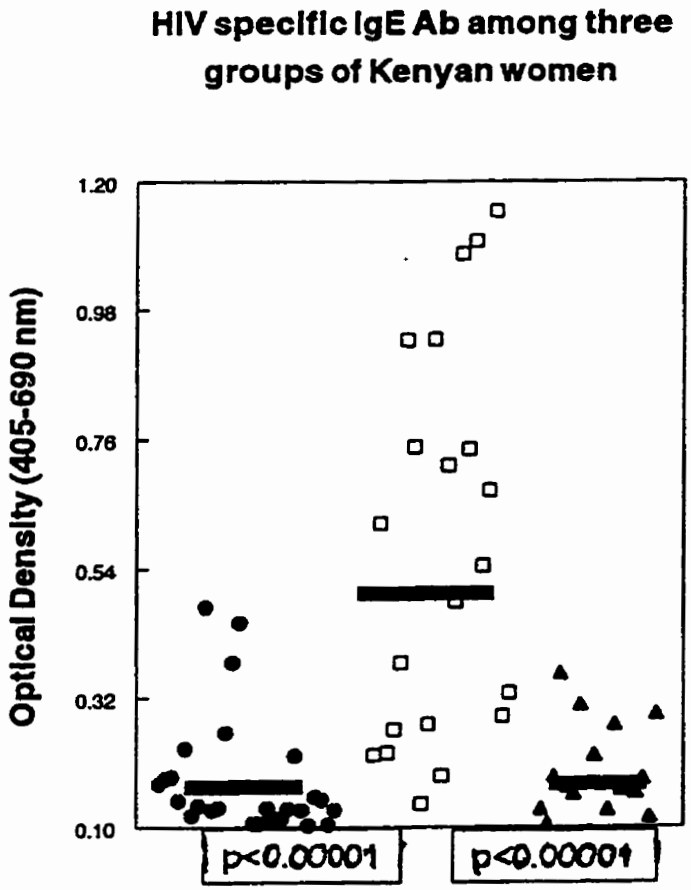

Figure 50. HIV-1 resistant Kenyan women do not exhibit detectable HIV-1-specific IgE Ab in the plasma. Plasma samples from resistant women (●, n=27), susceptible women (□, n=21) and low risk women (▲, n=18) were assayed for HIV-1 specific IgE antibody by ELISA as described. Each symbol represents one subject. The Y axis represents the optical density (OD) values at 405-690 nm. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test (resistant women versus susceptible women, P<0.00001; susceptible women versus low risk women, p<0.00001; resistant women versus low risk women, p=ns).

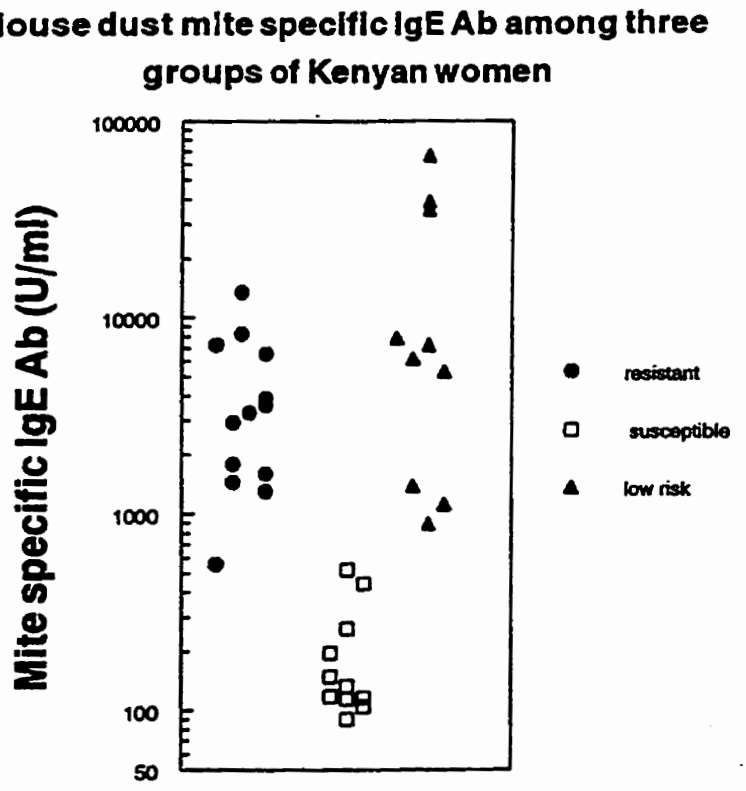

Figure 51. HIV-1 resistant Kenyan women produce similar levels of IgE Ab to the allergen House Dust Mite (HDM) as low risk women. Plasma samples from resistant women (●, n=13), susceptible women (□, n=11) and low risk women (▲, n=10) were assayed for HDM specific IgE antibody by ELISA as described. Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test (resistant women versus low risk women, p=ns; resistant women versus susceptible women, p=0.004; susceptible women versus low risk women, p=0.003).

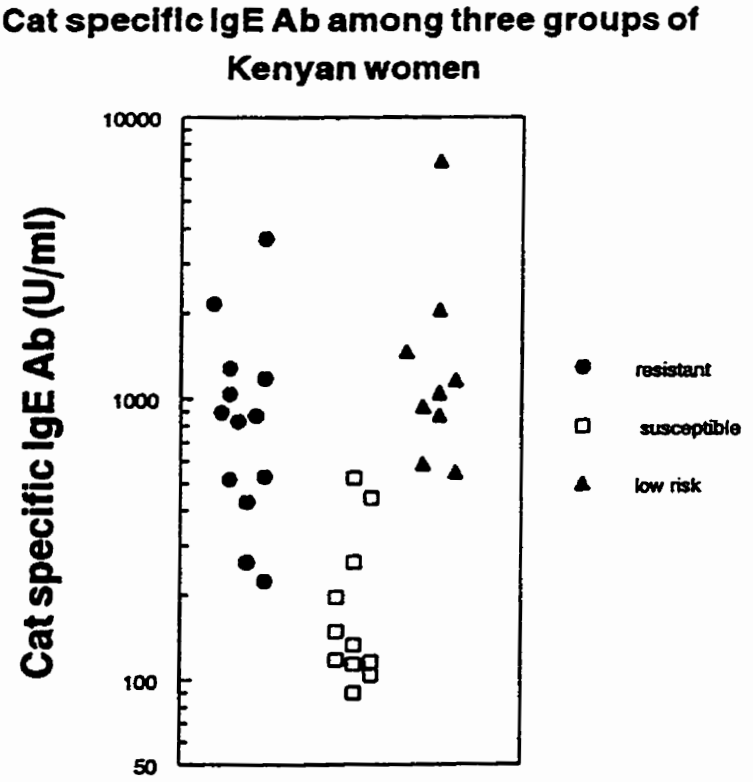

Figure 52. HIV-1 resistant Kenyan women produce similar levels of IgE Ab to cat allergen as low risk women. Plasma samples from resistant women (●, n=13), susceptible women (□, n=11) and low risk women (▲, n=10) were assayed for cat allergen specific IgE antibody by ELISA as described. Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test (Resistant women versus low risk women, p=ns; resistant women versus susceptible women, p=0.0002; susceptible women versus low risk women, p=0.0003).

compared to that of low risk women (median 3266, range 554-13404 U/ml vs median 6683, range 894-66365 U/ml; Mann-Whitney p=ns). In contrast to the subjects in the above two groups, HIV-1 susceptible women showed significantly reduced levels of both HDM specific IgE antibodies (median 712 U/ml, range 340-2386 U/ml; Mann-Whitney p=0.004 comparing resistant vs susceptible women; p=0.003 comparing susceptible vs low risk women).

Levels of cat specific IgE Ab in the plasma of resistant women (median 849, range 224-3680 U/ml) was similar to that in low risk women (median 990, range 161-6941 U/ml; Mann-Whitney p=ns). In contrast, the susceptible women exhibited significantly reduced levels of cat specific IgE Ab in the plasma (median 133, range 90-521 U/ml; Mann-Whitney p=0.0002 comparing susceptible vs resistant women; p=0.0003 comparing susceptible vs low risk women).

These data suggested that whereas HIV-1 resistant women do not differ from low risk women in plasma levels of allergen specific IgE Abs, HIV-1 susceptible women exhibit significantly reduced IgE Ab levels to two common environmental allergens.

4.3 HIV-1 Resistant Women Do Not Exhibit a Generalized Defect in IgE Isotype Synthesis

Results of the analysis of total plasma IgE levels among the three groups of Kenyan women are shown in the Figure 53. As evident, the range as well as the magnitude of IgE levels in the plasma was similar among the three groups of women (resistant women: median 447, range 4.8-5040 ng/ml; susceptible women: median 216, range 4.8-7091 ng/ml; low risk women: median 167, range 4.8-5673 ng/ml; Mann-Whitney p=ns in all comparisons). These data suggested that HIV-1 resistant women do not exhibit a generalized defect in IgE isotype synthesis.

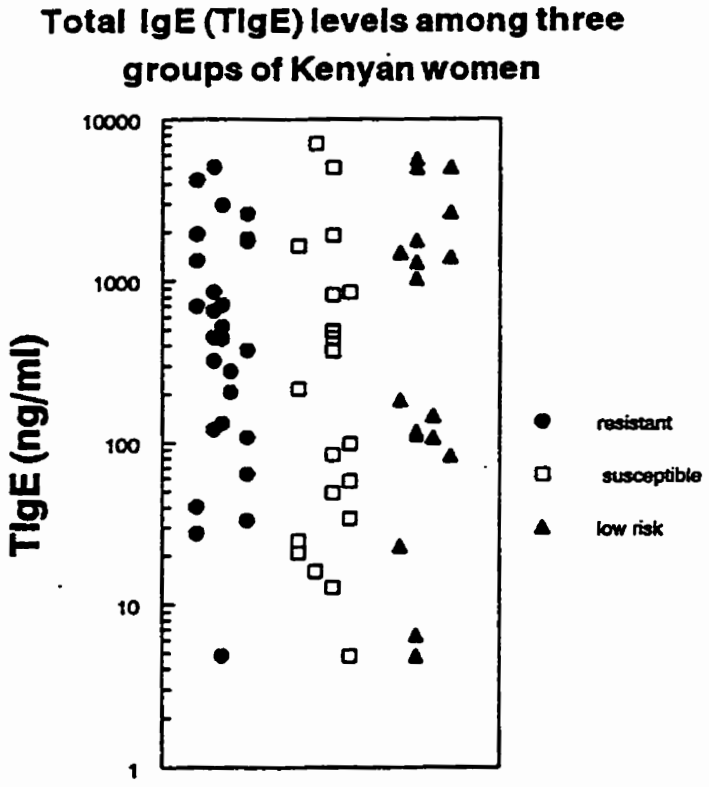

Figure 53: HIV-1 Resistant women do not exhibit a generalized defect in IgE isotype production. Plasma samples from resistant women (●, n=27), susceptible women (□, n=21) and low risk women (▲, n=18) were assayed for Total plasma IgE levels by ELISA as described. Each symbol represents one subject. Horizontal bars represent median values. Statistical significance was determined by Mann-Whitney U test (resistant women versus susceptible women, p= ns; susceptible women versus low risk women, p=ns; resistant women versus low risk women, p=ns).

DISCUSSION

1. CHARACTERIZATION OF TYPE-1/TYPE-2 CYTOKINE RESPONSES OF HIV-1 RESISTANT KENYAN WOMEN

The potential role of type-1/type-2 cytokine response in resistance versus susceptibility to HIV-1 infection was examined in the present study. Initially, it was hypothesised that differential virus specific type-1/type-2 cytokine responses were associated with clinical resistance versus susceptibility to HIV-1 infection among Kenyan women. HIV-1 specific cytokine responses in HIV-1 resistant and HIV-1 positive CDC stage 1 Kenyan sex workers were examined following short-term culture with the virus. All subjects (resistant and susceptible women) exhibited HIV-1 stimulated responses following primary culture with inactivated HIV-1_{IIIB}. Detailed kinetic studies indicated that peak cytokine synthesis occurred on the sixth day of culture in both groups. The nature of the HIV-1 specific cytokine response differed markedly in the resistant and susceptible populations. The resistant group exhibited significantly higher IFN-γ production than did the susceptible group in response to the virus-mediated stimulation (Mann-Whitney p=0.004). IL-2 was not examined as it is an unreliable indicator of type-1 cytokine synthesis (O'Garra, 1998; Abbas et al, 1996). In marked contrast, median HIV-1_{IIIB} driven IL-4 production was >20-fold higher in susceptible women compared to the resistant population (Mann-Whitney p=0.005). Healthy, low risk presumably unexposed women (n=11) did not exhibit detectable cytokine responses following culture under the same conditions. When the data are examined as the ratio of virus driven IL-4:IFN-γ production, a widely used strategy for evaluation of type-1 versus type-2 dominance of immune responses (Romagnani, 1997; Abbas et al, 1996),

resistant subjects exhibited patterns commonly termed "type-1 dominance" while susceptible women displayed "type-2 dominance" (Mann-Whitney $p<0.0002$).

A novel aspect of this study was the analysis of recall cytokine responses to whole inactivated HIV-1_{IIIB}. While the hypothesis that HIV-1 disease progression is associated with a TH1 to TH2 switch has been widely investigated, studies have primarily been conducted using polyclonal or HIV-1 peptides rather than whole virus. Although, use of HIV-1 peptides is a better approach than using polyclonal activators, HIV-1 infected subjects may not show detectable responses because of MHC restriction, as has been demonstrated recently using gp160. In one study, only 34% of patients examined exhibited proliferative responses to gp16, suggesting that MHC restriction may interfere with the analysis of immune responses using proteins/peptides as opposed to the use of the whole pathogen itself (Kim et al, 1997).

Most studies of human type-1 or type-2 dominance to HIV-1 used cytokines (i.e. IL-2, IL-10) are poor type-1/type-2 indicators (Clerici et al, 1997, 1996 and 1994). Given that most immune responses and individual T cells likely exhibit a much more complex spectrum of cytokine responses than the bimodal pattern initially proposed (Kelso, 1995; Mossman, 1996; Allen and Maizels, 1997), and given that several investigators have utilized cytokines such as IL-10 which is a poor representative of type-2 activity in humans (Clerici et al, 1997, 1996 and 1994; Daftarian et al, 1997), HIV-1 driven response of several other "type-2 associated" cytokines was evaluated. Notwithstanding the 20-fold lower median of IL-4 synthesis seen upon HIV-1 mediated activation in resistant women, virus stimulated IL-13 production was intense and equivalent in both groups. HIV-1 driven IL-5 responses were marginally higher in the resistant population exhibiting a median of 71 pg/ml response among resistant versus an undetectable (<3.9 pg/ml) responses in seropositive subjects

($p=0.046$). Also, IL-5:IFN-γ and IL-13:IFN-γ ratios were comparable in resistant and infected groups ($p>0.05$,), arguing against a broad type-2 association with resistance or susceptibility. IL-10 production not associated with either human TH1 or TH2-like T cell clones *in vitro* nor type-1 or type-2 responses *in vivo* (Del Prete et al, 1993; Abbas et al, 1996), was produced to a similar extent in both resistant and susceptible populations ($p=0.34$). Similarly, HIV-1 driven IL-10:IFN-γ ratio was also comparable between the two groups.

Use of polyclonal activators as surrogate Ags elicit intense, hence readily quantified, responses. However, it frequently activates different intracellular signalling events and elicits expression of different patterns of cytokine synthesis from those seen following MHC-dependant T cell activation (Cantrell, 1996). Previous comparisons seeking to identify associations between differential cytokine responses and disease progression have usually relied on a variety of polyclonal activators or, in some cases, HIV-1 peptides (≤20 amino acids) to evaluate the net HIV-1 specific immune response (Clerici et al, 1997 and 1996; Alonso et al, 1997; Meyaard et al, 1996). Thus, for comparison with previous studies, cultures were established using PHA. Cytokine responses were consistently evident in both resistant and seropositive subjects that were typically 5-50 fold more intense than those seen for that individual's recall response to HIV-1. However, whereas PHA stimulated type-1 (IFN-γ) synthesis was marginally higher among resistant women ($p=0.047$), none of the type -2 (IL-4, IL-13, IL-5 or IL-10) cytokine synthesis differed between the two groups. On the otherhand, PHA stimulated IL-4:IFN-γ ($p=0.006$) but not other type-2:type-1 ratios, and was significantly different between resistant and susceptible women. Comparisons of HIV-1 versus PHA driven cytokine responses clearly indicates that only HIV-1, but not PHA,

consistently distinguishes resistant women from susceptible women. Because, whether individual cytokines (IL-4 or IFN-γ) were examined or their ratio (IL-4:IFN-γ), only HIV-1 driven responses identified significant differences between the two groups. On the otherhand, PHA driven cytokine response was either not different (IL-4) or marginally different (IFN-γ) when individual cytokines were examined, or was significantly different when IL-4:IFN-γ ratio was compared. This suggests an inability of PHA to consistently distinguish between the two groups. This again underlines the importance of using conventional Ags for analysis of human cytokine responses.

While quantitative comparison of one cytokine with another is difficult, it appears that a key differentiator of resistant and stage 1 infected subjects is the nature and the intensity of the cytokine response elicited upon HIV-1 exposure. Specifically, clinical resistance and susceptibility to HIV-1 infection appear to be differentiated by virus-driven reciprocal differences in the intensity of IFN-γ and IL-4 cytokine production. The data also argue that resistance to HIV-1 is independent of IL-10 or IL-13 responses in these women. Furthermore, HIV-1 driven IL-5 responses were higher among resistant women. Thus, resistance versus susceptibility to HIV-1 infection in these subjects is not readily interpretable in terms of generalized TH1 or TH2-skewing of the HIV-1-specific response.

This study demonstrates, for the first time, an association of reciprocal synthesis of virus driven IFN-γ and IL-4 cytokines with resistance versus susceptibility to HIV-1 infection. This pattern of reciprocal synthesis of IFN-γ and IL-4 is reminiscent of the cytokine profile observed in human atopy. In fact, reciprocal synthesis of IFN-γ and IL-4 underlies clinical tolerance (resistance?) or susceptibility to atopic diseases. Thus, whereas

allergic individuals exhibit IL-4 dominated response to allergens, clinically tolerant non-allergic subjects exhibit an IFN-γ dominated response to the same allergen (Romagnani, 1997; Imada et al, 1995). However, such reciprocal pathogen driven synthesis of IFN-γ and IL-4 in relation to resistance versus susceptibility to infectious agents, has not been previously demonstrated in humans.

Findings from this study are consistent with animal models of resistance versus susceptibility to disease. The relationship of reciprocal synthesis of IFN-γ and IL-4 to disease susceptibility or resistance has been more clearly elucidated in animal models of infectious diseases, atopy and autoimmune diseases. Thus, resistance or susceptibility to *L. major* in the mouse model is associated with reciprocal production of IFN-γ and IL-4, although later studies using IL-4 knockout mice revealed that reduced IFN-γ, rather than enhanced IL-4, *per se*, determined the susceptibility in this model (Murphy, 1998). In murine models of candidiasis (Romani et al, 1994), trypanosomiasis (Nickell et al, 1993; Perito et al, 1992), influenza (Graham et al, 1994), chlamydia (Rank et al, 1992), and Lyme disease (Pride et al, 1998), it has been shown that TH1 responses/cells confer protection from infection. Also, extensive animal model studies over the past five years provide evidence for reciprocal IFN-γ and IL-4 responses with allergic immune responses (Romagnani, 1997, HayGlass, 1995). Furthermore, a number of studies reveal reciprocal synthesis of IFN-γ and IL-4 in relation to resistance or susceptibility to insulin-dependent diabetes mellitus in humans as well as in animal models (Solimena et al, 1996; Wilson et alm 1998). Thus, in essence, some human diseases and several animal studies provide clear evidence for association of reciprocal IFN-

γ and IL-4 synthesis with resistance versus susceptibility to infectious, autoimmune and, atopic diseases.

Since the resistant women exhibited an IFN-γ dominated cytokine response to HIV-1, the question whether they have a general tendency of enhanced Ag driven IFN-γ (type-1 like) cytokine production was addressed. To test this possibility, IFN-γ response of HIV-1 resistant women was compared with a group of HIV-1 negative low risk women. This comparison was based on the following rationale: both groups are HIV-1 negative (sero-negative, PCR negative); however, resistant women are sex workers who are intensely exposed to HIV-1 whereas the low risk women are not sex workers and hence are presumably at lower risk for exposure to HIV-1. Furthermore, they did not elicit recall cytokine response to HIV-1 *in vitro* in the present study.

IFN-γ cytokine responses were compared using a panel of common recall Ags (SK and PPD) and mitogens (PHA, anti-CD3 mAb). However, both Ag driven as well as mitogen driven IFN-γ synthesis was consistently similar between the two groups (Mann-Whitney $p > 0.05$ for all comparisons). These data argue that HIV-1 resistant women do not exhibit a general tendency to synthesize elevated levels of IFN-γ cytokine compared to low risk women following Ag or mitogen mediated activation. This also suggests that enhanced virus driven IFN-γ response of resistant women is likely an HIV-1 restricted phenomenon.

Subsequently, the hypothesis that HIV-1 resistant women may have a general tendancy to synthesize reduced Ag driven IL-4 was examined. Thus, recall Ag (SK and PPD) driven type-2 cytokine synthesis was compared between resistant and low risk women. Remarkably, IL-4 production to both Ags examined revealed a significantly lower

production in resistant women when compared to low risk women (Mann-Whitney p=0.01 for SK and p=0.001 for PPD). Furthermore, IL-13 production was either marginally or significantly lower among resistant women for SK (p=0.042) and PPD (p=0.02), respectively. IL-5 production to PPD was significantly lower in resistant women compared to low risk women (p=0.03), but not to SK (p>0.05). These data also raise the possibility of, but do not prove, a generalized tendency of reduced Ag driven IL-4 responses among the resistant women because, in this study, only two environmental Ags were examined. In order to test a general tendency, it may be required to examine a large panel of Ags derived from different sources because there may be differences in cytokine responses with other types of Ags.

The PBMC from resistant women, when stimulated with PHA, exhibited significantly lower frequency as well as intensity of IL-4 responses than the low risk women (Mann-Whitney p=0.006). In addition, resistant women also exhibited lower levels of IL-13 compared t low risk women (Mann-Whitney p<0.02). In contrast, both groups secreted comparable levels of IL-5 and IL-10 to PHA stimulation (Mann-Whitney p>0.05). As opposed to PHA, anti-CD3 mAb driven cytokine responses (all cytokines examined including IL-4) were similar between the two groups. Therefore, it is highly unlikely that resistant women are totally deficient in IL-4 protein synthesis. Comparing recall Ag versus mitogen driven cytokine responses together suggests that reduced Ag driven IL-4 synthesis among resistant women is not due to an intrinsic inability to synthesize IL-4 protein, since resistant women synthesised similar levels of IL-4 protein compared to low risk women on anti-CD3 mAb activation.

There are several reports examining recall Ag (influenza, PPD) driven IL-2 responses comparing HIV-1 positive with HIV-1 unexposed control subjects (Clerici et al, 1989 and 1993; Schulick et al, 1993; Golleti et al, 1998). However, I am unaware of studies examining type-1/type-2 cytokine response to recall Ags (SK and PPD Ag) among resistant compared to low risk subjects.

The data from the present study is consistent with a model that prostitutes exhibiting reduced HIV-1 driven IL-4 responses may be resistant to HIV-1 infection. It is not clear at this time whether clinical resistance to HIV-1 is the result or the cause of reduced IL-4 responses observed in this study. The possibility that a transient HIV-1 infection of the resistant women downregulated their IL-4 responses remains to be clarified. However, there is no evidence that HIV-1 infection can downregulate IL-4 responses; on the contrary, HIV-1 positive individuals in other studies and positive women in this study exhibit enhanced IL-4 responses. Since it has been demonstrated that HIV-1 replicates poorly *in vitro* in TH1 relative to TH2/TH0-like clones (Maggi et al, 1994; Vyakaranam et al, 1995), it seems reasonable to speculate that the unavailability of a sufficient number of TH2/TH0-like cells for HIV-1 to replicate in resistant women might have contributed to the clearance of HIV-1 by the default TH1 dominated responses and CTL generation. Furthermore, it was recently shown that IL-4 induced expression of functional CXCR4 (a coreceptor for T tropic strains of HIV-1) on CD4+ T cells, making them vulnerable to HIV-1 infection (Jourdan et al, 1998). In light of these recent data, one can speculate that the unavailability of IL-4 in resistant women for HIV-1 mediated activation may interfere with infection of cells with T tropic HIV-1. Collectively, these observations raise the possibility that resistant women, who exhibit markedly lower HIV-1-driven IL-4 synthesis *in vitro*, may be characterized by lower

levels of infectious HIV-1 production at initial exposure combined with enhanced clearance of HIV-1 that is attributable, in part, to intense IFN-γ dominated responses and CTL generation.

Following are the limitations of the present study: this study was conducted using an inactivated virus with the belief that it would simulate the *in vivo* immune response to an intact pathogen. However, *in vivo*, the immune response would be to an infectious virus rather than an inactivated virus. Use of clade B virus for analysis of recall cytokine responses is another limitation of this study because the use of currently prevalent Kenyan virus clade would have been ideal to study recall responses. Currently, the dominant Kenyan isolates belong to clades A, C, and D (Rowland Jones et al, 1997; Zhu et al, 1998). However, recent studies on an ancestor virus named ZR59, isolated from Africa (Congo), shows that clades B and D may have evolved from a single ancestral virus introduced into the African population around 1959 (Zhu et al, 1998). There is also preliminary data from our laboratory suggesting the presence of clade B virus in Kenya (Murray et al, 1998). Nevertheless, in the present study, virus-specific activation was carried out *in vitro* using a clade B virus to readily allow others to replicate our work in other populations. Thus the finding that HIV-1_{IIIB} readily evokes cytokine responses among PBMC from women with presumably minimal exposure to clade B viruses is consistent with the epidemiologic finding that these women, who have occupational exposure to a broad variety of HIV-1 variants within and between clades, continue to display resistance despite ongoing HIV-1 exposure. Moreover, from previous studies in these resistant women, it has been observed that they have a high frequency of CTL to HIV-1 peptide epitopes that are highly conserved between HIV-1 viral clades (Rowland-Jones et al, 1997). Thus, resistance to HIV-1 in these women appears to be

broadly cross protective. In conclusion, this data not only illustrates a novel mechanism associated with natural immunity to HIV-1, but also may have implications in the prevention and treatment of HIV-1 infection and AIDS. This data provide evidence that, for the design of any succesful vaccine or therapy against HIV-1, efforts should be made to reduce IL-4 production at the site of the immune response.

2. CHARACTERIZATION OF IL-12 AND IP-10 RESPONSIVENESS OF HIV-1 RESISTANT KENYAN WOMEN

That resistance or susceptibility to HIV-1 infection among Kenyan women examined was strongly associated with differential virus driven IFN-γ synthesis *in vitro* raised the possibility that these women may also exhibit differential responsiveness to agents that are known to promote IFN-γ dominated type-1-like response in humans. Thus, the responsiveness of these women to IL-12 (a prototypic IFN-γ promoting cytokine) and IP-10 (an alpha chemokine which was recently shown to promote dominance of human IFN-γ response *in vitro*) were examined (Trinchieri et al, 1996; Gangur et al, 1998).

Resistant women exhibited a significantly enhanced constitutive IL-12 responsiveness compared to susceptible women ($p=0.01$). More significantly, resistant women exhibited significantly higher IL-12 responsiveness compared to susceptible women on HIV-1 mediated activation ($p=0.001$). Analysis of constitutive IP-10 responsiveness in the two groups showed that they were similar. However, resistant women exhibited significantly enhanced IP-10 responsiveness ($p<0.05$) on virus mediated activation relative to the susceptible women. These findings together suggest that: 1) differential virus driven IFN-γ synthesis observed among resistant women and susceptible women *in vitro* may be

secondary to differential IL-12 and IP-10 responsiveness on virus mediated activation of PBMC from these women; 2) ongoing HIV-1 replication may have led to reduced *in vitro* IL-12 and IP-10 responsiveness as evident in susceptible women; and 3) despite continuous chronic exposures to HIV-1, resistant women have retained a strong IL-12 and IP-10 responsiveness *in vitro.*

Exogenous rIL-12 (as little as 25 pg/ml) or IP-10 (0.1 ng/ml), when added to the virus stimulated cultures, could enhance IFN-γ response among susceptible women to similar levels found among resistant women with virus alone. However, it is not clear at this time whether the IL-12/IP-10 enhanced IFN-γ response observed in susceptible women is T cell derived, because IL-12 and IP-10 can also act on NK cells to induce IFN-γ synthesis (Trinchieri et al, 1996; Maghazachi et al, 1997). Nevertheless, this data suggest that rhIL-12 and rhIP-10 may represent potentially suitable therapeutic agents for promoting IFN-γ responses among HIV-1 infected subjects.

I am not aware of studies examining IL-12 or IP-10 responsiveness in relation to resistance versus susceptibility to HIV-1 infection. There are, however, previous reports examining the potential of rIL-12 to reconstitute *in vitro* proliferative and/or cytokine responses of HIV-1 positive subjects. Thus, Clerici et al (1993) reported that proliferative IL-2 responses to HIV-1 peptides and influenza Ag was restored *in vitro* by the addition of rIL-12 to cultures. Others found that rIL-12 enhanced proliferative and IL-2 response to HIV-1 peptides, recall Ag (tetanus) and PHA (Seder et al, 1995). However, the effect on IFN-γ production following Ag mediated activation was not reported in these studies. Results from the present study demonstrate that the addition of rIL-12 to cultures of HIV-1 positive subjects restored IFN-γ responses, a novel finding. However, notably there are the following

differences between this and the previous two studies: 1) inactivated whole virus was used here as opposed to env peptides; 2) restoration of IFN-γ instead of IL-2 response was examined here using rhIL-12; and 3) most importantly, restoration of IFN-γ responses among positive subjects was compared with those of HIV-1 resistant women in this study whereas the previous studies compared IFN-γ response among positive subjects before and after addition of rIL-12 in cultures. In addition, many studies report restoration of proliferative responses of HIV-1 positive subjects to HIV-1 proteins or several recall Ags (SK, tetanus, candida) by IL-12 (Uherova et al, 1996; Blauvelt et al, 1996; De Fransesco et al, 1996; Landay et al, 1996).

IL-12 responsiveness has not been previously examined in relation to resistance versus susceptibility to HIV-1 or other infectious diseases in humans. However, there are some recent studies examining IL-12 responsiveness in human allergic disorders. Van der Pouw Krann et al (1997) reported that where non-atopic control subjects exhibit significantly enhanced IFN-γ response to IL-12, subjects with allergic asthma exhibited significantly reduced IL-12 responsiveness. Furthermore, HayGlass et al (1997) reported that constitutive, but not Ag driven, IL-12 responsiveness was significantly higher among clinically tolerant non-allergic subjects compared to allergic rhinitis subjects. In principle, results from the present study (i.e., HIV-1 resistant women exhibit enhanced type-1 [IFN-γ] response as well as IL-12 responsiveness on virus mediated activation) are consistent with a previous report that non-allergic subjects exhibit elevated type-1 response and IL-12 responsiveness on allergen mediated activation (Van der Pouw Krann et al, 1997).

In contrast to humans, a series of reports implicate IL-12 responsiveness in resistance to *Leishmania major* infection in mice. Evidence for the association of genetic susceptibility

to *L. major* infection with a defect in IL-12 responsiveness was reported in a mouse model (Guller et al, 1996). Thus, the susceptible Balb/c mouse exhibited a loss of IL-12 responsiveness (as assessed by IFN-γ synthesis) on anti-CD3 mAb mediated activation. They concluded from this study that the susceptibility of Balb/c mice to infection with *L. major* may derive from the loss of ability to generate IL-12 induced TH1 responses (Murphy, 1998). Furthermore, a genetic locus controlling this phenotype has been mapped to mouse chromosome 11 (Gorham et al, 1996). In essence, differential IL-12 responsiveness seems to underlie resistance versus susceptibility to *L. major* infection in mice. Thus, findings from the present study that resistance versus susceptibility to HIV-1 infection is associated with differential IL-12 responsiveness is consistent with these previous reports on *L. major* infection in mice.

Whereas IL-12 responsiveness has been examined in much detail in mice and to a lesser extent in humans (as discussed above), differential IP-10 responsiveness has not been reported in humans or mice in relation to diseases. Thus, to my knowledge, the present study represents the first examination of IP-10 responsiveness in relation to HIV-1/AIDS.

Since HIV-1 resistant women exhibited enhanced IL-12/IP-10 responsiveness compared to susceptible women, the hypothesis that resistant women may have a general tendency to exhibit enhanced IL-12/IP-10 responsiveness on Ag mediated activation was tested. For this, IL-12/IP-10 responsiveness of HIV-1 resistant women was compared with that of low risk women. However, results from these experiments provided evidence that resistant women do not differ from low risk women in either constitutive IL-12/IP-10 responsiveness or on Ag (SK or PPD) mediated activation. As described in previous sections, these results are consistent with the findings that resistant women do not differ from low risk

Kenyan women in Ag (SK or PPD) driven IFN-γ responses, although they do exhibit enhanced IFN-γ response to HIV-1 compared to susceptible women.

In summary, these data argue that: 1) resistance versus susceptibility to HIV-1 infection among Kenyan women is associated with differential IL-12 and IP-10 responsiveness *in vitro*; 2) enhanced IL-12/IP-10 responsiveness do not seem to represent a special feature of resistant women because they do not differ from low risk women in this phenotype on SK or PPD Ag mediated activation; and 3) rhIL-12 and rhIP-10 may represent potential therapeutic agents to promote IFN-γ responses among HIV-1 infected subjects.

3. ANALYSIS OF ANTIGEN SPECIFIC IGE AB AND TOTAL PLASMA IGE LEVELS OF HIV-1 RESISTANT KENYAN WOMEN

Among the imunoglobulins (Ig) present in the plasma, IgE is the most minor component (~0.003% of all Ig) (Janeway C 1996). However, IgE mediated immune defense is vital for protection against helminth parasites, which are endemic in Africa. It is now well established that IgE synthesis is reciprocally regulated by IFN-γ (negative regulator) and IL-4/IL-13 (positive regulators) cytokines (Romagnani, 1997; Bacharier et al, 1998). In particular, IL-4 was the original class switch factor discovered for IgE isotype synthesis via induction of germ line epsilon transcripts (Delphin et al, 1995), although later studies have identified a similar function for IL-13 (Bacharier et al, 1998; Punnonen et al, 1997). The previous analysis of cytokine responses to HIV-1 among Kenyan women provided evidence for association of resistance versus susceptibility with reciprocal synthesis of IFN-γ and IL-4 cytokines (Figures 13 and14). Therefore, it was of obvious interest to characterize IgE profile of these women, especially their systemic levels of HIV-1 specific IgE Abs.

The hypothesis that differential production of virus specific IgE Ab may be associated with resistance versus susceptibility to HIV-1 infection was addressed in this component of the study. As evident from the analysis of HIV-1-specific IgE Ab levels in the plasma, most of the resistant women (24/27 [89%] examined) did not exhibit HIV-1 specific IgE Ab. Absence of HIV-1 specific IgE Ab among the low risk women was expected because these subjects are at low risk for acquiring HIV-1 infection. In contrast, HIV-1 resistant women, who are continuously exposed to HIV-1 due to their profession and high risk behaviour, exhibited markedly reduced frequency of HIV-1 specific IgE Ab in the plasma relative to susceptible women. The absence of HIV-1 specific IgE Ab in both resistant as well low risk women is unlikely related to their not being infected with HIV-1, but rather because resistant women exhibit HIV-1 specific CTLs which implies past HIV-1 replication (Fowke et al, 1996 and 1994). Therefore, an alternative possibility is that marked deficiency of HIV-1 specific plasma IgE Ab may be associated with resistance to HIV-1 infection. This is further supported by the presence of high frequency as well as intensity of HIV-1-specific IgE Ab detectable in the susceptible women (who are also sex workers). These data provide evidence that resistance, but not susceptibility, to HIV-1 infection is associated with marked deficiency of HIV-1-specific plasma IgE Ab despite continuous exposure and evidence of past HIV-1 replication. This data is also consistent with the HIV-1 specific IL-4 and IFN-γ synthesis *in vitro* which has been described in the previous chapter.

Since resistant women did not exhibit HIV-1 specific IgE Ab in the plasma, the hypothesis that they may have a generalized defect in class switching to IgE isotype was addressed. This hypothesis was tested by examining plasma IgE Ab levels to common environmental allergens. Mite and cat allergens were examined because these are ubiquitous

and well characterized environmental Ags (allergens) which induce IgE Ab synthesis *in vivo*, bind specifically to IgE, and frequently precipitate allergic diseases. They represent the two most common allergies worldwide. The results revealed that resistant women exhibited similar levels of allergen specific IgE Ab to that of low risk women. Consistent with specific IgE Ab levels, total plasma IgE levels were also found to be highly similar between resistant and low risk women. Together, these data suggested that resistant women do not exhibit a generalized defect in class switching to IgE isotype and, despite chronic ongoing exposure to HIV-1, seem to have intact T cell help required for IgE class switching.

Recent studies have shown that: 1) in addition to IL-4, IL-13 can help as a switch factor for IgE synthesis (Bacharier et al, 1998); 2i) it was recently demonstrated using IL-4 -/- mice in the absence of IL-4 and IL-13 can help in IgE synthesis (Bancroft et al, 1998; Urban Jr. et al, 1998); and 3) more importantly, using IL-4-/- and IL-13 -/- mice, a critical role for IL-13 in resistance to intestinal nematode infection (which was independent of IL-4) was demonstrated (Bancroft et al, 1998; Urban Jr. et al, 1998). These studies are consistent with the present data that HIV-1 resistant women, although they exhibit significantly reduced recall Ag (SK and PPD) driven IL-4, their ability to produce IgE Ab to allergens or their total plasma IgE is not compromised. Therefore, it remains to be examined whether HIV-1 resistant women exhibit IL-4/IL-13 responses to environmental allergens such as dust mite and cat allergens.

I am unaware of any previous study examining HIV-1-specific IgE Ab responses in the context of resistance versus susceptibility to HIV-1 infection. However, there is a deluge of information on IgE levels in relation to disease progression and AIDS. In these studies, total serum IgE as well as specific IgE Ab to environmental Ags (air borne and food

allergens) was compared between HIV-1 positive and negative control groups. In most cases, serum IgE levels are increased in HIV-1 positive subjects as they progress towards AIDS (Dolececk et al, 1995; Koutsonikolis et al, 1996; Lucey et al, 1990; Shor-Posner et al, 1995; Vigano et al, 1995). Others did not find such changes in serum IgE levels (Wright et al, 1990). However, for specific IgE Ab, there is no consensus. Most studies found no increase in specific IgE Ab (Wright, 1990; Goetz et al, 1997; Vigano, 1995); others found selective increase in IgE Ab to fungal but not other Ags examined (Maggi et al, 1989). In particular, studies examining HDM and cat Ag specific IgE Ab find reduced levels of specific IgE Ab to these allergens in their subjects as they progressed to AIDS (Goetz et al, 1997). This is consistent with the present study where reduced mite and cat specific IgE Ab were detected in HIV-1 infected subjects relative to low risk women. It should be noted that the above studies did not examine HIV-1-specific IgE Abs. There are very few studies which examined HIV-1-specific IgE Ab in HIV-1 infected subjects: Khalife et al (1988) found the presence of *gag*-specific IgE Ab in hemophiliacs but not in homosexuals, and suggested that this may be related to the route of exposure to HIV-1; recently, Miguez-Burbano et al (1997) found HIV-1 specific IgE Ab in newborn babies and suggested that IgE based assays may be useful for early detection of HIV-1 infection in infants since IgE does not cross the placenta; and Secord et al (1996) reported the presence of HIV-1-specific IgE Ab in a subgroup of HIV-1 positive children who were clinically healthy, and suggested that IgE may play a protective role against disease progression. In contrast to these studies which examined HIV-1 specific IgE Ab in HIV-1 infected subjects, I am unaware of reports examining HIV-1 specific IgE Ab in resistant subjects.

In summary: 1) HIV-1 resistant women exhibit marked deficiency of HIV-1 specific plasma IgE Ab despite ongoing exposure to HIV-1 and evidence of past HIV-1 replication; 2) HIV-1 resistant women do not exhibit a generalized deficiency in IgE class switching or plasma levels of IgE; 3) the inability of resistant women to produce HIV-1 specific IgE Ab seems to be limited to HIV-1 because they made significant levels of IgE Ab to other Ags, such as dust mite and cat allergens; 4) the ability of resistant women to make similar levels of IgE Ab to environmental allergens suggests that the T cell is required for IgE Ab synthesis and is functionally intact, despite chronic ongoing exposure to HIV-1; 5) HIV-1 susceptible women exhibited reduced allergen (HDM and cat) specific IgE Ab suggesting the impaired functional status of their T cell system, perhaps due to ongoing HIV-1 infection; and 6) this data, together with recent findings that resistant women exhibit HIV-1 specific IgA Ab in the mucosa (Kaul R et al 1998), suggest that the B cell system of resistant women do not have an intrinsic defect in Ig isotype class switching, despite chronic ongoing exposures to HIV-1.

4. SUMMARY

The present study was conducted to examine the type-1/type-2 cytokine response as a potential immune mechanism associated with clinical resistance to HIV-1 infection among Kenyan women. Use of inactivated whole virus to examine recall cytokine responses represents a novel aspect of the approach used for this investigation. Major findings from this study are as follows. Clinical resistance versus susceptibility to HIV-1 infection among Kenyan women is associated with: (a) reciprocal *in vitro* synthesis of virus driven IFN-γ and IL-4 cytokines; (b) marginally enhanced virus driven IL-5 (among resistants) but similar IL-10 and IL-13 responses; (c) selective imbalance in virus driven IL-4:IFN-γ but not in other

type-2:type-1 cytokine ratios; (d) differential IL-12 and IP-10 responsiveness on virus mediated activation; and (e) differential virus specific IgE Ab levels in the plasma.

HIV-1 resistant women do not differ from low risk Kenyan women in : (a) recall Ag (SK and PPD) driven IFN-γ responses; (b) IL-12 and IP-10 responsiveness on Ag (SK and PPD) mediated activation; and (c) plasma levels of allergen (cat and HDM) specific IgE Ab or total IgE. However, HIV-1 resistant women exhibit markedly reduced recall Ag (SK and PPD) driven IL-4 responses.

The present study has generated the following hypotheses which remain to be tested: 1) HIV-1 resistant women may have a generalized defect in Ag driven IL-4 responses; and 2) this defect may underlie protective immune response to HIV-1 infection among resistant Kenyan women.

PART-II. CHARACTERIZATION OF NEONATAL TYPE-1/TYPE-2 CYTOKINE RESPONSES

INTRODUCTION

1. OVERVIEW

The study on the neonatal immune system is of vital importance because, during this period, a) individuals acquire the ability to distinguish self from non-self (Schwartz, 1989); b) tolerance to self Ag occurs in the peripheral immune system to prevent potential autoimmune diseases (Schwartz, 1989); and c) the development of memory cells required to fight against infectious diseases develop (Hayward et al, 1989; Lewis et al, 1991). Studies on the neonatal immune system also provide information to define the normal pathways of expansion and differentiation of immune cells, and this information can give insight on the observed enhanced susceptibility of neonates to infectious and allergic diseases (Fallon, 1989; Lewis and Wilson, 1995).

An effective strategy to prevent and control infectious and atopic diseases among children is to induce protective immune responses against various infectious agents and allergens *in utero* or at birth by prophylactic vaccinations. However, lack of a clear understanding on the abilities of the fetal or neonatal immune system, in terms of its maturity to generate protective immune responses, remains a major problem.

Humoral immune responses of neonates has been widely studied and the data suggest that neonates can generate specific Ab responses which are mainly of the IgM class (Lewis and Wilson, 1995). However, the status of cell mediated immunity, especially cytokine

responsiveness, remains to be defined. It is an important area to investigate, especially because of the proposal that cellular immunity at birth may influence the incidence, progression and prognosis of pediatric AIDS (Clerici et al, 1993). Thus, it has now become increasingly important to define the developmental timetable of immune responses in neonates to delineate whether the lack of a given response is a normal ontogeny or if it reflects an underlying disease state.

2. REVIEW OF LITERATURE

2.1 Review of Neonatal T Cells

2.1.1 Neonatal T cell phenotype

The T (thymus) lymphocytes are a class of lymphocytes that originate from the stem cells in bone marrow, but undergo maturation and differentiation in the thymus. These cells are the main mediators of cellular immune responses. These responses are necessary for protection against infectious diseases. The T cells can not recognize the Ag directly, but they recognize Ag derived peptide fragments bound to the MHC molecules on Ag presenting cells (Janeway, 1996).

The T cells express receptors (i.e., TCR) that recognize both MHC and the antigenic peptide. The TCR is comprised of two chains, each with a constant and a variable portion. The TCR is associated with the CD3 molecules on the cell surface. Once the TCR is engaged with the MHC-peptide complex, the signal is transduced into the T cells via the CD3 complex (Janeway, 1996).

The T cells of adults express a number of surface molecules, some of which are present on all T cells (pan T cell marker, eg., CD3), while others characterize

subsets (for example, CD4 for T helper cell and CD8 for cytotoxic T cells) (Janeway, 1996). The development of T cells expressing these and other important surface molecules has been widely studied and is reviewed below.

2.1.1.1 CD3 + T cells: The CD3 molecule is a pan T cell marker. Functionally, CD3 is important because it transduces signal from the TCR into the cytoplasm of the T cell. From the second trimester onwards, the number of T cells in the fetal circulation gradually increases during pregnancy (Hohlfeld et al, 1990). This increase continues for about six months after birth, followed by a gradual decline to adult levels during childhood (Erkeller-Yuksel et al, 1992). The levels of CD3 on neonatal T cells are also reported to be similar to that of adult T cells (Wilson et al, 1985).

2.1.1.2 CD4 and CD8 + T Cells: T cells are phenotypically and functionally classified into two main types - helper and cytotoxic T cells. This is based upon the presence of cell surface markers CD4 and CD8. Thus, CD4+ T cells are called the helper lymphocytes because they secrete cytokines and help the B cells to produce antibodies. They also mediate the DTH response and activate macrophages to kill the intracellular pathogens. CD8+ T cells are known as the cytotoxic T cells. The main function of CD8+ T cells is to kill virus-infected or neoplastic cells (Abbas et al, 1996; Janeway, 1996). The physiological ratio of CD4:CD8+ T cells in adults is ~ 2:1 (Stites 1996).

In humans, by 14 weeks gestation, CD4 and CD8 T cells have been demonstrated in fetal liver and spleen (Asma et al, 1983). The ratio of CD4 to CD8 T cells in the circulation is high during fetal life (about 3.5) and gradually declines with age and reaches the adult ratio of 2 by age 4 years (Campbell et al, 1974). The levels of CD4 and CD8 proteins on neonatal T cells is similar to that of adult T cells (Wilson, 1985).

2.1.1.3 Naive and memory T cells in neonates. The immune system has a remarkable capacity to remember the Ag it had encountered before. This immunological memory helps an individual to respond more quickly and more intensely if the same pathogen is encountered again. Immunological memory is maintained by the memory T cells which are generally characterized by the expression of the surface molecule CD45RO. On the other hand, naive T cells generally express CD45RA (Clement et al, 1988; Pierenne et al, 1992).

The presence of such markers has been studied in neonates. There is a common consensus from different phenotypic studies that circulating neonatal T cells predominantly express a surface phenotype similar to the virgin subset of T cells in adults (CD45RA+CD45RO-CD29lo) (Teder et al, 1985; Gerli et al, 1984; Sanders et al, 1988). However, the CD45RO + cells (memory cells) increase with age after birth, presumably due to cumulative antigenic exposures and T cell activation (De Paoli et al, 1988; Hayway et al, 1989). Unlike adult virgin T cells, virtually all peripheral fetal and neonatal T cells express the CD38 molecule (Wilson, 1985). This molecule is present

on most thymocytes, suggesting that the peripheral T cells in the fetus and neonates may be in the transitional phase of development. It was reported that CD40 ligand, which interacts with CD40 on B cells to provide a co-stimulus essential for Ig isotype switching from IgM to IgG, A or E (Fuleihan et al, 1995), is weakly expressed on activated cord blood T cells compared to adult T cells, and is fully expressed only after *in vitro* priming (Brugnoni et al, 1994; Durnandy et al, 1995; Nonoyama et al, 1995; Fuleihan et al, 1994).

In essence, based on surface marker analysis, whereas there is no major difference in CD3, CD4 or CD8 T cell number between the neonate and the adult peripheral circulation, neonatal T cells have been regarded as naive or virgin cells (Lewis and Wilson, 1995). However, these studies are phenotype-based and therefore do not necessarily provide detailed information on the function of neonatal T cells.

2.1.2 Neonatal T helper cell functions

T helper cell functions have been classically studied by two methods: 1) proliferative responses; and 2) cytokine responses. Whereas the former estimates the ability of memory T cells to respond by proliferation on *in vitro* challenge, the latter elucidates the quality of memory T cell recall response (for example, whether the response is IFN-γ, IL-4, or IL-10 dominated).

2.1.2.1 T cell proliferation studies. The proliferative responses of neonatal T cells have been examined using a number of stimuli: (i) mitogens (like PHA, concavelin A); (ii) alloantigens; (iii) superantigens; and (iv) conventional antigens/allergens (Lewis and Wilson, 1995). Proliferative

responses to these stimuli have been reported to be negligible (Harris et al, 1992), less than (Rosenberg et al, 1983; Granberg et al, 1986; Ayoub et al, 1971), equal to (Clerici et al, 1993l Campbell et al, 1974; Deacock, 1992; Risdon et al, 1995) or more than (Deacock, 1992; Risdon et al, 1994; Campbell et al, 1974; Carr, 1972) the response of adult PBMCs. Thus, there is no consensus on the status of T cell proliferative responses of neonates to the above mentioned stimuli.

In contrast to phenotype-based studies as discussed before, which suggested that neonatal T cells are functionally naive, there are reports on the proliferative responses of cord blood mononuclear cells (CBMC) to Ags derived from microbes such as PPD, *Candidasis* and *Paracoccidoides* (Munk et al, 1995; Shiratsuchi and Tsuyuguchu, 1981) as well as allergens derived from HDM and ovalbumin (Holt et al, 1997). Although these studies suggest the presence of memory (primed) T cells in CBMC, the underlying mechanism(s) of this phenomenon are under intense investigation.

In essence, in contrast to the consensus on phenotype of neonatal T cells, the functional studies based on proliferation of neonatal T cells have remained largely inconclusive.

2.1.2.2 Type-1/type-2 cytokines in neonates. Cytokines are molecular messengers that orchestrate the various cells involved in an immune response such as APC, T and B cells. They are produced by many nucleated cells in the body and act on a variety of target cells. Cytokines play an important role in normal physiological development of an individual, and

modulate inflammation and immune responses (Mosmann et al, 1996; Romagnani, 1997; Abbas et al, 1996). Based on the pattern of cytokine production, T helper cells have been classified into two functional subsets: (i) TH1 (produce predominantly IFNγ, TNFβ and IL-2); and (ii) TH2 (produce mainly IL-4, IL-13 and IL-5) (Mosmann et al, 1996). It has been demonstrated that whereas TH1 like cells play an important role in cell mediated immune responses (such as DTH and CTL responses), TH2-like cells are crucial for humoral immune response. Thus, TH1-like cells play an important role in protection against intracellular pathogens and in autoimmune diseases and the TH2-like cells play a role in protection against both intra and extra cellular pathogens and also underlie atopic diseases such as common allergies and asthma (Mosmann et al, 1996; Romagnani, 1997; Abbas et al, 1996). Since non-T cells also contribute to the production of cytokines characteristic of TH1 and TH2 cells, it has recently been proposed to classify these cytokine responses as type-1 and type-2 respectively (Clerici and Shearer, 1996). Details on type-1/type-2 cytokine responses has been reviewed in Part-I of this thesis. In contrast to the widely studied proliferative responses, the type-1/type-2 cytokine responses of neonates have not been extensively examined. Furthermore, studies on cytokine production in neonatal cells have mostly used polyclonal simulators such as PHA, anti-CD3 mAb, phorbolesters and ionomycin, or superantigens which may not necessarily reflect the physiologically relevant Ag-driven cytokine responses (Imada et al, 1995).

IL-2 and IFN-γ cytokines have been extensively examined in neonates. It is widely reported that neonatal T cells synthesize IL-2 in amounts comparable to adults following mitogen stimulation (Lewis and Wilson, 1995; Bodeker et al, 1982) or are reduced following alloantigen stimulation (Clerici et al, 1993). In contrast to IL-2, the production of IFN-γ to polyclonal stimulation (with PHA or PMA-ionomycin) has been reported to be markedly reduced in neonates (Wilson, 1986; Lewis and Wilson, 1995; Miyawaki et al, 1985; Wakasuyi et al, 1985; Lewis et al, 1991). Also, production of IL-4 to mitogen stimulation was reported to be diminished in CBMC (Lewis et al, 1991; Lewis and Wilson, 1995). Examination of mRNA for IL-5 revealed marked reduction in the number of cells expressing IL-5 in CBMC (Lewis and Wilson, 1995). There is a recent report examining IL-4 and IL-10 cytokine gene expression at the mRNA level in neonates using conventional antignes (PPD, HDM and ovalbumin). They also reported the synthesis of IL-10 by CBMC on Ag mediated activation (Holt et al, 1997). However, this study did not compare neonates with adults.

There are some limitations that should be borne in mind when interpreting the above studies. Most of these studies have examined cytokine responses primarily following polyclonal activation (Lewis and Wilson, 1995). However, there is compelling evidence that polyclonal and Ag mediated activation elicit different intracellular signalling pathways (Cantrell, 1997) and consequently distinct patterns of cytokine responses (Imada et al, 1995; Gangur et al, 1998). For instance, where PHA mediated stimulation of

human PBMC elicits a type-2 dominated cytokine synthesis, anti-CD3 mAb or superantigen (toxic shock syndrome toxin-1) mediated activation elicits a type-1 dominated cytokine response (Gangur et al, 1998). Also, many of the studies measured message for the cytokine rather than the proteins, and it is arguable that the presence of messenger RNA for a cytokine may not necessarily correlate with protein production (Bamford et al, 1996). Thus, the overall T cell function of neonates, in terms of specific cytokine responses, remains to be clearly defined.

2.2 Review on Neonatal Antigen Presenting Cells (APC)

APCs play a key role in immune responses. Thus, on encounter with a pathogen, these cells initially phagocytose the pathogen and processes it into small peptides of 8-15 amino acids long. This is called Ag processing. The processed peptide is then displayed at the cell surface in association with the MHC molecules for recognition by the T cells, which is referred to as Ag presentation. In addition to Ag-MHC class II bimolecular complex, at least one additional co-stimulatory signal (cell surface molecule or soluble factor like cytokines) delivered by the APC is essential for driving the T helper cell response (Janeway, 1996).

The APC population include mononuclear phagocytes (macrophages and monocytes), (DC), B cells and Langerhans cells. All these major professional APC have been reported to be present in the fetus around 12 weeks of gestation (Foster et al, 1989; Harvey et al, 1990). The expression of class I and class II MHC molecules by a variety of fetal tissues is also evident by 12 weeks of gestation (Hofman et al, 1984; Oliver et al, 1989).

2.2.1 Costimulatory molecules

B7.1 (CD80) and B7.2 (CD86) are the best defined co-stimulatory molecules expressed on APC, with their ligands CD28 and CTLA-4 acting as the primary positive and negative co-stimulatory receptors on T cells. However, it is now evident that any number of molecules may provide accessory function and that efficient responses are only generated following multiple interactions (Lenschow et al, 1996; Croft et al, 1997). The level and nature of costimulatory molecule expression by neonatal APC is largely unknown.

Hunt et al (1994) isolated low-density, nonadherent, nonphagocytic, HLA-DR+ cells with the morphology of DCs from the cord blood of full-term newborn infants. Flow cytometric studies showed that the density of intercellular adhesion molecule-1 (ICAM-1; CD54) and MHC class I and II Ags on cord blood DC were significantly lower than those in adult blood. However, the levels of B7.1 (CD80) or B7.2 (CD86) expression were not studied.

Production of cytokines has been investigated in some detail with human neonatal monocytes and macrophages. Whereas IL-1, IFNα (Ray et al, 1970; Handzel et al, 1983; Kohl et al, 1983), GM, M, G-CSF (English et al, 1992) production by monocytes and macrophages from neonates appears to be equivalent to that of adults, IL-6 and TNF-α production by cells from neonates appears to be equal to or modestly less than that by monocytes from adults.

Thus, detailed analysis of neonatal APC, especially their efficiency in Ag presentation, their ability to stimulate cytokine synthesis, and the expression of costimulatory molecules, remains to be performed.

3. SCOPE OF THE PRESENT STUDY

Human neonates are relatively more vulnerable to many infections and often exhibit a prolonged illness. A number of hypotheses have been proposed to explain this increased susceptibility including: 1) a lack of pre-existing Ab ; 2) decreased complement levels (Lewis and Wilson, 1995; Baker et al, 1976); 3) deficient phagocytic cell function (Weston et al, 1977); 4) immature T cell function (Lewis and Wilson, 1995; Haemey, 1994; Zola et al, 1995); and 5) decreased numbers of $CD45RO^+$ cells (Pirenne et al, 1992; Clement et al, 1988). It is believed that such deficiencies may lead to defective innate and/or adaptive immunity in neonates, thus making them more susceptible to frequent and severe infections.

The inability of neonates to respond to Ag stimulation *in vitro* has been proposed to be due to the decreased numbers of $CD45RO^+$ Ag-experienced or memory cells (Lewis and Wilson, 1995). However, others argue that T cell hyporesponsiveness in neonates reflects an inherent functional immaturity of T cells, independent of CD45 isoform expression (Pirenne et al, 1992; Clement et al, 1988). Most of these have been attributed to the presence of antigenically naive T cells in the neonate. However, reports do exist that neonates can respond to candida, PPD, environmental allergens and paracoccidoides, arguing for the presence of Ag primed T cells. Furthermore all major APC populations, such as macrophages, dendritic, Langerhan's and B cells, are present in neonates and express MHC class II at levels generally comparable to adults. Thus, the increased clinical susceptibility is clearly demonstrable but the underlying immunological reasons remain unclear.

To date, the dominant experimental strategy used to assess immunologic capability in the human neonate has been polyclonal activation using mitogens or antibodies to cell

surface receptors expressed on T cells (Pirenne et al, 1992; Ayoub et al, 1971; Carr et al, 1972; Wakasugi, 1985; Byrne et al, 1994). Although this approach provides a rapid, technically simple assessment of immune potential, it differs in substantial ways from physiologic immune activation. The implicit assumption underlying these studies is that polyclonal activation elicits intense, easily quantified responses which mirror those elicited in response to Ag. However, as alluded to earlier, there is strong recent evidence that polyclonal and Ag mediated activation of cells can elicit distinct intracellular signalling pathways and consequently different patterns of cytokine responses (Cantrell, 1996; Nishizuka, 1984; Imada et al, 1995; Gangur et al, 1998).

In this study, as an alternative approach to overcome these difficulties, the relative capacity of neonatal and adult cells to respond to alloantigen was analyzed. Alloantigens are useful for examining neonatal immune potential because such responses do not require priming but do require HLA mediated Ag recognition. While differing in important aspects from conventional exogenous Ag-driven cytokine production, they are much more likely to reflect immune capacity than activation with stimuli, such as anti-CD3 or PMA plus calcium ionophore. At the same time, the allo-response, although much more physiologic than PHA, anti-CD3 or PMA plus ionomycin mediated activation, still differs in several respects from conventional exogenous Ag driven T cell activation. Notably, the precursor frequency of responding and cross-reactive T cells is much higher than is found for conventional Ags, and Ag presentation differs from that occurring in normal *in vivo* responses where the MHC between APC and T cell is identical and the exogenous Ag provides the stimulus. However, conventional Ags (such as tetanus and influenza) do not elicit proliferative or cytokine responses in most neonates. Allergens, such as HDM and ovalbumin, can induce cytokine

gene expression (in ~40 % of the cases) but the cytokine levels in general are not measurable (Holt PG et al 1997). In view of these problems, an alloantigen driven *in vitro* culuture system was used in the present study. Since, cord blood is the most easily obtainable, non-invasive blood sample from the neonate, the immune function of CBMC was analyzed in comparison to adult PBMC.

4. HYPOTHESIS AND OBJECTIVES

The present study was undertaken to address whether or not the human neonate exhibits defects in: (i) T cell function (with respect to cytokine synthesis); and/or (ii) APC function (with respect to the ability of APC to stimulate cytokine synthesis). To test these hypotheses, the study was undertaken with the following specific objectives: 1) to compare the T cell function between neonates and adults by proliferation and type-1/type-2 cytokine responses to mitogen (PHA) and alloantigens. This would test whether the neonates have a defect in T cell function (both proliferative as well as type-1/type-2 cytokine responses); 2) to compare the APC function between neonates and adults in MLRs to stimulate proliferation and type-1/type-2 cytokine synthesis. This would test whether the neonates have a defect in APC function with respect to stimulating proliferation and Type-1/Type-2 cytokine synthesis.

MATERIAL AND METHODS

1. SUBJECTS

Cord blood was collected at the time of elective Caesarean sections of healthy mothers delivering at term in the Health Sciences Centre, Winnipeg, Canada. Use of placental products was approved by the University of Manitoba Faculty Committee on the Use of Human Subjects in Research. Following delivery of the placenta, the external surface of the cord was washed to avoid contamination with maternal cells, and blood was then collected by cordocentesis into heparinized tubes. Caesarean section samples were chosen deliberately to minimise the change in the cytokines that occur during the labour. Adult peripheral blood was collected from healthy volunteers into heparinized vacutainer tubes. All samples were processed within one hour of being collected.

2. MONONUCLEAR CELL PREPARATION

The mononuclear cell fraction was prepared using a density gradient technique as described previously by Imada M et al (1995). Briefly, fresh blood was diluted with an equal volume of sterile saline, carefully layered onto Histopaque-1077 (Sigma Chemical Company, St. Louis, MO) and centrifuged for 30 minutes (at 600g) at room temperature. The cells harvested from the interface were washed in serum free saline, then resuspended into 2-3 ml of RPMI with 10% FCS (Gibco BRL, Life Technologies Inc., New York, USA) and the number of viable cells determined using trypan blue exclusion. CBMC staining was also performed with Turk's stain to exclude nucleated erythrocytes from cell counts and to determine the degree of contamination. In the subjects studied, nucleated red blood cells made up 3-11% (median 7%) of the total cell count used for culture. In all experiments,

responder cells were adjusted to a final concentration of $1x10^6$ leukocytes/ml (viability ≥98%) in RPMI-10 medium for culture.

3. T HELPER CELL PROLIERATION ASSAY USING PHA

3.1 Optimization of the Concentration of PHA and Days of Stimulation

To optimize assay conditions for PHA-P (Sigma Chemical Company) mediated activation, assays were performed using both CBMC (n=2) and adult PBMC (n=2). Cells were cultured in triplicate using 96-well flat bottom plates in a final volume of 200 µl/well at a final concentration of 1×10^6 leukocytes/ml. Initially, experiments were performed to determine the optimal concentration of PHA. For this, triplicate cultures were set up with CBMC (n=2) and PBMC (n=2) stimulated with and without PHA at various concentrations (0, 1, 2, 5, 10, 15 and 20 µg/ml). Optimized PHA concentration was used in subsequent experiments to determine the optimal days of stimulation. Thus, following stimulation of cells with PHA for 2, 3, 4 and 6 days, cultures were pulsed with [3H] thymidine for 18 hours prior to harvest and counts were determined.

3.2 T Cell Proliferation Assays Using CD4+ and CD8+ T Cell Enriched CBMC

To determine the phenotype of CBMC proliferating in response to PHA activation, cultures were established with intact CBMC, CBMC enriched for CD4+ T cells, or enriched for CD8+ T cells. CD4 and CD8 T cell enrichment was performed using immunomagnetic beads (Dynal, Oslo, Norway) following the manufacturer's protocol. Briefly, anti-CD4 or anti-CD8 monoclonal Ab coated magnetic beads were washed 4 times with medium, mixed with the appropriate number of cells, and incubated on ice (30 minutes for CD4 enrichment and 60 minutes for optimal CD8 enrichment). After incubation, adherent cells were separated

with the help of a magnet. Median viability was 97% in the three experiments carried out. The leukocyte concentration was adjusted to $1x10^6$/ml and proliferative assays of intact CD4+ and CD8+ T cell enriched CBMC carried out as described above.

4. MIXED LEUKOCYTE REACTION (MLR)

The following parameters were optimized before performing the primary MLR assays. All cultures were set up in triplicates.

4.1 Optimization of Number of Stimulator Cells for Measuring Allo Response in MLR Assays

The number of stimulator cells required to obtain maximal primary MLR responses was determined as follows. A CBMC-pool of four unrelated neonates was treated with mitomycin C (50 μg/ml for 30 minutes at 37°C), washed extensively and counted. Aliquots of the pool were prepared at different concentrations between 1 to 5 x 10^6/ml and used as stimulators. This pool was kept the same for all experiments. Fresh CBMC were used as responders at 1 x 10^6 cells/ml. Cultures were set up in triplicate in 96-well flat bottom culture plates (Corning, Rochester, NY), pulsed with 1 μCi tritiated thymidine on the sixth day, and harvested 18 hours later. Counts were determined using a scintillation counter.

5. ANTIGEN PRESENTING CELL (APC) FUNCTION ANALYSIS

The relative APC function of neonates was examined by determining the capacity of irradiated neonatal leukocytes pooled from four unrelated cord blood samples to act as stimulators of proliferation in MLR. The APC function provided by CBMC was compared to that of adult PBMC in a criss-cross manner with both adult and neonate responders. Thus,

neonatal cells were used as APC to stimulate both neonatal and adult responders and, independently, irradiated adult cells were used to stimulate neonatal and adult responders over a 6-day culture period in triplicates. The stimulator pool was kept constant for all experiments. Cultured cells were pulsed with [3H] thymidine and cells were harvested after 18 hours and counts were determined. For cytokine measurements, all MLR assays were performed using irradiated stimulators (5000 rads) and supernatants were collected on days 1,2,3 and 6. Each assay contained controls of neonatal and adult stimulators and responders cultured alone. For proliferation assays mitomycin-C treated (50 ug/ml for 30 minutes at 37°C) stimulators were used.

6. CYTOKINE ASSAYS

PHA and alloantigen driven IFN-γ, IL-4, IL-5 and IL-10 cytokine responses were measured in CBMC (n=10) and PBMC (n=10) derived culture supernatants using ELISA assays as described below. Peak values for individual subjects were determined from the means (±SEM) of values obtained by analysis of four 2-fold dilutions prepared for each of the triplicate cultures per condition that were harvested on the above different days.

6.1 IL-4 ELISA

A sandwich ELISA using the human IL-4 Duoset Kit (Genzyme Diagnostics, Genzyme Corporation, Cambridge, MA) was performed according to the manufacturer's instructions, with the exception that streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) was used. Recombinant IL-4 standard, supplied with the kit, was included in each ELISA. The lower limit of detection was ~15 pg/ml of the manufacturer's standard.

6.2 IL-5, IL-10, IFN-γ ELISA

Details on these methods have been described in Part-I of this thesis.

7. STATISTICAL ANALYSIS

Differences between the groups were analysed using non-parametric statistical tests (Mann-Whitney U test and Fisher's exact test).

RESULTS

1. OPTIMIZATION OF EXPERIMENTAL CONDITIONS

1.1 Optimization of PHA Driven Proliferation

Preliminary experiments, carried out using PHA over a range from 1-20 ug/ml, demonstrated that neonatal and adult cells are activated over a virtually identical time course and PHA concentration range. Both neonatal and adult cells responded maximally at the same concentration of 5 ug/ml (Figure 1). Furthermore, the day cell harvest was optimized and day 3 was found optimal for peak proliferation of both groups of subjects (Figure 2).

1.2 Optimization of MLR Assays

The optimal concentration of allo stimulator cells was determined by titrating the proliferative response over a range of concentrations – 0-5x10^6/ml. As evident from Figure 3, the proliferative response reached a plateau from 2x10^6/ml of stimulator cells. Results were consistent between the two subjects studied. Therefore 2x10^6 concentration of stimulator cells was used in all experiments.

2. ANALYSIS OF NEONATAL IMMUNE RESPONSES

2.1. Analysis of PHA Driven Immune Responses of Neonates

2.2.1 PHA-driven proliferative responses are comparable between neonates and adults

Using the optimized experimental conditions (5 ug/ml of PHA and day 3 harvest), PHA stimulated proliferative responses of neonatal (n=16) and adult (n=5) cells were examined in detail. CBMC and PBMC were cultured with PHA for 3 days,

Fig.1. Optimization of PHA concentration for proliferation

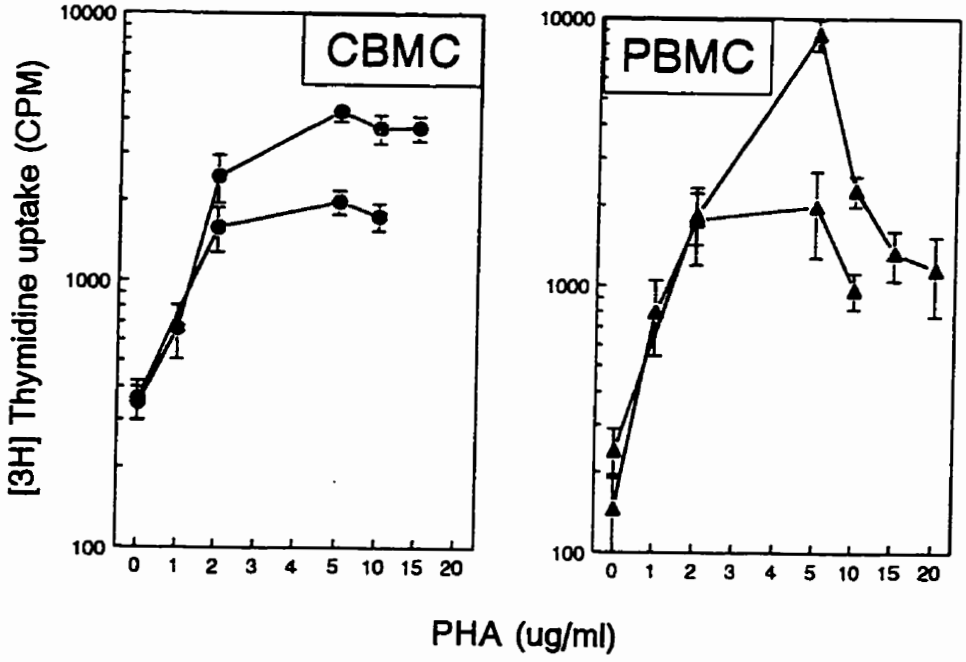

Figure 1. ***Optimization of PHA concentration for proliferation.*** Cultures were set up in triplicates using CBMC (left panel) and PBMC (right panel) ($1x10^6$/ml) with and without indicated concentrations of PHA. Cells were harvested on day 3 and proliferation was measured by [3H] thymidine uptake as described in methods section. Each line represents one subject. Data are represented as mean CPM (+/- SEM).

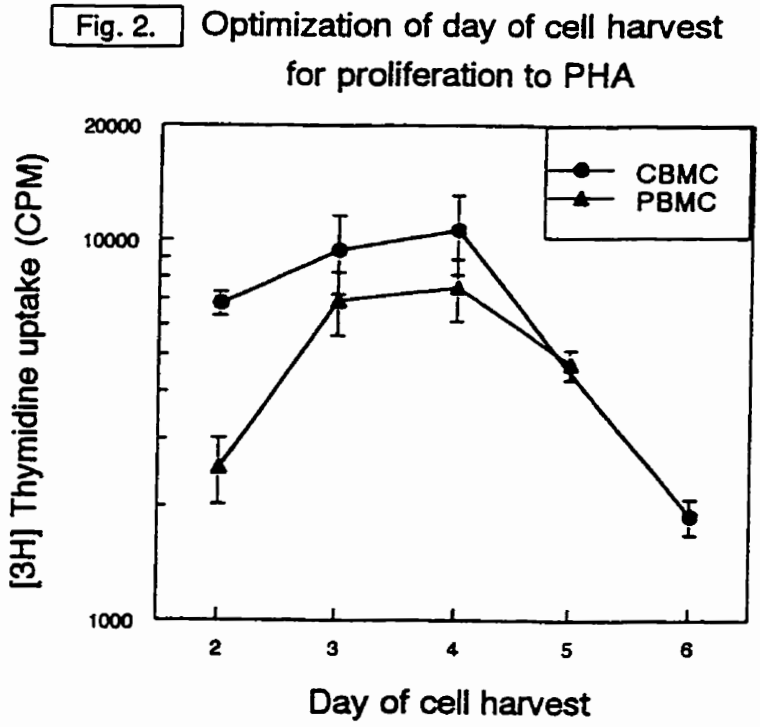

Figure 2. Optimization of day of cell harvest for proliferation to PHA. Cultures were set up in triplicates using CBMC and PBMC ($1x10^6$/ml) as indicated with and without 5 ug/ml of PHA. Cells were harvested on indicated days (X axis) and proliferation was measured by [3H] thymidine uptake as described in methods section. Each line represents one subject. Data are represented as mean CPM (+/- SEM).

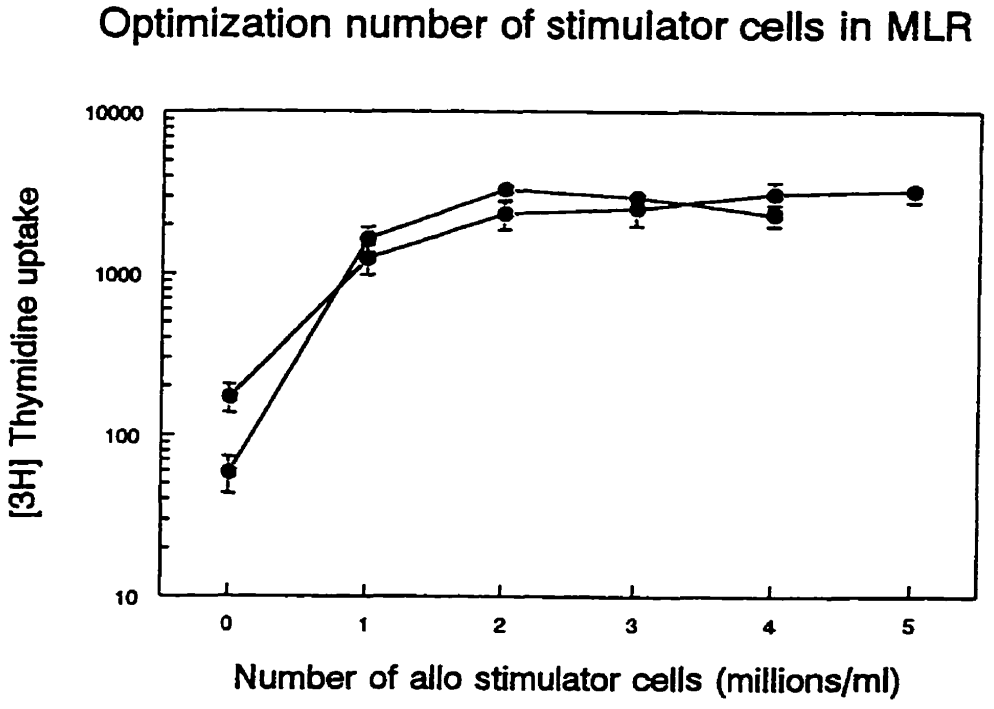

Figure 3. ***Optimization of number of stimulator cells for mixed leukocyte reaction assay.*** Cultures were set up in triplicates using CBMC ($1x10^6$/ml) with and without mitomycin treated allo stimulator cells at various concentraions as indicated (X axis). Cells were harvested on day 6 and proliferation was measured by [3H] thymidine uptake as described in methods section. Each line represents one subject. Data are represented as mean CPM (+/- SEM).

pulsed with [3H] thymidine, cells were harvested after 18 hours and counts were measured.

In the absence of PHA stimulation, the background proliferation among neonates and adults was 56-1278 CPM and 94-249 CPM, respectively. As seen in Figure 4, neonatally derived cells and adult cells exhibit very similar proliferative responses following PHA-mediated activation (CBMC median 8487 CPM, PBMC median 7810 CPM; Mann-Whitney $p>0.05$).

2.1.2 PHA preferentially activates neonatal CD4+ over CD8+ T cells. In order to characterize the responding cells to PHA stimulation, experiments were performed using intact CBMC, CBMC enriched for CD4+ cells (by depleting CD8 cells), and CBMC enriched for CD8+ cells (by depleting CD4+ cells). Results from three neonates examined are shown in Figure 5. Use of highly enriched neonatal CD4+ cell and CD8+ cell cord blood populations indicates that PHA preferentially activates CD4 T cell proliferation.

2.2 Analysis of PHA Driven Cytokine Responses of Neonates

2.2.1 Neonates exhibit severely impaired ability to secrete IFN-γ to PHA stimulation. CBMC (n=16) and PBMC (n=5) were cultured with and without optimal concentrations of PHA (5 ug/ml) for two days and culture supernatants were collected and assayed for cytokines (IFN-γ, IL-4, IL-5 and IL-10) by ELISA. As evident from the results (Figure 6, left panel), PHA stimulation indicated a markedly impaired capacity to generate IFN-γ responses among neonates compared to adults (CBMC median 2.56 U/ml, PBMC median 41 U/ml; Mann-Whitney $p=0.0008$).

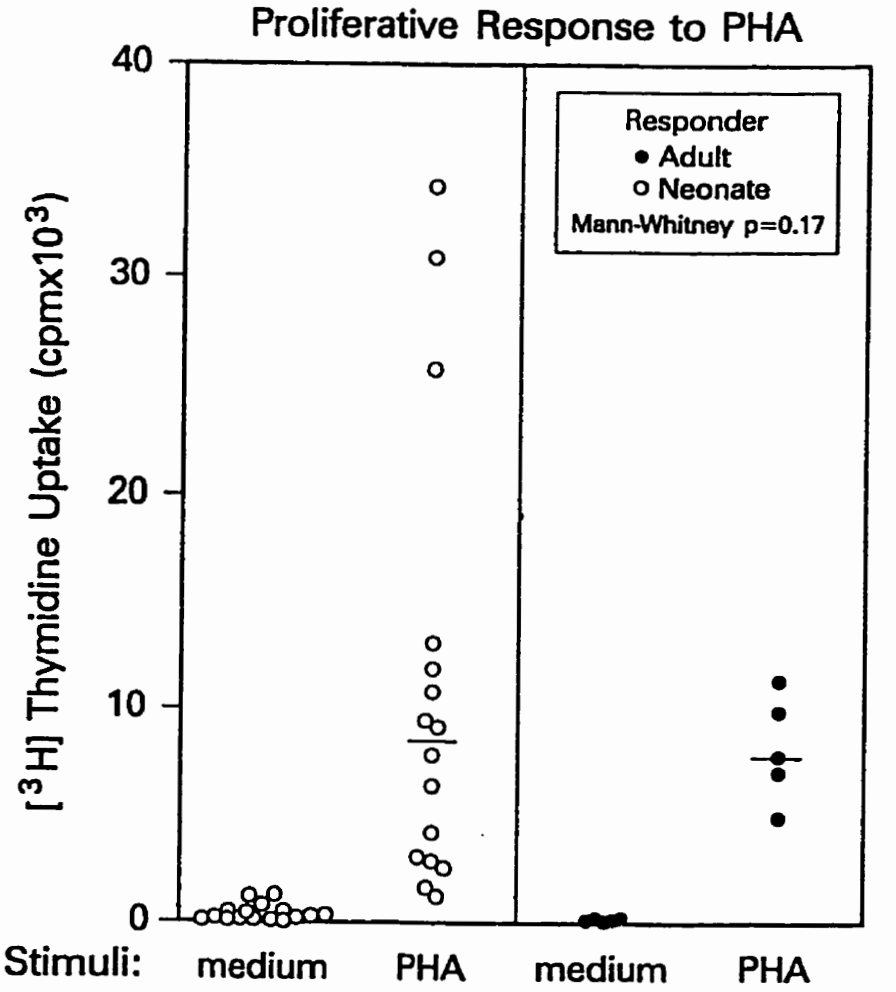

Figure 4. PHA driven proliferative responses are compabarable between neonates and adults. Proliferation was determined in three day cultures set up with PHA at 5 ug/ml. The mean response of triplicate cultures set up with CBMC (n=16) and PBMC (n=5) is shown. The median SEM (not shown) was 5%.

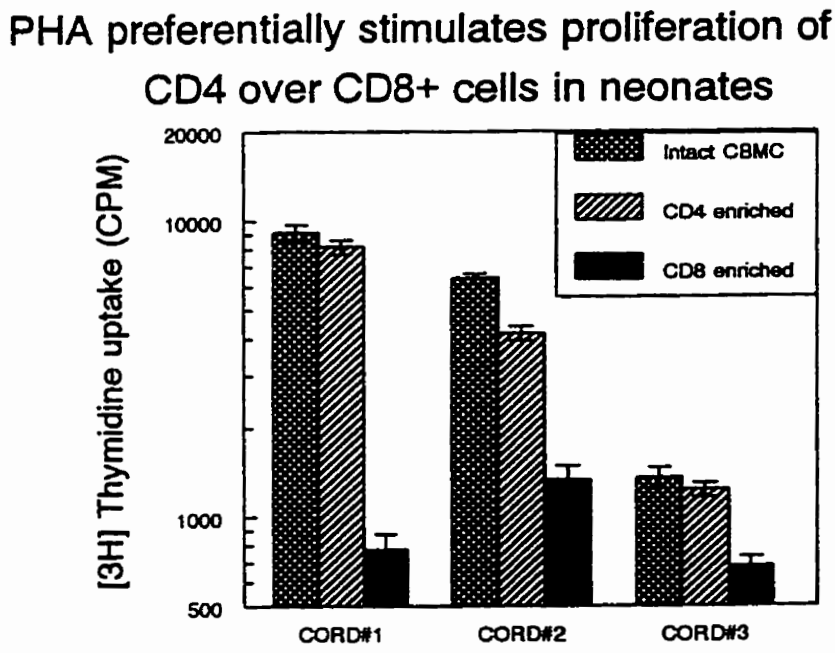

Figure 5. ***PHA preferentially activates neonatal CD4+ over CD8+ T cells.*** Unseparated and highly enriched cord blood CD4 and CD8 populations were cultured with and without PHA as described at Materials and Methods. Mean CPM and stimulation indices (in brackets) were determined from triplicate cultures independently established for each of the three subjects. Unstimulated cultures exhibited 57-398 cpm.

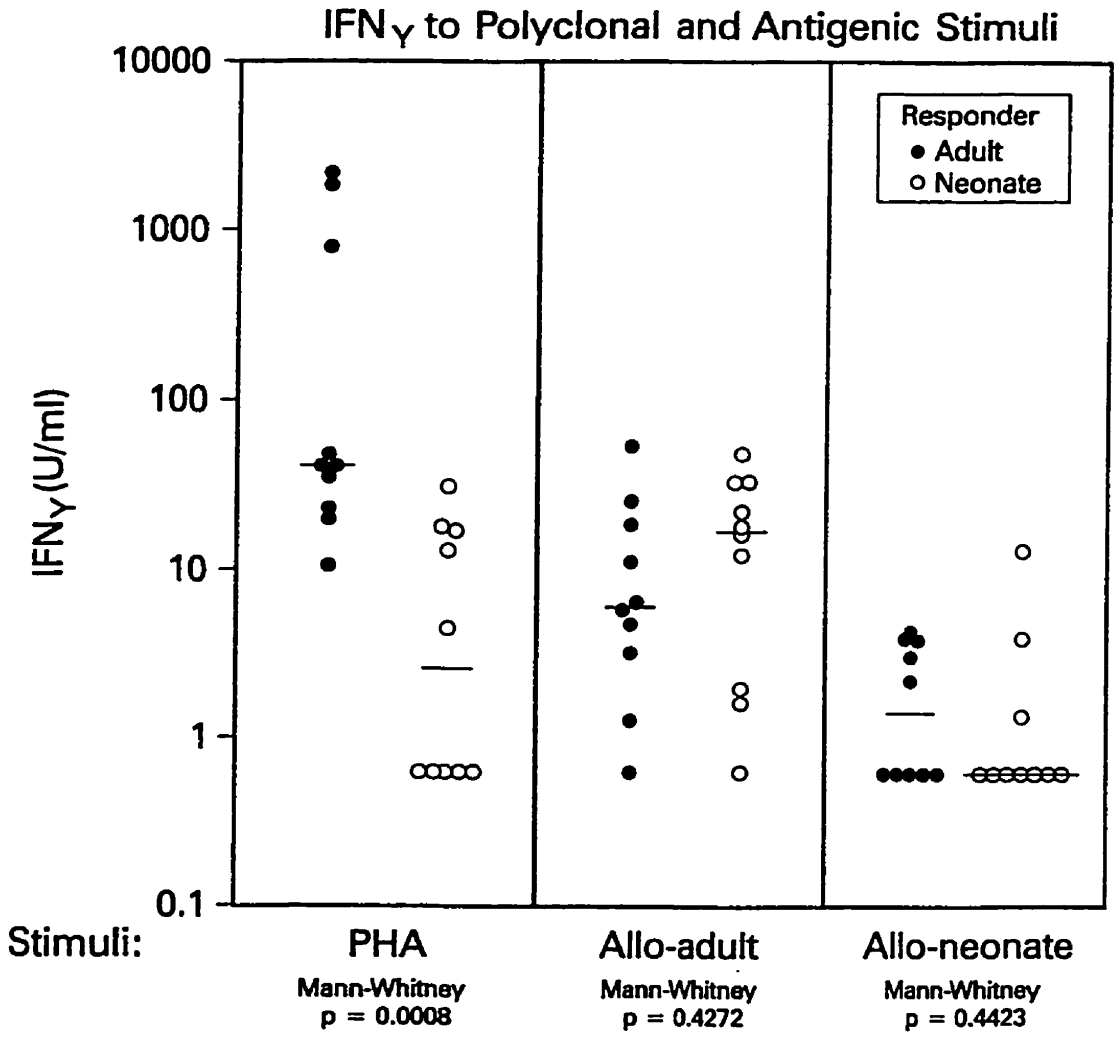

Figure 6. Neonates exhibit markedly impaired IFN-γ production to PHA, but not to antigenic (irradiated allo-adult or allo-neonate) stimuli. Mean (of triplicates SEM ~7%) IFN-γ production for neonates and adults is shown. Solid lines indicate median values within each population. Allo-adult: a pool of four unrelated irradiated adult PBMC; allo-neonate:a pool of four unrelated irradiated neonatal CBMC.

Cells cultured in the absence of PHA exhibited IFN-γ responses below the limits of detection (<0.31 U/ml).

2.2.2 The intensity of IL-4, IL-5 and IL-10 cytokine responsiveness to PHA is similar in neonates and adults. Examination of other cytokines revealed a trend towards reduced IL-4 production by neonates that was not statistically significant (CBMC median 15 pg/ml, PBMC 85 pg/ml; Mann-Whitney p=0.25; Figure 7, left panel) with essentially similar intensities of the IL-5 (CBMC median 38.5 pg/ml, PBMC median 33.50 pg/ml; Mann-Whitney p=0.85; Figure 8, left panel) and IL-10 responses (CBMC median 627.5 pg/ml, PBMC median 1116.5 pg/ml; Mann-Whitney p=0.17; Figure 9, left panel).

Collectively, analysis of cytokine responsiveness to PHA indicate that, while the neonatal repertoire is profoundly impaired in its capacity to generate IFN-γ responses, mitogen-driven TH2-associated cytokine production (IL-4, IL-5 and IL-10) is similar in neonates and adults.

3. ANALYSIS OF ALLOANTIGEN DRIVEN IMMUNE RESPONSES OF NEONATES

3.1 Alloantigen Driven Proliferative Responses are Comparable Between Neonates and Adults

As an independent approach to characterizing T cell function, alloantigen induced proliferative responses by neonates (n=12) and adults (n= 5) were compared in MLR assays. The proliferation of cells in the absence of stimulator cells among neonates and adults were 56-1278 and 94-249 CPM, respectively. As with PHA, no difference was evident in

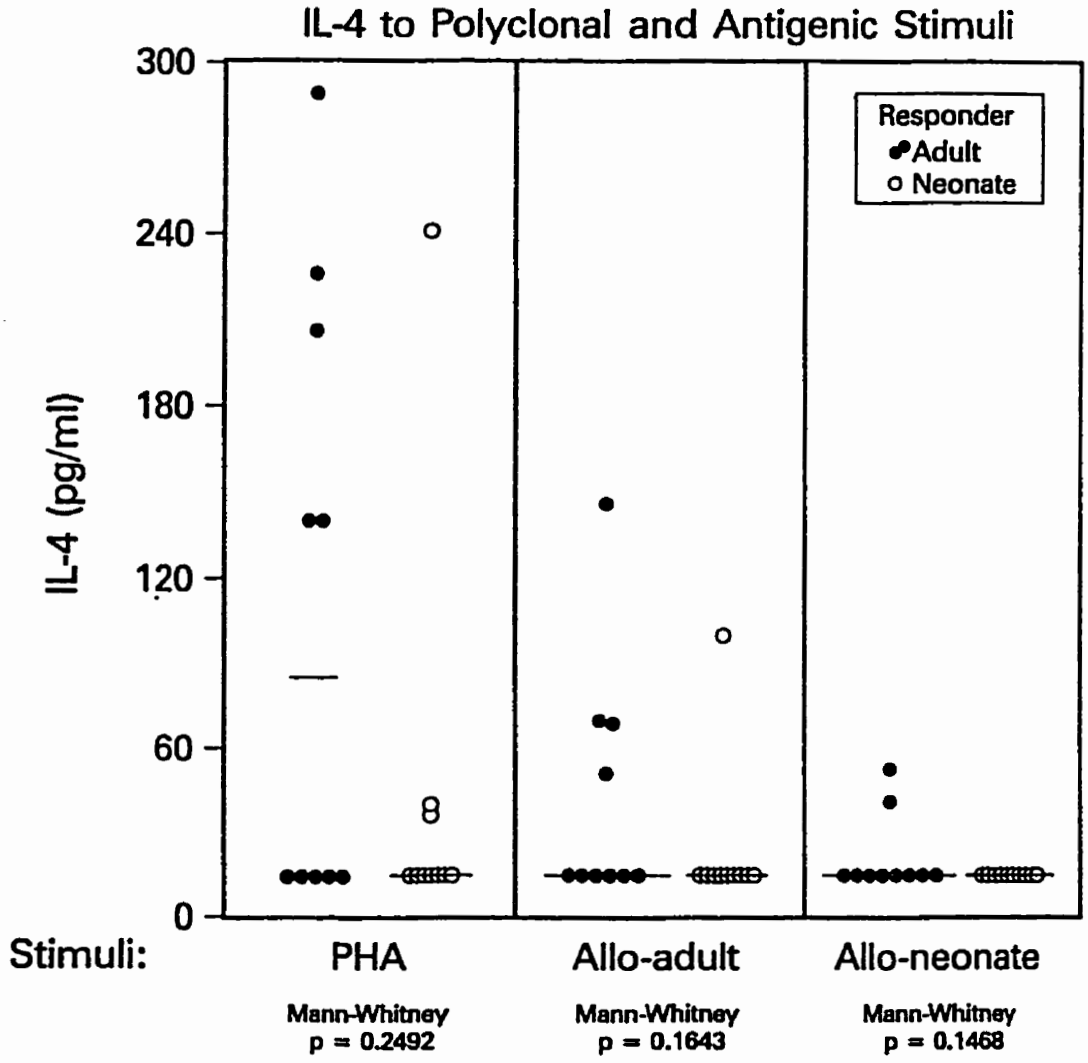

Figure 7. Neonates do not significantly differ in IL-4 production to polyclonal (PHA) or antigenic (allo-adult, allo-neonate) stimuli compared to adults. Peak IL-4 production over a three day culture period for neonates and adults is shown here with SEM for any given subject less than 15%. Solid lines indicate median values. Irradiated neonatal and adult stimulators are as described for figure 5.

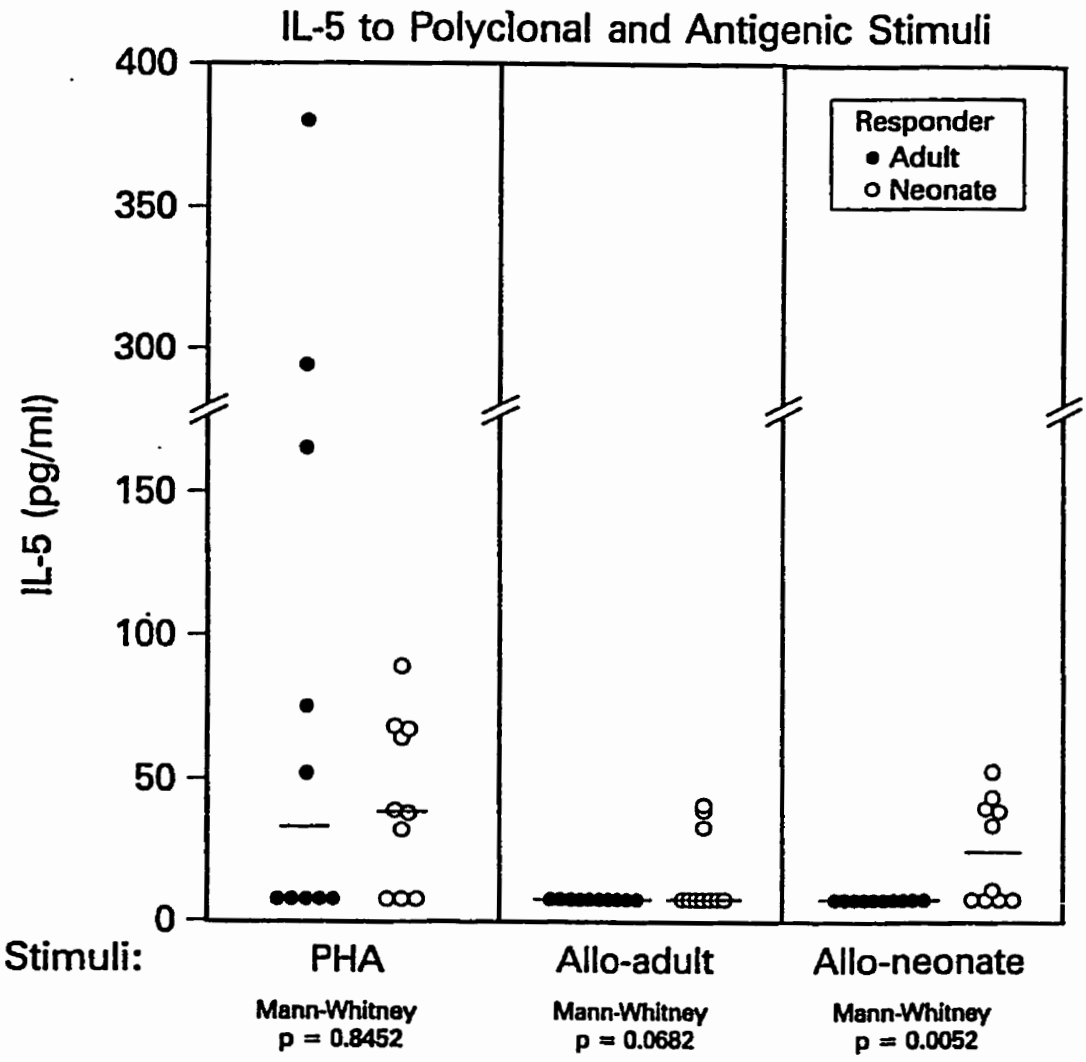

Figure 8. Neonates exhibit significantly higher IL-5 production to antigenic (allo-adult, allo-neonate) but not to polyclonal (PHA) stimulation in comparison to adult responses. Peak IL-5 production over a three day culture period for neonates and adults is shown here with SEM for any given subject less than 15%. Solid lines indicate median values. Irradiated neonatal and adult stimulators are as described for figure 5.

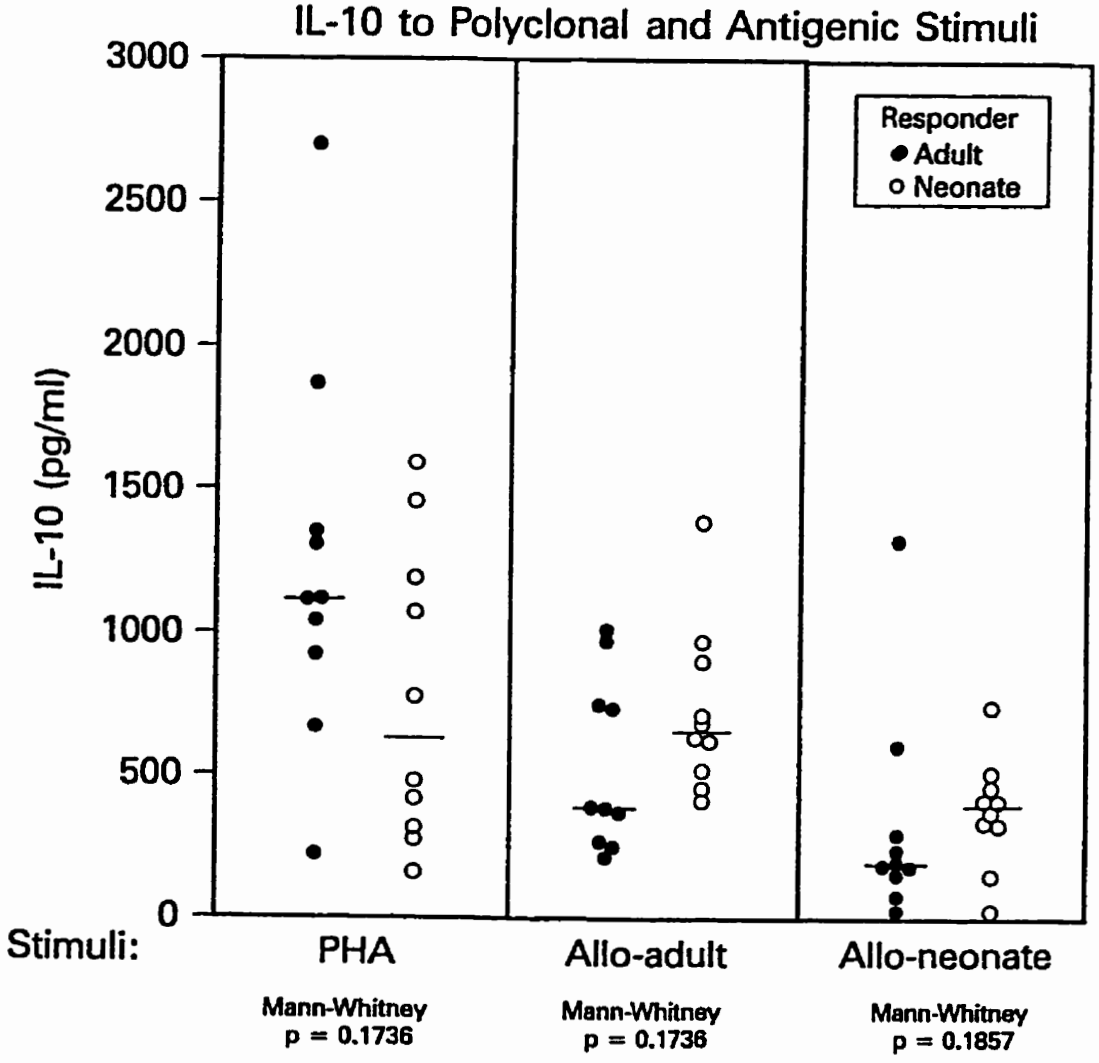

Figure 9. Neonates do not significantly differ in IL-10 production to polyclonal (PHA) and antigenic (allo-adult, allo-neonate) stimuli compared to adults. Peak IL-10 production over a three day culture period for neonates and adults is shown here with SEM for any given subject less than 15%. Solid lines indicate median values. Irradiated neonatal and adult stimulators are as described for figure 5.

alloantigen driven proliferation between neonatal and adult responder populations (median: CBM 5310 CPM, PBMC 3405; p=0.75; Figure 10).

3.2 Alloantigen Driven Cytokine Responses in Neonates and Adults

In order to assess quality of T helper cell response, alloantigen driven cytokine responses were examined in neonates (n=10) in comparison to adults (n= 10). Cultures were set up and supernatants were harvested and assayed over a period of six days for cytokines (IFN-γ, IL-4, IL-5 and IL-10) by ELISA as described in the Methods Section. Peak levels of cytokine responses were used in all analysis.

3.2.1 Neonates exhibit profound IFN-γ response to alloantigen that is indistinguishable in intensity from that of adults. In marked contrast to results obtained following PHA-driven activation where neonatal responders are markedly deficient relative to adults (p=0.0008), alloantigen stimulated IFN-γ responses by adult and neonatal responders did not differ (Figure 6, center and right panels; Mann-Whitney p=0.43 and 0.44, respectively). This was equally evident independent of whether irradiated allogeneic adult cells (Figure 6, center panel) or neonatal cells (Figure 6, right panel) were used as stimulators.

3.2.2 Antigen-driven IL-5 responses are higher in neonates, while IL-4 and IL-10 responses are comparable to adults. Analysis of alloantigen driven cytokine revealed that IL-4 production was low and similar in both groups in primary MLR (Figure 7, center and right panels) and was not affected by the type of stimulator used. The pattern of antigen-driven IL-5 production that was observed also differed markedly from that seen in response to mitogenic stimuli. In contrast to PHA driven responses, IL-5 responses were higher among neonates (Figure 8, centre and

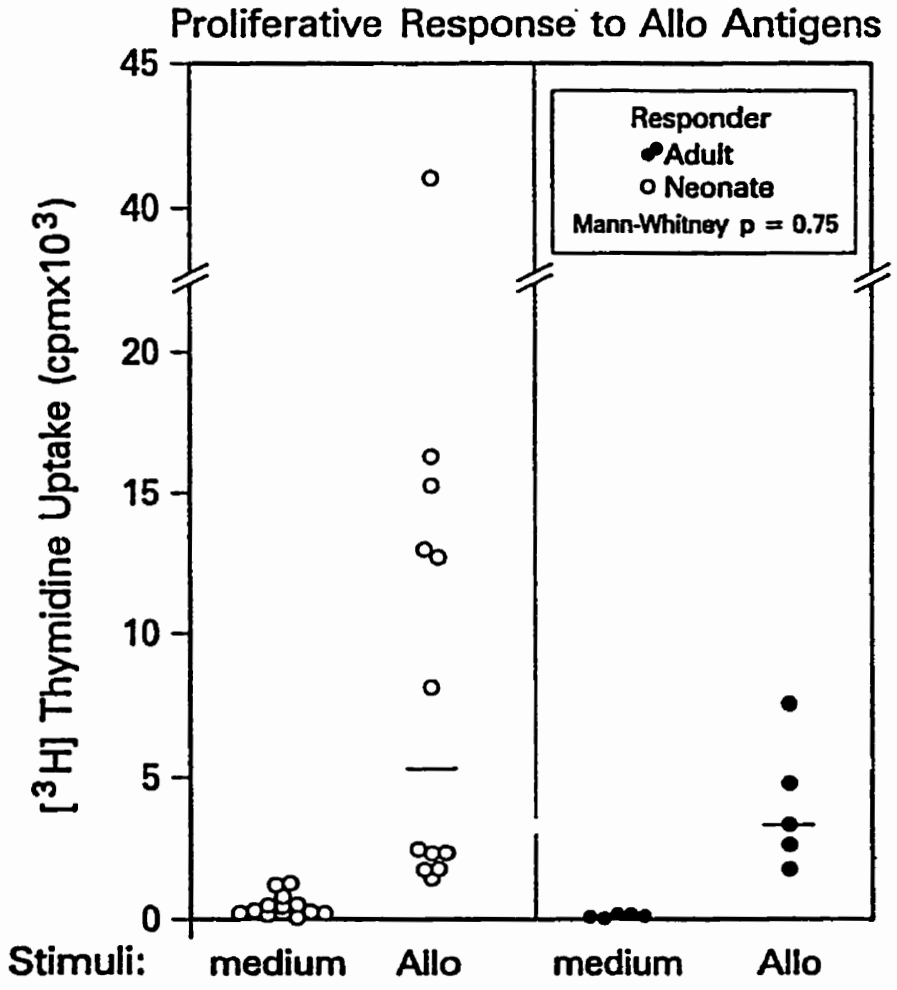

Figure 10. T cell proliferation to allo(neonate) antigens in neonatal and adult populations is of similar intensity. The T cell proliferation assay was set up using the optimal concentration of a pool of mitomycin treated, unrelated allo CBMC (2 x 10^6/ml). The proliferative response of triplicate cultures of CBMC (n=12) and PBMC (n=5) at six day culture period was measured by thymidine uptake and is shown in cpm. Median SEM was 7%.

right panels). Thus, 5/10 neonatal responders generated IL-5 responses (versus 0/10 amongst adults; Fisher's $p<0.005$) to neonatal allo-stimulation. As well as a higher frequency of responsive individuals, neonates also exhibited substantially higher levels of IL-5 production compared to adults, with the adult population producing median values of <7.5 pg/ml (ie., consistently undetectable) while neonates produced a median response of 36.5 pg/ml (Mann-Whitney $p=0.0052$).

Alloantigen stimulated IL-10 synthesis exhibited somewhat slower kinetics in neonates (~24 hours) but highly similar peak intensity independent of whether irradiated adult or neonatal APC were used (Figure 9, center and right panels).

In the absence of PHA or allo stimulation, controls (neonatal or adult responders alone, neonatal or adult stimulators alone) yielded IL-5 and IFN-γ responses that were below the limits of detection (7.5 pg/ml and 0.6 U/ml, respectively). IL-10 responses ranged from 15-167 pg/ml for neonatal responders (median 70 pg/ml) and 15-375 for adult responders (median 244 pg/ml). IL-4 was not determined.

4. ANALYSIS OF ANTIGEN PRESENTING CELL (APC) FUNCTION OF NEONATES

4.1 Neonatal and Adult APC Stimulate Similar Levels of Proliferative Responses

The relative APC function as defined by the capacity of irradiated neonatal and adult pools to act as stimulators of proliferation and cytokine synthesis in MLR was examined. As shown in Figure 11, irradiated neonatal cells elicited proliferative responses comparable to those stimulated by irradiated adult cells. Further, it should be noted that equivalent

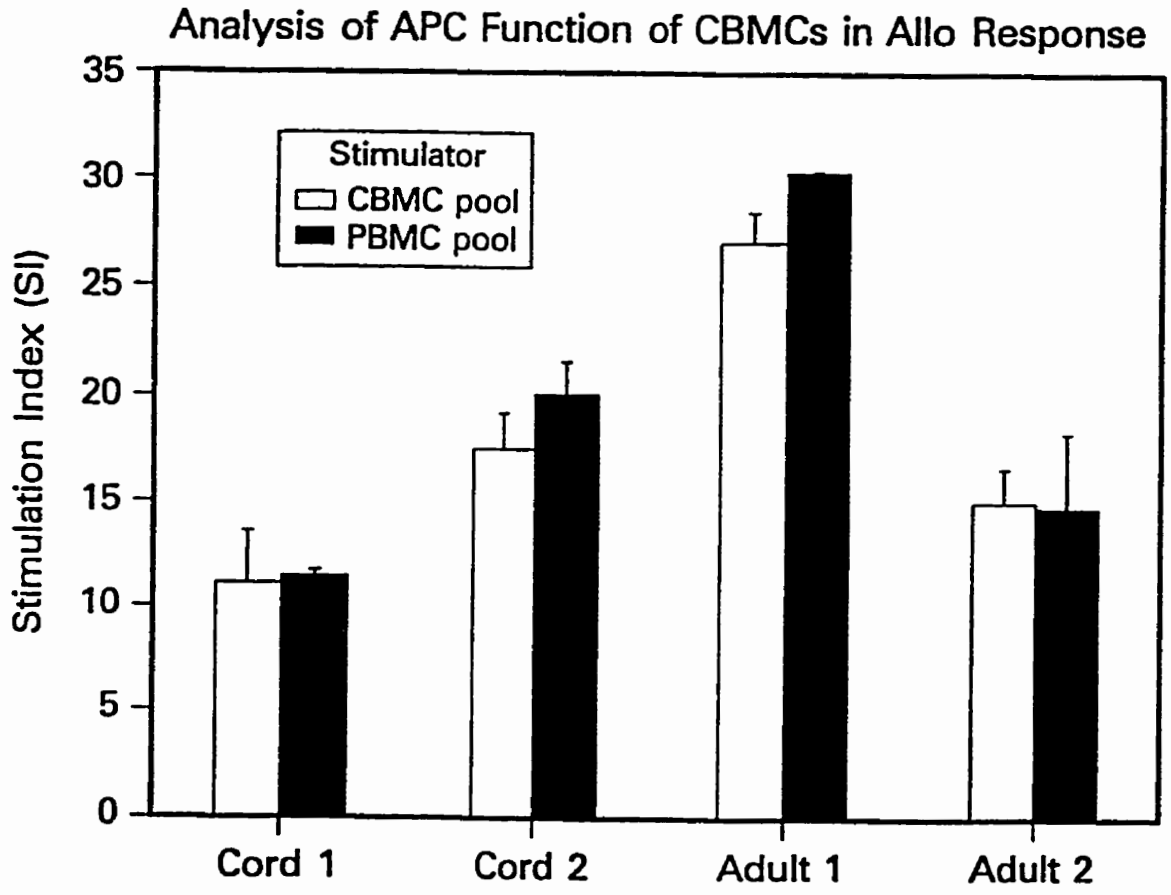

Figure 11. Comparison of APC function of neonates with that of adults. Using PBMC and CBMC as stimulators in MLR, the proliferative response of neonates and adults was measured in a criss-cross manner using each type of stimulator to activate both types of responder populations. The results are shown in SI (calculated as described in materials and methods). SEM ranged from 0.2-11.2%.

proliferation ($p>0.05$) was observed by both adult and neonatal responders for any given stimulator type.

4.2 Neonatal APC Exhibit a Defect in Stimulating IFN-γ and IL-10 but not IL-4 and IL-5 Cytokine Synthesis

Examination of cytokine responses, summarized in Table 1, indicated that while the capacity of neonatal and adult responders to produce most cytokine responses did not differ detectably, the relative capacity of adult versus neonatal cells to act as stimulators of cytokine synthesis was clearly different. Thus, both the number of individuals who exhibited detectable IFN-γ synthesis and the magnitude of their cytokine production was markedly lower when alloactivation was carried out using neonatal stimulator cells. Eight of 20 subjects had detectable levels of IFN-γ synthesis with neonatal stimulators, while 18 of these 20 exhibited IFN-γ in response to irradiated adult allo stimulators (Fisher's $p<0.001$). The median intensity of the neonatal stimulator driven IFN-γ response was <0.62 U/ml (range <0.62-13.2 U/ml), while that elicited by exposure to adult allo stimulation was 17 U/ml (range <0.62-48.3 U/ml, Mann-Whitney $p<0.003$) in neonates.

The frequency of subjects capable of generating IL-10 responses following alloactivation was similar for both adult and neonatal responder cells. However, in neonates, the median intensity of the neonatal stimulator driven IL-10 response was 393 pg/ml (range 30-742 pg/ml) while that elicited by adult stimulators was 654 pg/ml (range 408-1385 pg/ml, Mann-Whitney $p<0.005$). Among adult responders, the median intensity of the neonatal stimulator driven IL-10 response was 193 pg/ml (range 30-1322 pg/ml), half of that obtained using adult stimulators (median 383 pg/ml, range 209-1009 pg/ml; Mann-Whitney $p<0.02$). These data indicate a profoundly reduced capacity of neonatal APC to elicit IL-10 synthesis

TABLE 1. Comparison of APC function of neonates and adults to induce cytokine synthesis*.

Cytokine	Responder	Neonatal Stimulators Median (range)	Adults stimulators Median (range)	Mann-Whitney P
IFN-γ U/ml	Neonate	<0.62 (<0.62-13)	17 (<0.62-48)	0.003
	Adult	1.4 (0.62-4.4)	6.1 (0.62-54)	0.08
IL-4 pg/ml	Neonate	<15 (all <15)	<15 (<15-100)	0.32
	Adult	<15 (<15-53)	15 (<15-146)	0.23
IL-5 pg/ml	Neonate	25 (11-53)	7.5 (7.5-41)	0.17
	Adult	<7.5 (all <7.5)	<7.5 (all <7.5)	0.32
IL-10 pg/ml	Neonate	393 (30-742)	654 (408-1385)	0.005
	Adult	192	383 (209-1009)	0.02

*APC function was measured in MLR assays using irradiated neonatal and adult stimulator cells to independently stimulate both neonatal and adult responders. Ten subjects were used for each group. Cytokine responses observed in triplicate cultures over a 6-day period were determined by ELISA. Values in the table represent the mean (range) of cytokine production for each combination.

by either neonate or adult responders. In contrast to the IFN-γ and IL-10 responses, IL-4 and IL-5 production did not differ in these comparisons of the capacity of irradiated neonatal and adult cells to act as stimulators.

DISCUSSION

The present study was undertaken to evaluate the immune function of healthy neonates. This study used normal healthy neonates, as we wanted to characterize first the cytokine producing as well as inducing capacity of neonatal cells. Here cord blood samples were used. Although it is debatable whether cord blood represents maternal or neonatal blood, it is nevertheless the most easily available, non-invasive sample from neonates and hence widely used. In contrast to many previous approaches of using mitogens (Lewis and Wilson, 1995; Pirenne et al, 1992; Ayoub et al, 1971; Carr, 1972), the relative capacity of neonatal and adult cells to respond to alloantigen was evaluated in this study. Alloantigens are useful for examining neonatal immune potential because such responses do not require priming but do require HLA mediated Ag recognition. While differing in important aspects from conventional exogenous Ag-driven cytokine production, they are much more likely to reflect immune capacity than activation with stimuli such as anti-CD3 or PMA plus calcium ionophore.

The data from this study, that the proliferative response of neonates to both PHA and alloantigen do not differ from those of adults, is in agreement with previous reports (Clerici et al, 1993; Beacock et al, 1992; Risdon et al, 1995). Furthermore, PHA stimulation primarily resulted in CD4+ T cell activation with substantially weaker stimulatory capacity for CD8+ T cells as has been reported in studies of adult PBMC (Reinherz et al, 1979 and 1980). The proliferation assay is a relatively crude estimate of T cell function because it measures only whether the immune cells can respond to the Ag and does not give

information on the nature of the response. Therefore, the cytokine responses to these stimuli were examined to more fully delineate the functional potential of neonatal T cells.

The present data confirm numerous previous reports that the capacity of neonates to generate IFN-γ responses upon polyclonal activation is markedly impaired relative to that of adults (Lewis and Wilson, 1995; Wakasugi et al, 1985; Byrne et al, 1994; Takahashi et al, 1995; Lewis et al, 1991; Miyawaki et al, 1985; Wilson et al, 1986; Muller et al, 1996). The consensus that neonatal T cells are deficient in their capability to generate IFN-γ responses has been attributed to factors ranging from intrinsic abnormality of neonatal T cells (Wilson et al ,1986), increased sensitivity to prostaglandins (Wakasugi and Virelizier, 1985; Wakasugi et al, 1985), and the low frequency of CD45RO expressing T cells present in neonates (Byrne et al, 1994; Takahashi et al, 1995).

When the hypothesis that the type of stimulator selected (ie., PHA, a polyclonal stimulator versus allo-antigenic stimulation) is responsible for the apparently deficient IFN-γ response, rather than inherent T cell immaturity observed in neonates was pursued, the capacity of neonatal responder cells to produce IFN-γ was found to be essentially similar in adults and neonates. This is consistent with recent studies indicating that other neonatal T cell functions are often comparable to those of adults (Splawski et al, 1996).

PHA induced IL-5 production did not differ detectably in neonates and adults, but IL-5 production to alloantigen (as measured by both the intensity of response and frequency of responding individuals) was substantially elevated in neonates.

Taken together, the data suggest that while neonatal T cells are deficient in some of their responses to mitogen, their capacity to respond to more physiologic stimuli, such as alloantigen, is comparable to that of adults. At the same time, it needs to be emphasized that

the allo-response, although much more physiologic than PHA, anti-CD3 or PMA plus ionomycin mediated activation, still differs in several respects from conventional exogenous Ag driven T cell activation. Notably, the precursor frequency of responding and cross-reactive T cells is much higher than is found for conventional Ags, and Ag presentation differs from that occurring in normal *in vivo* responses where the MHC between APC and T cell is identical and the exogenous Ag provides the stimulus.

Previous studies examining the ability of neonatal cells to act as APC have utilized cord blood cells along with extensively purified adult responder cells (Clerici et al, 1993; Deacock et al, 1992; Risdon et al, 1995). An inherent difficulty in the interpretation of such assays is the knowledge that even very low levels of contamination with adult APC makes it impossible to evaluate the function of neonatal accessory cells. I circumvented this problem by stimulating neonatal responder cells with irradiated or mitomycin C treated neonatal cord blood as accessory cells. The alloimmune response can be mediated via three pathways: 1) self-MHC-CD4; 2) allo-MHC-CD8; and 3) allo-MHC-CD4 (Via et al, 1990). In the MLR assays reported here, stimulator CBMC provide the alloantigenic stimuli, while accessory cell function is provided by the alloAPC, allo-peptides on self APC present in the responding CBMC population, or both. In all cases, a positive proliferative/ cytokine response in this assay measures the APC function of fetal APC in the absence of confounding effects introduced by contaminating adult APC. Using this system, I compared the APC function of neonates and adults to stimulate proliferation by both neonatal and adult responders. These criss-cross experiments revealed that the APC function of neonates is comparable to that of adults insofar as their capacity to stimulate proliferation. This is in agreement with previous reports where the APC function of CBMC was analyzed in complex

depletion experiments using adult PBMCs as responders (Risdon et al, 1994; Clerici et al, 1993; Deacock et al, 1992; Risdon et al, 1995).

The data in this report clearly indicate that pooled neonatal APC are less effective stimuli for the induction of cytokine synthesis, with the IFN-γ and IL-10 data suggesting immaturity of APC function in neonates. The difference in the capacity of APC from CBMC compared with adult PBMC to stimulate cytokine production, as opposed to proliferation, was unexpected. IFN-γ, a prototypic Th1 cytokine, is critical for the generation of CTL and DTH responses which are the mediators of cellular immunity. These results suggest that the cell mediated immune response in neonates may be inadequately generated, a finding that can translate into an increased susceptibility to intracellular pathogens. IL-10, produced by T cells, B cells and macrophages/monocytes, is a growth factor for thymocytes, B cells and mast cells (Roussel et al, 1992; Lucey et al, 1996). It also may provide a costimulatory signal for the proliferation and differentiation of B cells into IgG and IgA secreting plasma cells (De-France et al, 1992). The decreased ability of neonatal APC to induce IL-10 production suggests that the costimulus necessary for B cell differentiation into these Ab isotype secreting plasma cells may be inadequate in neonates, and hence may contribute to the observed decreases in Ig synthesis at this age.

An obvious candidate to explain differences in APC function between neonates and adults is differences in costimulatory molecule expression. Initial activation of naive T cells requires at least one Ag non-specific costimulatory signal in addition to the Ag-specific interaction resulting from TCR/MHC interaction. B7.1 (CD80) and B7.2 (CD86) are the best defined co-stimulatory molecules expressed on APC with their ligands, CD28 and CTLA-4, acting as the primary positive and negative co-stimulatory receptors on T cells. However, it

is now evident that any of a number of molecules may provide accessory function and that efficient responses are only generated following multiple interactions (Lenschow et al, 1996; Croft, 1997).

The level and nature of costimulatory molecule expression by neonatal APC is largely unknown. On resting adult human APC or effector cells, the level of CD80 expression is very low to undetectable (Lenschow et al, 1996). CD86 expression is more complex with B and T cells at undetectable levels, monocytes at low to moderate levels, and DCs at the highest relative levels. Regulation of CD80/86 expression is strongly influenced by cell-cell interactions and cytokines. Such regulation is likely to be complex as different cytokines upregulate (ie IFN-γ) or inhibit (ie IL-10) costimulator expression and a broad variety of potential APC (Langerhans/DC, monocytes, B cells) express CD80/86 in response to different forms of activation (Lenschow et al, 1996; Takamizawa et al, 1997). Similarly, CD80/86 expression of human Langerhan's cells appears to be differently regulated by IFN-γ or IL-4 (Yokozeki et al, 1997; Stack et al, 1994) and may reflect different functions (Fleicher et al, 1996). Hunt et al (1994) isolated low-density, nonadherent, nonphagocytic, HLA-DR+ cells with the morphology of DCs from the cord blood of full-term newborn infants. Flow cytometric studies showed that the density of intercellular adhesion molecule-1 (ICAM-1; CD54) and MHC class I and class II Ags on cord blood DC were significantly lower than those in adult blood. Levels of CD80/CD86 expression were not determined. Thus, detailed analysis of the relative maturity (phenotypic and functional) of neonatal APC remains to be performed.

In light of the present observation of increased IL-5 synthesis among Ag-stimulated neonatal responders, a finding most pronounced when neonatal APC were used ($p<0.005$;

Figure 4), the findings of Delespesse and colleagues (1995 and 1997) are of particular interest. Their studies indicate that the intensity of B7 co-stimulation at priming markedly affects the lymphokine-producing phenotype. T cells primed on CD32-B7 double L transfectants produced much more IL-4 and IL-5 and slightly less IFN-γ than did those primed on B7 deficient cells. The enhanced IL-4/IL-5-producing capacity of cells primed on CD32-B7 L fibroblasts was speculated to be related to increased IL-4 production during priming which suggests the the maturation of naive T cells along the Th2 or Th1 pathway may be regulated by the level of B7 expressed on APC.

Cord blood has been recently demonstrated to be a rich source of CD34+ haemopoetic stem cells, which are key cells used for bone marrow transplantation (BMT) and in gene therapy. Clinical demonstrations of reduced immunoreactivity and graft rejection have made cord cells a valuable source of such stem cells (Thomson, 1995). However, the immunological basis underlying the success of cord transplantation is not well understood. Increased IFN-γ cytokine production is associated with severe transplantation-related complications in BMT patients (Tanaka et al, 1995). Furthermore, there is an association between intragraft IL-10 mRNA expression and acute renal allograft rejection (Xu et al, 1995). These reports, together with this data that cord cells are relatively poor inducers of IFN-γ and IL-10 is consistent with suggestions that cord blood may have the advantage of reduced potential to induce graft rejection when used in allogenic BMT.

Thus, in summary, this study provides evidence that neonatal T cells are as immunocompetant as adults in the production of IFN-γ, IL-4, IL-5 and IL-10 cytokines when tested using a physiologically relevant stimulus. However, neonatal APC are functionally immature in inducing IFN-γ and IL-10 cytokines. This, rather than inherent deficiencies in

neonatal T cell capability, may play a significant role in the increased vulnerability of neonates to infection.

This study was undertaken in healthy neonates with an overall aim to study the cytokine producing and inducing capacity in HIV infected and non-infected children, but for reasons of non availability of sufficient viable cells, it could not be pursued further. Nonetheless, results from this study can serve as the fundamental basis to study such functions in any infection of neonates.

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IMAGE EVALUATION
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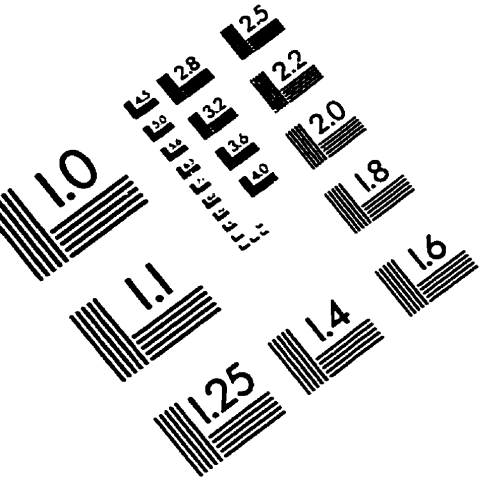

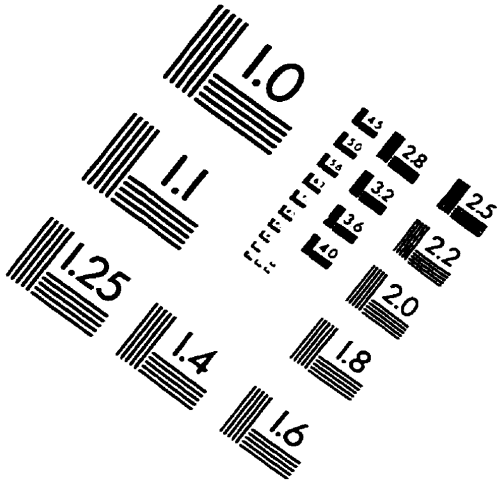

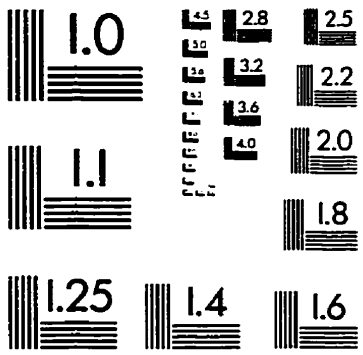

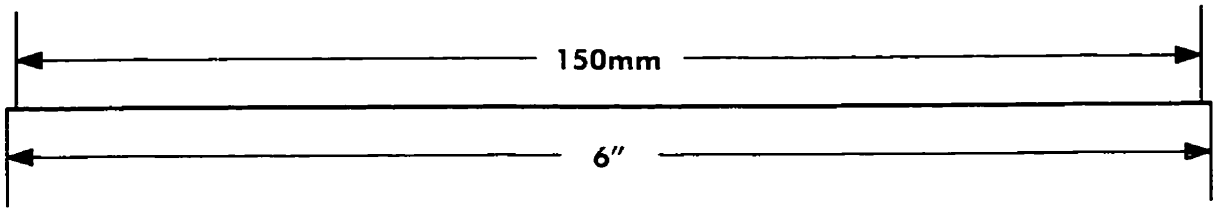

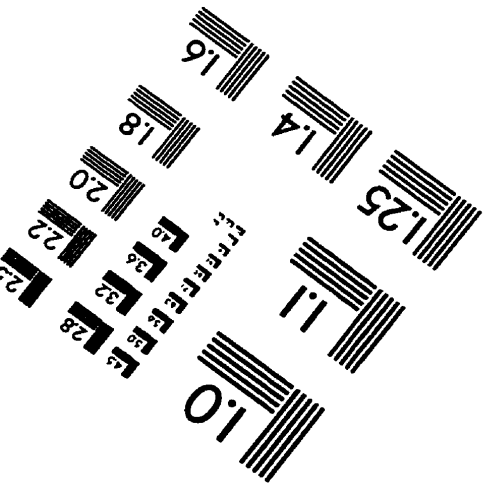

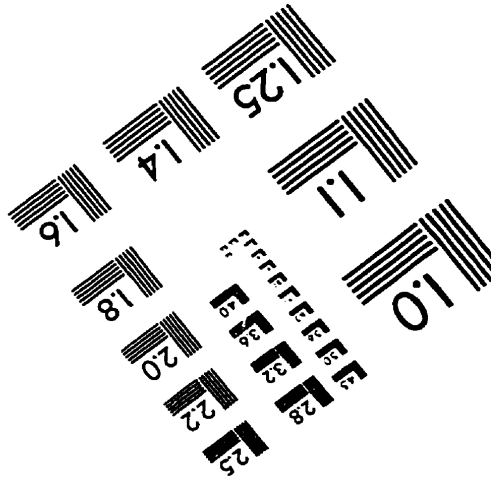

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